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The Reovirus FAST Proteins:
A New Perspective on Protein-Mediated Membrane Fusion

by

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Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
August 2006

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This work is dedicated to the discovery of truth, in all its hiding places.

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ABSTRACT

The fusogenic reoviruses are the only examples of non-enveloped viruses that encode membrane fusion proteins. The non-structural fusion-associated small transmembrane (FAST) proteins are the smallest known membrane fusion proteins and are the only viral gene products required to mediate extensive cell-cell fusion and syncytium formation in infected and FAST protein-transfected cells.

Analysis of four different FAST proteins (ARV-p10, NBV-p10, BRV-p15 and RRV-p14) in transfected cells revealed that they indirectly alter membrane permeability to small molecules (e.g. uridine and hygromycin B) by producing large syncytia that lose membrane integrity when they die by apoptosis. These observations support a dual role for syncytium formation in the reovirus replication cycle. Fusion at early infection times allows the infection to spread locally and safely access the replication machinery of neighbouring cells. Extensive syncytium formation late in infection leads to apoptosis-induced membrane permeability, facilitating virus release and dissemination of the infection. These results also indicate that the FAST proteins are dedicated cell-cell fusion proteins, but the mechanism of FAST protein-mediated fusion remains unclear.

The small size of the FAST proteins, and their lack of structural similarity to other viral fusion proteins, suggest they may mediate fusion through a novel mechanism. The larger viral fusion proteins use complex triggered conformational rearrangements to supply the energy necessary to drive membrane fusion. Analysis of the FAST proteins reveals that they lack specific receptor-binding capability and instead they exploit the closeness provided by cadherin-dependent cellular adhesion machinery to mediate efficient cell-cell fusion. In addition, other cell factors, such as membrane cholesterol and actin remodelling, are critical for FAST protein function, further indicating the importance of the cellular environment in dictating the success of FAST protein-mediated fusion.

The FAST proteins are the only fusion proteins that have uncoupled receptor binding from membrane fusion, resulting in an untriggered and unregulated fusion reaction. This unique scenario has allowed the FAST proteins to adopt novel and alternate strategies for mediating membrane merger and as such they offer a new perspective on the process of biological membrane fusion.

LIST OF ABBREVIATIONS AND SYMBOLS USED

aa	amino acid
Ab	antibody
ADAM	a disintegrin and a metalloproteinases
ADIP	afadin DIL domain interacting protein
Ala (A)	alanine
AP1-3	adaptor protein complex 1-3
Arg (R)	arginine
Arp2/3	actin-related protein 2/3 complex
ARV	avian reovirus
ARV-p10	avian reovirus fusion protein (10 kDa)
Asn (N)	asparagine
Asp (D)	aspartic acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BRV	baboon reovirus
BRV-p15	baboon reovirus fusion protein (15 kDa)
CCR5	CC chemokine receptor 5 (HIV gp120 co-receptor)
CD4	cluster of differentiation antigen 4 (HIV gp120 receptor)
CD44	cluster of differentiation antigen 44
cDNA	complementary DNA
CHO	Chinese hamster ovary cells
Ci	Curie
cLPDS	complete lipoprotein deficient serum
CMAC	7-amino-4-chloromethylcoumarin
CMTMR	5-(and-6) -(((4-chloromethyl)benzoyl)amino)-tetramethylrhodamine
CMV	cytomegalovirus
CO_2	carbon dioxide
COP	coat protein complex
cpm	counts per minute; unit of measurement for liquid scintillation counters
CRAC	cholesterol recognition/interaction amino acid consensus sequence
CTB	cholera toxin B subunit
CXCR4	CXC chemokine receptor 4 (HIV gp120 co-receptor)
Cys (C)	cysteine
d	day
DAG	diacylglycerol
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
DC-SIGN	dendritic cell
DIC	differential interference contrast microscopy
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DRM	detergent resistant membrane
duf	dumfounded protein (*Drosophila*)

E	envelope proteins of togaviruses and flaviviruses
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EEV	poxvirus extracellular enveloped virus
EF-2	elongation factor 2
EFF-1	epithelial fusion failure-1 protein (*C. elegans*)
EL	L-cells expressing E-cadherin
ER	endoplasmic reticulum
EtOH	ethanol
ex/em	excitation and emission maxima for fluorophores
F	paramyxovirus fusion protein
F(Ab) H+L	heavy and light chain antibody fragments
FACS	fluorescence activated cell sorting
FAST	fusion-associated small transmembrane proteins
FBS	foetal bovine serum
FRET	fluorescence resonance energy transfer
G	paramyxovirus glycoprotein
GAP	GTPase-activating protein
GDI	guanine nucleotide dissociation inhibitor
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
Gln (Q)	glutamine
Glu (E)	glutamic acid
Gly (G)	glycine
GM1	a ganglioside
GPI	glycosylphosphatidylinositol
GSL	glycosphingolipid
GTPase	guanosine triphosphatase
h	hours
H	paramyxovirus hemagglutinin protein
HA	influenza virus hemagglutinin protein
hbs	hibris protein (*Drosophila*)
HBSS	Hank's balanced salt solution
HBsAg	Hepatitis B virus surface antigen
HeNe	helium neon laser
HHV-6	human herpesvirus 6
His (H)	histidine
HIV	Human immunodeficiency virus
HMDS	hexamethyldisilazane
HMG-CoA	hydroxy-methylglutaryl-coenzyme A
HN	paramyxovirus hemagglutinin/neuraminadase protein
hpi	hours post-infection

HPO	horseradish peroxidase
hpt	hours post-transfection
HS	horse serum
HVEM	herpesvirus entry mediator
ICAM-1	intercellular adhesion molecule 1
Ile (I)	isoleucine
IMV	poxvirus intracellular mature form
IP3	inisitoltriphosphate
JAM	junction adhesion molecule
kDa	kiloDaltons
Leu (L)	leucine
LFA-1	leukocyte function antigen-1
Lys (K)	lysine
MβCD	methyl-β-cyclodextrin, cholesterol depleting agent
MDCK	Madin-Darby canine kidney
MEM	minimal essential medium
MeOH	methanol
Met (M)	methionine
min	minutes
MFR	macrophage fusion receptor
MMP	matrix metalloproteinase
MOI	multiplicity of infection; MOI of 1 = 1pfu/cell
MRV	mammalian reovirus
MuLV	Murine leukemia virus
n	number of experiments used in SE calculation
NA	numerical aperature
NaCl	sodium chloride
NaDOC	sodium deoxycholate
NBT	nitroblue tetrazolium
NBV	Nelson Bay virus
NBV-p10	Nelson Bay virus fusion protein (10 kDa)
NMR	nuclear magnetic resonance
NRS	normal rabbit serum
NSF	N-ethylmaleimide-sensitive factor
ORF	open reading frame
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine

PEG	polyethylene glycol
pfu	plaque-forming unit
Phe (F)	phenylalanine
PI	phosphatidylinositol
PI	propidium iodide
PI3K	phosphatidylinositol-3-kinase
PIP	phosphorylated derivatives of PI
PIP_2	phosphotidylinosititol-4,5-bisphosphate
PLA_2	phospholipase A_2
PLAP	placental alkaline phosphatase
PMA	phorbol 12-myristate 13-acetate
PPO	2,5-diphenyloxazole
Pro (P)	proline
PS	phosphatidylserine
PVDF	polyvinylidene difluoride
RIPA	radio-immunoprecipitation buffer A; cell lysis buffer
RNA	ribonucleic acid
RRV	reptilian reovirus
RRV-p14	reptilian reovirus fusion protein (14 kDa)
rst	roughest protein (*Drosophila*)
RSV	respiratory syncytial virus
s	seconds
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electorphoresis
SE	standard error of the mean
SEM	scanning electron microscopy
Ser (S)	serine
SH3	Src-homology 3 domain
SI	syncytial index
SM	sphingomyelin
SNAP	soluble N-ethylmaleimide-sensitive factor attachment protein
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
sns	sticks and stones protein (*Drosophlia*)
TCA	trichloroacetic acid
TEM	transmission electron microscopy
Thr (T)	threonine
t-RNA	transfer RNA
Trp (W)	tryptophan
t-SNARE	SNARE associated with target membrane
Tyr (Y)	tyrosine
U18666A	3β-(2-(diethylamino)-ethoxy)-androst-5-en-17-one

Val (V)	valine
v-SNARE	SNARE associated with vesicle membrane
VSV	vesicular stomatitis virus
VSV-G	vesicular stomatitis virus fusion glycoprotein
X	any amino acid

ACKNOWLEDGEMENTS

This research could never have been completed without the many people who have donated cells, reagents and technical expertise to my cause over the years. I thank them now:

Raphael Garduno	Mark Nachtigal	David Hoskin	Todd Hatchette
Neale Ridgway	Victor Rafuse	Damien Yohn	Gary Faulkner
Mary Ann Trevors	Patrick Lee	Ping Li	Don Stoltz
Richard Hill	Drew Leidal	David Conrad	Andrea Makkay
Michael Way	Steven Wharton	Judy White	Masatoshi Takeichi
Earl Brown	Colin Parrish	Jennifer Gruenke	Deborah Brown

I would to extend a special thank you to Stephen Whitefield and the staff of the Cellular Microscopy and Digital Imaging suite for giving me the skills to see such amazing things.

I must also acknowledge the faculty and administrative staff of the Department of Microbiology and Immunology for providing such excellent undergraduate and graduate programs and for taking very good care of their students.

I would like to thank my supervisory committee, Dr. David Byers, Dr. Roger McLeod and Dr. Rick Singer for all of their guidance, insightful comments and useful discussions over the years. Thank you as well to my external examiner, Dr. Robert Doms, for taking the time to critique my work and travel to Dalhousie University for my defence. Your efforts are greatly appreciated.

I also acknowledge the funding for this work provided by the Natural Sciences and Engineering Research Council of Canada (NSERC), the Nova Scotia Heath Research Foundation (NSHRF) and Cancer Research and Education Nova Scotia (CaRE).

And now for my boss. Roy, you are an excellent teacher and wonderful mentor. Your undergraduate virology classes rekindled my love of science and led me to research. To have ended up doing graduate school in your lab is an honour. "Thank you" is an ineffective way to express my gratitude, but many thanks follow anyway.
-Thank you for letting us find our way as researchers while encouraging our strengths and providing opportunities to correct our weaknesses.
-Thank you for the opportunity to TA virology repeatedly; it was one of my most rewarding experiences as a graduate student.
-Thank you for your guidance and giving me a push whenever I needed it.
-Thank you for saying the right thing whenever I feel it's all coming apart.
-Thank you for letting me help you move...and cut wood after the hurricane.
-Thank you for all the wonderful parties. Sorry about the BBQ lighter.
-Thank you for getting that paper out before I leave ☺.
-Finally, thank you for accepting me into your lab and filling it with excellent scientists, and even better friends. I will be hard pressed to find a work environment as enjoyable and rewarding as the Duncan Lab.

"The floggings will continue until morale improves"

For my coworkers and friends...
Jing, you are the glue that holds the lab together. Thank you for all of your help when I first started in the lab and thank you more for being such a wonderful person to see first thing in the morning everyday. Dr. Bob, your professionalism, precision and organization are an example to us all. But don't let these kind words make you think I have forgiven you for that crack about my hair. Jenn and Sam, you both set a high standard for scientific integrity and social ridicule. You have inspired us all in both respects.

Deniz, when you share a bench with someone for four years, you get to know a thing or two about them. You wear glasses and quit your job a lot. For making me laugh, getting so excited about graphs, teaching me about proteins and being a great friend, I bequeath to you my pipettes and first pick of my buffers when I am gone. Eileen, your funny stories amuse me and your honest opinions are always respected, as brutal and untactful as they can sometimes be. Chuck, I don't think it's too soon to say how glad I am that you joined our lab. Because of you, Roy didn't ask me to "take another stab" at those antibodies. Mr. Teenie, thank you for letting me call you that, and for always having a post-it handy when I need one. To my students and colleagues, Tara, Bert and Erin, I hope your time in our lab was as enjoyable and rewarding as it was for me to supervise you. To all the Duncanites, past and present, you all make coming into work everyday an absolute joy. Thank you. I never would have made it through five years of research without you guys. And I never would have made it home some nights if were not for you either.

I must also express my gratitude to my family. You have been a part of this journey the longest and your love and support over that last 28 years has been invaluable and always appreciated. Mom, you have been an inspiration and a role model in every regard my entire life. I wouldn't be where I am if not for your encouragement and confidence when I was lacking. Thank you for everything. To my dad and grandmother, thank you for your patience, friendship and counsel through the last 10 years of post secondary education. David, I consider myself lucky to have a big brother like you. You are probably unaware, but your wisdom and no-nonsense advice has helped me weather numerous storms. It almost makes me feel bad for breaking so many of your toys.

Suzanne, never underestimate my genuine gratitude and love, earned by your empathy and endless support. Thank you for always believing in me. You make everything worthwhile.

And finally...thank you to fusogenic reoviruses, the FAST proteins (especially p14) and the ~1 million syncytial nuclei who made this all possible.

CHAPTER 1
Introduction

1.1. Overview

Membrane fusion is a process critical to eukaryotic life. At the cellular level it is responsible for maintaining and regulating subcellular compartments by controlling vesicle trafficking (555). At the organismal level it is required for fertilization, muscle and bone development, placenta formation and virus infections (42, 86, 115, 280, 575). Due to their primary role of maintaining compartmentalization, biological membranes are naturally resistant to membrane fusion and require specialized proteins to mediate the energetically unfavourable event of membrane merger. The majority of our body of knowledge on membrane fusion proteins comes from experiments conducted on the proteins isolated from enveloped viruses and cellular trafficking machinery. While these proteins are diverse in nature, they are believed to function in a fundamentally similar manner by using triggered protein conformational rearrangements to drive the fusion reaction (22, 42, 59, 555). It is unclear how this mechanism extends to cell-cell fusion events, as no cellular fusion proteins have been positively identified (86). The only membrane fusion proteins identified with the primary function of causing cell-cell fusion are the reovirus FAST proteins (111, 125, 494). These unusual fusion proteins lack the structural elements necessary to facilitate fusion in the traditional manner and may have evolved a novel method for executing the steps of membrane fusion. Insights gleaned from these simple little proteins may contribute to a greater understanding of the process by which proteins mediate membrane merger.

The following sections will provide a brief overview of the process of membrane fusion and the proteins known or predicted to execute it. In addition, attention will be given to illustrating the complexity of biological membranes and identifying the numerous factors that can affect the outcome of a fusion reaction. Finally, a summary of the current knowledge about the FAST proteins will be provided, to better establish a context for the ensuing studies. We begin with a description of biological membranes.

1.2. Biological membrane composition

In general, eukaryotic membranes contain three types of lipids (phospholipids, sphingolipids and sterols) asymmetrically distributed between the bilayers (561). Lipids are amphipathic molecules with hydrophilic head groups and hydrophobic tails. In an aqueous environment they align themselves tail-to-tail to produce a bilayer with solvent-exposed head groups and a hydrophobic interior (561). The lipid composition of a membrane alters its physical properties and can greatly influence its ability to perform biological processes, including membrane fusion (62). This introduction will describe the general plasma membrane components of animal cells, as this is most relevant to the ensuing discussions on membrane fusion. However, important exceptions will be noted when appropriate.

1.2.1. Phospholipids

Phospholipids are by far the most abundant membrane lipid and consist of a glycerol backbone to which one of several phosphate-containing head groups and two acyl chains of varying length and saturation are attached (561). Phospholipids not only provide the basic building blocks of membranes, they also play important roles in cell signalling and metabolism (527, 561). Generally, phospholipid acyl chains, or tails, are heterologous with one being completely saturated and the other containing various degrees of unsaturation. Acyl chain length can range from 10-26 carbons, (16-24 is typical) and chain length is a major determinant of membrane thickness (477, 561, 589). Introduction of double bonds between carbons in the acyl chain (desaturation) causes the chain to kink and bend out of plane, disrupting the lateral packing of lipids and increasing membrane fluidity (268, 527).

The most prevalent lipid in eukaryotic membranes is phosphatidylcholine (PC), comprising ~40% of the total membrane lipid (477, 561, 589). PC is predominantly found on the outer leaflet of the plasma membrane of mammalian cells. Phosphatidylethanolamine (PE) and phosphatidylserine (PS) are major inner-leaflet membrane lipids and comprise ~10-30% and ~4-10%, respectively, of cell membranes (477, 589). PS and PE differ from PC in their headgroup modification and their tendency to contain more unsaturated acyl chains (406). PS is a negatively charged lipid and is

important in apoptotic signalling when it is externalized to the outer leaflet of the plasma membrane (163, 346). Another important signalling lipid is phosphatidylinositol (PI) and its phosphorylated derivatives (PIPs). PIPs contain a cyclic inositol ring for a head group that can be differentially phosphorylated to produce a variety of effector molecules (247). PIPs are exclusive to the cytoplasmic-facing leaflets of cellular membranes and compose a very small percentage of total membrane lipids (477, 561, 589). However, despite their low abundance, PIPs are very important molecules for cell signalling, serving as precursors for inositoltriphosphate (IP3) and diacylglycerol (DAG), two potent cell activators (140, 327). Further, each organelle has a different subset of the PIP derivatives that serve as membrane markers; thus they play a significant role in identifying organelle membranes to trafficking and cytoskeleton remodelling machinery (247). PIPs are integral to several signalling events, especially those resulting in cell adhesion and motility and, as will be elaborated on later, this may influence the fusion potential of the PIP-containing membrane (247, 253).

1.2.2. Sphingolipids

There are more than 300 types of sphingolipids that are derived from a ceramide backbone and include sphingomyelin (SM) and several different glycosphingolipids (561). SM is a major outer-leaflet constituent on the plasma membrane (~10-20%) and is synthesized primarily in the Golgi and to a lesser extent at the plasma membrane (477, 561, 589). SM is a particularly noteworthy sphingolipid as it has a propensity to interact with membrane cholesterol. Glycosphingolipids (GSL) comprise a large family of ceramide-derived lipids that contain one or more diverse sugar (e.g. glucose and galactose) residues as a head group (561). Of particular relevance to this study are the gangliosides, especially GM1, as its physical properties allow it to be used as a tool when studying plasma membrane heterogeneity (described later). GSLs are outer membrane leaflet residents that are synthesized in the Golgi in a tissue- and cell type-specific manner (561). All sphingolipids are highly saturated because of their ceramide backbone, and thus promote membrane rigidity.

1.2.3. Sterols

Cholesterol is the most abundant sterol (~20-40%) in animal plasma membranes and is generated *via* a complex biosynthetic pathway involving over thirty enzymes, including hydroxy-methylglutaryl-coenzyme A (HMG-CoA) reductase and squalene synthase (406, 561, 589). Cholesterol biosynthesis begins in the endoplasmic reticulum (ER) but it is predominantly found in abundance only at the plasma membrane. Endocytosis of surface-localized cholesterol results in its recycling back to the plasma membrane to maintain adequate cholesterol levels at that location. Cholesterol also has a propensity to migrate between bilayer leaflets and establishes equilibrium with approximately half the total cholesterol in each leaflet (386, 520). Cholesterol is a roughly planar molecule with a tetracyclic fused ring body and a small hydroxyl head group (406). This structure adds rigidity to membranes, reducing transmembrane permeability, inhibiting lateral protein movement and restricting lipid mobility (406, 561).

1.3. Membrane microdomains

The last fifteen years have seen a flurry of papers attempting to describe and define an unusual property of biological membranes. Lipids in bilayers have the capacity to adopt several states that are influenced by temperature and lipid packing. The liquid-disordered phase is typical of membranes containing unsaturated lipids that cannot pack tightly together. These lipids have more lateral mobility and must rearrange constantly to prevent solvent exposure of hydrophobic acyl chains (9). Since the majority of plasma membrane lipids have unsaturated acyl chains, the cell membrane is believed to adopt the disordered phase (331). Conversely, lipids with saturated acyl chains, including SM and GSLs, can form a liquid-ordered phase that is more rigid and less permeable (9, 434). The planar structure of cholesterol allows it to behave similarly (406, 607). When mixed with unsaturated lipids in a membrane, cholesterol and SM will associate directly with one another to form a microdomain with a liquid-ordered phase in a membrane predominantly disordered (501). GSLs and saturated lipids also prefer the ordered to the disordered environment and are incorporated into these ordered microdomains, referred to as lipid rafts (220, 434, 501). These interactions primarily occur in the outer leaflet of

the plasma membrane, where SM and GSLs reside, and it is unclear if lipid rafts are unilamellar or bilayer structures (218). It is noteworthy however, that inner leaflet lipids purported to be microdomain-associated include the less abundant phospholipid species such as PIPs, PE and PS, suggesting that microdomain formation may serve to accumulate these lipids in sufficient concentration for them to exert their biological activities (136, 347, 422). Thus, there may be inner and outer membrane rafts and/or rafts that span both layers. This hypothesis remains unproven and controversial (151, 218, 223, 387).

The identification of lipid rafts has been a difficult process, and it is only recently that techniques have been developed to confirm their existence in living cells at physiological temperatures. For years, there was, and still is, concern over the process by which rafts have been analyzed. The ordered structure of rafts renders them less sensitive to solubilization with cold, non-ionic detergents such as Triton X-100 (54, 56). Once extracted in this way, rafts can be isolated by centrifugation on a sucrose gradient and the lipid and protein content analyzed. Many proteins are now known to associate with these detergent-resistant membranes (DRMs) and localization to microdomains is correlated with the function of these proteins. An example of this is the regulation of γ-secretase enzymatic activity by its association with membrane microdomains (571). However, detergent extraction has come under criticism because results can vary greatly depending on the detergent used (144, 458, 479). Also, the effects of detergents on membranes are numerous and many reports of DRM association may be artefactual (223). Often DRM data are inconsistent with other methods, such as microscopy (387).

Various analytical approaches suggest membrane microdomains range in size from about 20 to 200 nm (129, 416, 422, 433, 565), requiring an array of sophisticated methodologies with nanometer resolution, such as fluorescence resonance energy transfer (FRET), two-photon microscopy, laser trapping and single particle tracking, to detect lipid rafts in live cells (189, 266, 275, 276, 422, 433, 511). The resolution of most optical microscopes is only about 200 nm; thus standard immunofluorescence imaging techniques that can identify "co-localization" between two molecules offer limited information about whether they reside in the same microdomain. Strong support for the existence of microdomains comes from microscopic imaging of rafts in live cells under

physiological conditions (200, 276). Current views on lipid rafts suggest that they, like the lipids that comprise them, are quite heterogeneous and dynamic (191, 423). The proposed biological function of these microdomains is to serve as platforms for recruiting and aggregating signalling and adhesion molecules, allowing them to better exert their cellular functions (reviewed in (198, 222, 265, 502). The influence of lipid rafts on membrane fusion is still unclear, but possibilities will be discussed later.

1.3.1. Lipid Summary

The historical view of biological membranes as a fluid mosaic of protein and lipid moving unhindered in a homogenous membrane (503) must be modified to account for the heterogeneity of lipid rafts and complex membrane dynamics of motile cells (156). The diversity and ratio of membrane lipids generates unique membrane compositions for each cell type and organelle. Thus, each biological membrane will have unique biophysical properties. These properties can greatly affect the fusion ability of a given membrane. Parameters such as membrane fluidity and thickness are highly variable, not only between membranes, but even within a given membrane in the form of membrane microdomains. The discussion thus far has described membranes as a heterogeneous sea of diverse lipids containing regions of increased order or disorder. However, biological membranes are never composed simply of lipids - they are surrounded and penetrated by proteins, which can compose about 60% of the mass of a plasma membrane (589). Therefore, in addition to lipids, integral and membrane-associated proteins can play a role in regulating membrane fusion.

1.4. Non-lipid membrane interacting components

To complete our picture of biological membranes, we must add the host of proteins that adorn a bilayer. Figure 1.1 shows the complexity of a membrane containing a variety of lipid, protein and carbohydrate components. There are many ways for proteins to associate with membranes. The most obvious, though probably not the simplest, is membrane-anchoring *via* insertion of one or more hydrophobic transmembrane domains. All known membrane fusion proteins fall into this category. Peripheral membrane proteins associate with a single leaflet of the bilayer by

amphipathic and/or electrostatic interactions. Proteins can also be anchored to membranes *via* modification with acyl chains (338). Common modifications include addition of a 14-carbon myristate or 16-carbon palmitate moiety (338, 508). Palmitoylation allows tighter membrane association than myristoylation due to the longer acyl chain length. As a result, protein myristoylation provides insufficient hydrophobic interactions to permanently anchor proteins to a membrane, and additional electrostatic interactions with anionic phospholipids are generally required for membrane association (356). Both palmitoylation and myristoylation have been implicated as raft localization signals and they can also be found on certain membrane fusion proteins. For example, the murine leukemia virus (MuLV) envelope protein (its fusion protein) requires palmitoylation for localization to membrane microdomains, but not for fusion activity (322). In addition, the influenza virus hemagglutinin (HA) fusion protein requires palmitoylation of its cytoplasmic domain for raft-association, fusion and coordination of virus assembly (467, 608). As well, a component of the cellular fusion machinery, soluble N-ethylmaleimide sensitive factor attachment protein (SNAP-25), is palmitoylated and required for fusion (208, 229). Proteins can also be modified with the isoprenoids farnesyl (15-carbon) or geranylgeranyl (20-carbon) (338). Like cholesterol, isoprenoid biosynthesis also uses the HMG-CoA reductase enzyme. Ras proteins, which are signalling and regulatory molecules, are palmitoylated and isoprenylated to enhance their membrane association and effectiveness as signal transductors (338). Other lipid-modified proteins include glycosylphosphatidylinositol (GPI)-linked proteins. GPI-linked proteins are covalently attached to phosphatidylinositol *via* an oligosaccharide and are thus anchored in the membrane (250). Many GPI-linked proteins, such as Thy-1 and prion protein, are raft-associated due to the saturated acyl chains on the GPI anchor (58).

In addition to the diversity of proteins found directly associated with the membrane are the host of other proteins surrounding the inner and outer membrane leaflets. Specialized proteins controlling cell signalling, adhesion and motility constantly modify the cytoplasmic facing leaflets of cellular membranes in response to various intracellular and extracellular events. For example, a lateral network of cortical actin lines the inner leaflet of the plasma membrane to strengthen the cell against osmotic pressure and shear stress as well as to assist in maintaining cell shape (586). Actin can

also assemble laterally and perpendicularly to the plasma membrane in response to cell-cell adhesion molecules, adding strength to the contacts and influencing cell shape and motility (154, 586). In addition, vesicle trafficking depends on protein scaffolds to alter membrane curvature and add vesicle stability (154, 402, 519). All of these processes can change the physical properties of a membrane, adding tension, stability or bending, which can influence the ability of that membrane to support fusion.

Similar complexity applies to the outside of the cell, where a combination of proteins and carbohydrates form structures such as the extracellular matrix (ECM) found between cells and the glycocalyx located on the lumenal side of endothelial and epithelial cells (reviewed in (261, 539, 540)). These structures vary greatly in their composition depending on their location and the cell type they are associated with, but serve similar functions, providing structural support and protection for the plasma membrane. In general, the structures found on the outside of cells present a physical barrier to membrane fusion by restricting the ability of membranes to come into close proximity.

From the above discussion, it is clear that biological membranes, especially plasma membranes, are more than just lipid bilayers. They should be viewed as networks of interconnected lipid, protein and carbohydrates that provide natural barriers to infection and offer strength and structural support to cells and organelles. All of the factors within a membrane and on either side of it help define what requirements must be satisfied to facilitate membrane fusion. However, despite the complexity of biological membranes, and the potential barriers to fusion, membrane merger is still a common occurrence.

1.5 Examples and instances of membrane fusion

Before describing how the membrane and membrane-associated components described above can influence membrane fusion, an introduction to the various physiological fusion events will be provided. There are essentially three main types of fusion reactions in nature: virus-cell fusion, cell-cell fusion, and vesicle-organelle fusion. Each of these categories includes many different events requiring unique fusion proteins. The details of the individual fusion proteins will be discussed later in this chapter.

Enveloped viruses must encode a membrane fusion protein to gain access to a host cell's interior. There are many species of enveloped viruses, each of which must breach the plasma membrane either at the cell surface or once internalized by endocytosis. Each viral fusion protein must therefore be able to compensate for the lipid composition of target membranes and the various structures associated with them. For example, fusion at the cell surface might be complicated by the presence of cortical actin networks, whereas fusion from an endocytic vesicle would encounter clathrin coat proteins. Alternatively, initiating fusion from an endosomal compartment could render the fusion reaction free of cytosolic membrane stabilizing factors. Thus, virus entry is subject to spatial and temporal constraints and an infecting virus must regulate the activation of its fusion protein. Viruses generally encode a single fusion protein capable of binding to host cells and facilitating the fusion reaction. In a few instances, as with herpesviruses, these functions are assigned to multiple proteins that function as a fusion complex (516).

Membrane fusion proteins responsible for vesicle trafficking, which involves the generation, transport and fusion of cargo-containing membrane vesicles to target organelles, are also well understood. The lipid composition of a vesicle is determined by the organelle it is derived from; thus vesicles at different points in a trafficking pathway can have dramatically different lipid compositions. For example, vesicles derived from the ER will have low cholesterol content, while plasma membrane-derived endocytic vesicles will be enriched in this sterol (561). Further, the origin and destination of a vesicle will determine the type of coat protein it carries. ER-Golgi transport uses coat protein complex II (COPII) proteins, Golgi-ER retrograde transport uses COPI-coated vesicles and Golgi-endosome, endocytosis, and Golgi-lysosome transport uses clathrin-coated vesicles and different adaptor protein complexes (AP1-AP3) (11, 329). Clathrin-coated transport vesicles are about 50 nm in diameter; thus fusion of these vesicles occurs between a highly curved vesicle and a relatively planar target membrane (329). Like virus-cell fusion, vesicle fusion is highly regulated. The cell must ensure that cargoes are targeted to the correct subcellular compartments, and in the case of neurotransmitter release, at the correct time. These intracellular trafficking fusion events are coordinated by a related set of proteins termed SNAREs (soluble N-ethylmaleimide-

sensitive factor attachment receptors) that serve as one component of a multi-protein fusion complex (555).

Cell-cell fusion includes the broadest category of types of fusion events, but is the least understood. In most cases, cell-cell fusion involves the merger of plasma membranes of opposed cells, leading to formation of multinucleated syncytia. The exception to this trend is fertilization where cell-cell fusion between a sperm and an ovum results in the production of a single cell with a full genome complement. Syncytium formation is an essential and common process in animal life. The syncytiotrophoblast layer that forms the maternal-foetal interface is a large syncytium a single cell layer thick (431). Skeletal muscle is formed by the fusion of myoblasts into long, parallel myotubes, which synchronizes muscle contraction and enhances the efficiency of signal transduction through the shared cytoplasm (150). Macrophages fuse to form sheet-like giant cells that encompass pathogens or differentiate into osteclasts and fuse during bone development (117, 574). The eye lens requires fusion for formation and maintenance to promote clarity (313, 490). In the worm *Caenorhabditis elegans*, about one third of all cells fuse during development (598). Note that all the physiological examples of syncytia form three-dimensional structures, such as sheets and narrow tubes, which maintain a large surface area to volume ratio that is required to maintain homeostasis. While all of these examples require the fusion of plasma membranes, the diversity of lipid content and surface protein profiles and the extracellular environment surrounding these cells make each fusion scenario unique. The proteins involved in these reactions have not been positively identified, but the candidate proteins will be described in detail later. However, one can see that cell-cell fusion must be tightly regulated. Fusion with the wrong partner or at the wrong time during development could have disastrous consequences for the organism.

It is clear that each membrane fusion reaction occurs between membranes with very different lipid and protein compositions, degrees of membrane curvature and various membrane-associated proteins. The degree to which these factors affect membrane fusion will be discussed next.

1.6. Factors affecting biological membrane fusion

The previous sections have introduced the complexity of biological membranes and the diversity of membrane fusion reactions. Each of the variables introduced thus far can enhance or hinder the activity of membrane fusion proteins. Biological membranes are inherently stable and resist fusion (62). The process of merging lipids from opposing bilayers into one bilayer forces membranes to adopt non-planar bilayer intermediates (104). These intermediate structures are energetically unfavourable, and thus all membrane fusion reactions require energy to overcome the thermodynamic barriers that prevent spontaneous membrane merger (104). However, the physical properties of a bilayer and the proteins associated with it can alter the energy needed for execution of membrane fusion.

1.6.1. Lipid shape and saturation

The size ratio of a lipid's head group to its tail group generates three basic lipid shapes that can affect membrane curvature, lipid packing and susceptibility to fusion (Figure 1.2) (reviewed in (62, 212, 614)). Cylindrical lipids are said to have neutral curvature, meaning they promote the establishment of planar bilayers. Phosphatidylcholine (PC), cholesterol and sphingomyelin (SM) fall into this category. Neutral curvature lipids do not dramatically affect membrane fusion. Phosphatidylserine (PS) and phosphatidylethanolamine (PE) have relatively small head groups compared to their acyl chains and thus adopt cone-shaped structures, with the bases of the cones in the interior of the membrane. In addition, highly unsaturated acyl chains on any glycerophospholipid would be highly kinked and increase the relative diameter of the acyl chain relative to the headgroup. The cone shape forces neighbouring lipids to bend around PS or PE, to align head groups and prevent exposure of hydrophobic surfaces to the aqueous environment. Lipid rearrangements to accommodate the cone shape promote bending of the membrane in the direction of the head groups and is said to induce negative curvature (i.e. concave relative to lipid head groups) (Figure 1.2). Negative curvature is favourable for fusion as it can support the non-bilayer structures that form as fusion intermediates. Conversely, inverted cone-shaped lipids with head groups larger than tail groups promote positive membrane curvature (convex relative to lipid head

groups) and are inhibitory to fusion (Figure 1.2). Removal of an acyl chain, *via* phospholipase activity, can produce lysolipids that contain inverted cone-like structures. Lyso-PC is a potent inhibitor of membrane fusion as it blocks formation of negatively curved fusion intermediates (211).

1.6.2. Membrane curvature and tension

On a larger scale, the curvature of the bilayer can also affect fusion. Planar bilayers are more stable than highly curved ones. Depending on the lipid composition, spherical vesicles in excess of 100 nm - 400 nm behave as if they are planar bilayers with respect to fusion, thus cell-cell fusion and most virus-cell fusion events can be interpreted as fusion between planar bilayers (561). However, intracellular trafficking vesicles, and some enveloped viruses, are only about 50 nm in diameter, necessitating fusion of curved membranes (561). There are essentially five ways to introduce curvature in a membrane (reviewed in (357)). First, lipid shape, as discussed above, can promote negative or positive membrane curvature (Figure 1.2). Similarly, the shape of a protein transmembrane domain or protein oligomerization can influence lipid packing to promote curvature. As well, actin-filled membrane protrusions can force the membrane to adopt non-planar conformations. Specialized proteins involved in vesicle trafficking, like clathrin, can force a bilayer into a highly curved vesicle. Finally, amphipathic helices or other peptide structures can interact with membranes to alter lipid packing, forcing the membrane to curve (24, 158, 166). Membrane curvature induced by the physical properties of lipids is a spontaneous process whereby the membrane attempts to prevent the energetically unfavourable exposure of its hydrophobic interior (614). Alternatively, planar membranes can be forced to adopt curved structures through the action of proteins and peptides. Membranes bent in this way are prone to fusion because it presents an opportunity to relieve the curvature strain and return the membrane to a planar state.

1.6.3. Electrostatic repulsion

Electrostatic effects have only a minor influence on membrane fusion. Electrostatic repulsion is generated by the interactions of lipid head groups with like

charges when they come within close proximity of one another. These repulsive forces only weakly resist membrane fusion (114, 521).

1.6.4. Van der Waals interations

Van der Waals forces exist at short distances (~1 nm) between the hydrophobic tail portions of membrane lipids. The application of stress on a membrane such that the hydrophobic interior is exposed generates an attractive force between hydrophobic structures that can enhance fusion (225, 421).

1.6.5. Hydration repulsion and surface tension

The surface of a membrane bilayer has a layer of water that adheres to the polar head groups of charged lipids. This hydration layer is a significant barrier to fusion when membranes get within 1-3 nm of each other, and the energy required to remove the water increases exponentially the closer membranes get (424). Agents that can dehydrate a membrane surface can promote fusion. Polyethylene glycol (PEG) is commonly employed to promote membrane merger through surface dehydration. This allows membranes to come into very close proximity (219, 601). Similarly, high-affinity binding of divalent cations can displace water interactions with polar head groups and bridge negatively charged lipids in opposing membranes to close the intermembrane distance and promote fusion (29, 81, 149).

Dehydration-mediated bridging between membranes also increases the surface tension of affected membranes. Bridging with divalent cations causes attractive forces perpendicular to the membrane to pull lipids out of the membrane, exposing hydrophobic regions to the solvent (29, 81). This increase in surface tension is fusion promoting. Osmotic stress can have the same effect and promote fusion of artificial membranes (421).

1.6.6. Membrane fluidity

The lateral packing of membrane lipids can also affect membrane fusion. The degree of acyl chain saturation influences membrane curvature as well as membrane fluidity (219, 315, 594). Increased fluidity might support fusion by lowering the energy

required to initiate fusion (594). In other words, a fluid membrane is more disordered and in a higher energy state; therefore the membrane is closer to the activation energy barrier required to initiate fusion, so less energy needs to be introduced to the system to promote fusion. Increased fluidity might facilitate the lipid rearrangements required to form non-bilayer intermediates during fusion. One would therefore predict that lipid rafts might be resistant to fusion based on their organized structure. Surprisingly, cholesterol, which promotes membrane stability and rigidity, frequently enhances fusion (439). Conversely, sphingomyelin (SM) can be inhibitory to fusion by promoting fusion pore flickering, a process whereby fusion pores open briefly then rapidly close (439). Cholesterol and SM are major constituents of the membrane microdomains that have been associated with several fusion proteins (439). How this association influences the outcome of various fusion reaction will be discussed later.

1.6.7. Membrane-associated proteins

The information presented about membrane proteins and fusion is only intended as a brief overview of the types of ways in which proteins can influence a fusion reaction and to remind the reader that physiological fusion always occurs in a complex environment with multiple lipid and protein factors that can influence the efficiency of fusion at a particular site.

The number and diversity of membrane-associated proteins precludes a detailed account of them here. However, a few examples will be mentioned to illustrate the point that there are several ways in which membrane-associated proteins can influence a fusion reaction. The cortical cytoskeleton that lines the cytoplasmic face of the plasma membrane can pose a barrier to membrane fusion as it limits vesicle access to the membrane and could prevent the expansion of a fusion pore (153). Evidence indicates that transient disassembly of cortical actin is necessary for exocytosis (153, 505). As well, the protein scaffolds that surround transport vesicles must be disassembled before fusion can occur (47).

On the exterior of the cell there are many physical barriers to membrane fusion. The extracellular matrix, glycocalyx or mucus layer that surrounds cells prevent the close contact of opposing cell membranes. Viruses that infect mucosal epithelia usually

express a neuraminadase protein on their surface to help penetrate the mucus layer (475). Similarly, matrix metalloproteinases (MMP) are necessary to break down the extracellular matrix in order to promote cell motility and cell-cell interactions (563). By removing the extracellular matrix to promote cell-cell contact, MMPs are removing a physical barrier to fusion, and can thus be considered fusion-promoting factors.

1.6.8. Summary of factors affecting fusion

The parameters described in this section are forces and influences that a fusion protein must work with or against in each fusion reaction. Each fusion event is unique and many variables will be present or absent from any given reaction. Ultimately, each fusion reaction is similar in that the end result is the same - the fusion of two bilayers into one. Thus an important question is raised: if fusion reactions need to deal with different parameters, are all fusion proteins different? In other words, is there only one way to promote membrane fusion, or are there as many mechanisms as there are fusion events? The answers to these questions are not straightforward, and as with many things in nature, the truth likely lies somewhere in the middle. It appears that some features of a fusion protein are conserved, while others are tailored to the specific fusion reaction they execute.

1.7. Membrane fusion is a five-step process

The study of a variety of fusion proteins suggests that despite the diversity of fusion machines, there are several elements common to all fusion systems, which indicates that the fusion reaction may proceed *via* a universal multi-step process (22, 104, 318, 509). Although there is no consensus on how to divide the multiple steps of the fusion reaction, it will be described here as a five-step process (Figure 1.3).

1.7.1 Binding

The first step in the fusion process is binding, since membranes cannot fuse unless they are near each other. This process is essential to most physiological fusion reactions as it is the step that confers specificity to the fusion reaction and ensures fusion of the correct membranes. Binding refers to the bridging of opposing membranes, usually

from ~10 nm - 300 nm apart, with a protein tether (280, 528). Binding can be accomplished by the fusion protein itself or by accessory proteins (121, 280, 516, 528). For many fusion proteins, receptor binding can serve as a trigger for the fusion reaction. For others, it merely docks the two membranes in anticipation of some other activating trigger. The binding step can be inhibited by membrane-associated factors such as a mucus layer, glycocalyx or extracellular matrix on the exterior of the cell or by cortical actin networks on the interior of the cell (376, 475, 563).

1.7.2. Close membrane apposition

Prior to the actual merger of opposing membranes, they must first be brought into very close proximity, with less than 1-2 nm separating the opposing membranes. At this distance, the hydration layer is a significant repulsive force and traditional models for membrane fusion hold that energy is needed to overcome its fusion-inhibitory effects. In protein-free systems, this step of the fusion reaction can be accomplished using PEG to eliminate water from the intermembrane space, promoting membrane contact over a large area. In most protein-mediated membrane fusion systems, mechanical force is required to pull membranes into close proximity and/or contact (22, 555). Dehydration of the water layer between membranes, whatever the cause, can result in the formation of a fusion stalk (Figure 1.3A). In the more recent modified stalk structure (Figure 1.3B), lipids bend and tilt to produce a point-like contact between two membranes (104). This structure minimizes membrane curvature, exposure of the hydrophobic membrane interior and the area of headgroup contact, resulting in a relatively low-energy method for membranes to contact (104). Stalk formation is only one of the predicted models for membrane contact, but is important to protein-mediated membrane fusion because it presents a plausible structure from which subsequent fusion steps can proceed.

1.7.3. Hemifusion

The next two steps of the fusion reaction, hemifusion and pore formation, have been difficult to dissect and may not be able to be uncoupled. Hemifusion refers to the stage of the fusion reaction where lipids in the contacting (or outer) membrane leaflets mix but the inner membranes remain intact. The current modified stalk model for

hemifusion initiation suggest that progression from stalk formation to hemifusion may not require as much energy as previously thought (104, 226, 613). For most fusion proteins, hemifusion directly follows close membrane apposition with conformational rearrangements supplying the energy for both steps where needed (92, 360).

Hemifusion initiates at a point and is predicted to widen to form a hemifusion diaphragm with larger areas of inner leaflet membranes interacting through their tail groups (293, 315, 341). Both the hemifusion point and the hemifusion diaphragm structures are predicted to be unstable because of the forced membrane curvature and potential for void spaces (104). Lipids that promote negative curvature could stabilize the hemifusion structures and promote fusion, while lipids that promote positive curvature could inhibit fusion.

1.7.4. Pore formation

Hemifused membranes must quickly transition back to separate bilayers or continue on to form a fusion pore. The fusion pore refers to the actual fusion of two bilayers into one with an aqueous channel connecting the previously isolated compartments. Transition from hemifusion to the fusion pore is a critical step and also requires energy input. Models for the formation of a fusion pore are varied, but generally it is believed that a fusion pore might develop directly from the point of hemifusion without the formation of a hemifusion diaphragm (22, 91, 104). Other models suggest that pore formation is the result of the rupturing of the expanding hemifusion diaphragm (93, 293, 315). The energy to facilitate pore formation is also supplied from the mechanical energy released during the activated fusion protein's conformational reorganization (104, 360, 366).

1.7.5. Pore Expansion

The final step of the fusion reaction is pore expansion, which broadens the aqueous channel and completes the merger of the two membranes. Without rapid expansion, newly developed fusion pores will close in a process known as flickering. Recent studies describe this as the most energy-demanding step in the fusion reaction

(104). Membrane factors such as cholesterol and sphingomyelin are reported to affect fusion pore growth by stabilizing the fusion pore and resisting flickering (439).

1.7.6. Summary

It is important to note that the current models for membrane fusion described above are biased heavily toward the activity of fusion proteins from enveloped viruses and the cellular vesicle trafficking machinery. All of these fusion proteins appear to rely on mechanical energy released from conformational rearrangements to drive the fusion reaction. Little is known about cell-cell fusion, which may use a fundamentally different mechanism for mediating fusion. Thus, the universality of the above fusion steps is not guaranteed. However, biophysical studies with non-protein-mediated fusion suggest that it is most likely that the steps of fusion are universal but the process of proceeding through them may be variable (314).

1.8. Defining a fusion protein

As will be described in the following sections, the proteins involved in membrane fusion reactions comprise a diverse group, many of which function as multifunctional proteins or in multi-protein complexes that perform multiple functions. For our purposes, it is important to define what the terms "fusion protein" and "fusion machine" mean. A fusion machine is a protein or the minimal components of a protein complex that is necessary and sufficient to mediate all of the above five fusion steps. As will be described, regulatory elements or external triggers, such as low pH, often control fusion. A fusion protein is the individual component of a fusion machine that is necessary and sufficient to cause membrane merger once the triggering requirement has been satisfied. Alternatively, multiple proteins may be required to execute lipid mixing, in which case no one component is a fusion protein *per se*, but they act as "core fusion machinery" with the primary purpose of causing membrane merger. These distinctions are necessary to avoid the classification of receptor-binding and other regulatory proteins as fusion proteins. While these components are often integral and necessary parts of a fusion machine, they are generally not directly involved in the latter steps of the fusion reaction. Thus, a membrane fusion protein can be an individual protein that also functions as a

fusion machine, or it may be part of a multicomponent fusion machine (i.e. a fusion complex) that executes the lipid rearrangements during fusion. The importance of these distinctions in the nature of the fusion machinery will become evident as we examine the features of various fusion machines and compare them to the FAST proteins later.

1.9. Influenza HA and Class I fusion proteins

1.9.1 Influenza HA

The entry process of enveloped viruses is the best understood fusion reaction. Most viral fusion proteins can be classified as class I or class II fusion proteins. Class I proteins make up the largest group with the influenza virus hemagglutinin (HA) and the human immunodeficiency virus (HIV) gp41 proteins being the best-characterized fusion proteins (30, 185, 507). It is with HA that we will begin our discussion of membrane fusion proteins.

Influenza HA (Figure 1.4) is a large single-pass transmembrane glycoprotein consisting of a ~560 aa polypeptide that is cleaved to form HA_1 (~320 aa) and HA_2 (~220 aa) subunits linked together with a disulphide bond (507). HA monomers assemble into trimers prior to incorporation into the virion surface, where they extend about 10-13 nm from the membrane surface (596). The HA_1 subunit forms a globular head and is the receptor-binding domain of HA. The binding step of membrane fusion is accomplished by HA_1 interactions with cell surface sialic acid, thus docking the virion particle about 10 nm from the plasma membrane (7, 437, 507).

The HA_2 domain is the fusion domain and consists of predominately amphipathic α-helical structures with a hydrophobic N terminus (~20 aa) buried in the centre of the trimer, away from the aqueous environment (Figure 1.4A arrow) (87, 593). Virions bound to the cell surface are endocytosed where the low pH of the endosome triggers conformational rearrangements in HA to initiate the fusion reaction. Although estimates vary, at least three HA trimers are required for fusion initiation (28, 120, 365). Low pH alters the arrangement of the α-helices in the HA_2 domain causing them to adopt an extended conformation (61, 596). This conformational reorganization exposes the previously buried N-terminal hydrophobic domain, termed the fusion peptide. The sequence of events that follows fusion-peptide exposure is uncertain, but what is clear is

that the hydrophobic fusion peptide rapidly embeds itself in the nearest hydrophobic environment (258). This could be the donor membrane containing the HA protein, the target membrane bound by the HA_1 subunit, or both (Figure 1.5). Models for HA-mediated fusion have been proposed for each of these scenarios and will be discussed shortly. For the moment, the traditional model of target-membrane insertion will be discussed. The HA_1 domain is pushed aside during these rearrangements to allow the HA_2 domain to bridge the 10 nm between the donor and target membranes (280, 507). Thus, HA2 is now anchored in the donor membrane with its transmembrane domain and in the target membrane with the fusion peptide.

Exposure and embedding of the fusion peptide is immediately followed by further structural remodelling to form what is referred to as the six-helix bundle (27, 69, 485). The three extended α-helical rods of each HA trimer (one rod per monomer) jackknife back on themselves forming the six-helix bundle (Figures 1.4B and 1.5). Bundle formation pulls the donor and target membrane into close proximity (1-2 nm) with enough force to supply the energy for hemifusion and pore formation (22, 61). In addition, modification with palmitoylation in the transmembrane and cytoplasmic domains may assist in the transition from hemifusion to pore formation (467). It is likely the formation of the six-helix bundle also supplies the energy for pore expansion with the cooperation of the transmembrane and cytoplasmic domains (22, 104). This model is referred to as the "cast and retrieve" model, and is only one of several models for HA-mediated fusion (Figure 1.5).

An alternative model for HA-mediated fusion is referred to as the dimple theory, which postulates that the HA fusion peptides embed only in the donor membrane (Figure 1.5) (292). This model was proposed because in the absence of target membranes, HA activation leads to donor membrane insertion of the fusion peptide (584). Membrane insertion of the fusion peptides is predicted to occur before the α-helical extension or helix bundle formation. As the protein attempts to complete its structural remodelling with its fusion peptide anchored in the donor membrane, the membrane is pried out of plane to form a dimple under high curvature strain (292). The energy in the dimple is released into the target membrane to promote fusion to relieve the strain. A modification of the dimple theory is the hydrophobic defect model, which suggests the prying of the

donor membrane pulls lipids from the membrane, exposing the hydrophobic interior (27). This hydrophobic defect can be corrected by the addition of lipids from the nearby target membrane, thus promoting lipid mixing and fusion (27).

A final model for HA-mediated fusion suggests that the fusion peptides insert into both the donor and target membranes (Figure 1.5) to cause fusion by a mechanism incorporating elements from the above theories. Fusion peptides inserted in the target membrane allow membranes to be mechanically pulled together by HA refolding while the insertion of the fusion peptide in the donor membrane creates membrane deformities that increase lipid disorder and promote fusion (258, 521).

Another point in need of discussion is how fusion proteins like HA cause the transition from hemifusion to pore formation. This topic remains a matter of much debate, but the pertinent issues will be briefly discussed now. The central issue is whether the hemifusion intermediate allows lipid mobility. Traditional models for membrane fusion describe the formation of a hemifusion diaphragm that subsequently ruptures to form a fusion pore (93, 293, 315). With this model, hemifusion allows the free diffusion of lipids between the hemifused donor and target membranes, but prevents exchange between aqueous compartments until the hemifusion diaphragm is ruptured. This model is referred to as unrestricted hemifusion since lipid movement is not constrained. Detection of lipid mixing using fluorescent probes is a common assay to identify whether hemifusion has occurred (274, 374). In contrast, the restricted hemifusion model suggests that unrestricted hemifusion is not a component of the fusion reaction, but represents a dead-end pathway for fusion (91). Instead, the proteins that are mediating the fusion reaction pack tightly around the point of hemifusion preventing lipid exchange until after a fusion pore has formed and expanded (91). With this model, a hemifusion diaphragm does not form and the transition from hemifusion to pore formation is closely coupled (374).

Regardless of the precise mechanism, all models for HA-mediated fusion describe the mechanical energy released during structural remodelling as necessary for overcoming the thermodynamic barriers to fusion (22, 104, 258). Thus, HA is held in a metastable state prior to activation with low pH, which triggers the irreversible changes that drive fusion (69). The close membrane apposition, hemifusion and pore formation

steps, and probably pore expansion as well, are all connected and dependent on the conformational changes triggered by low pH (104). That is, they occur in succession. Most models for protein-mediated membrane fusion suggest that membranes are forced to fuse through structural refolding of the fusion proteins, which supply sufficient energy to overcome the activation barrier that prevents spontaneous lipid mixing (69, 258). It is only recently that the idea of fusion protein-mediated membrane destabilization has been introduced as part of HA fusion models (157, 301). It is becoming increasingly accepted that membrane fusion may occur through the combined efforts of membrane destabilization to lower the fusion activation barrier and mechanical force to overcome that barrier (301).

1.9.2. Retrovirus gp160

Other Class I fusion proteins contain similar structural elements as HA and include proteins from the *Orthomyxoviridae*, *Retroviridae*, *Paramyxoviridae*, *Filoviridae* and *Coronaviridae* families. The human immunodeficiency virus (HIV) gp160 protein, encoded by the *env* gene, serves as the receptor-binding and membrane fusion protein for HIV (599). Like HA, gp160 functions as homotrimers in which each monomer is proteolytically cleaved to form two non-covalently associated subunits, one for receptor binding (gp120) and one for fusion (gp41) (291). However, unlike HA, HIV entry occurs at the plasma membrane and is not pH-triggered. Instead, fusion is regulated and triggered by the multivalent binding of gp120 to its receptor and co-receptors, which activates conformational rearrangements in gp41 that lead to membrane merger (185). The primary receptor for HIV is CD4 on macrophages and T-cells (119). The conformational changes in gp120 mediated by receptor-binding prime gp120 for interactions with the co-receptor, CXCR4 or CCR5 (188, 299, 392). Conformational changes resulting from co-receptor engagement are translated to gp41, activating the fusion reaction and facilitating virus entry (185, 392). Since fusion does not require low pH as a trigger, expression of gp120/gp41 on the surface of infected cells can also mediate cell-cell fusion if neighbouring cells express the ligands for gp120 (499).

Another interesting feature of HIV is its ability to incorporate cellular adhesion molecules such as CD44 and intercellular adhesion molecule-1 (ICAM-1) into the viral

envelope, and the ability of gp120 to bind dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) on dendritic cells, increases the pathogenicity of the virus by increasing its ability to interact with target cells (19, 235, 298, 553). However, despite the advantage of multiple binding strategies, viral entry only occurs when triggered conformational rearrangements in gp120 activate the gp41 fusion subunit.

The fusion reaction is generally believed to proceed similar to that of HA, since the gp41 subunit contains a hydrophobic fusion peptide and α-helical domains (185). The reorganization of these helical domains into a six-helix bundle pulls membranes into close proximity and provides the energy required to drive membrane fusion (344). Thus, once again, the close apposition, hemifusion and pore forming steps of the fusion reaction are closely coupled and dependent on energy released from triggered, irreversible conformational changes.

One notable difference between gp41 and HA is the presence of a long cytoplasmic tail. HA, and most other viral fusion proteins, have relatively short cytoplasmic tails (20-40 aa) while gp41 has about 150 aa (78). The function of this long tail is generally believed to coordinate virus assembly through interaction with the structural protein, gag (78). However, isolates of HIV and simian immunodeficiency virus (SIV) containing truncations in the cytoplasmic domain show enhanced infectivity and fusogenicity (78, 236, 466). These observations suggest that the length of the cytoplasmic tail might influence fusion efficiency.

1.9.3. Paramyxovirus F proteins

Different members of the *Paramyxoviridae* family encode slightly different fusion proteins. Unlike HA or gp160, the receptor-binding and fusion elements are encoded by separate genes for individual transmembrane proteins. However, the fusion protein, F, is synthesized as a F_0 precursor and proteolytically cleaved to form F_1 and a short F_2 subunit (88). The F_1 portion is similar to the HA_2 or gp41 subunits of influenza or HIV and contains α-helical bundles, is arranged in trimers that extend about 12 nm from the membrane, and contains an N-terminal hydrophobic fusion peptide (88, 304, 461). Interestingly, some reports suggest F may have a second, internal fusion peptide as

well (415). Unlike HA, F protein-mediated fusion is not triggered by low pH, but likely results from direct interactions with the associated receptor-binding protein (209, 462, 536). This is similar to the situation with the pH-independent HIV gp160 protein, except binding and fusion functions are assigned to separate, but interacting, transmembrane proteins. The attachment protein varies depending on the species of paramyxovirus with most paramyxoviruses expressing hemagglutinin/neuraminadase (HN), while measles virus carries hemagglutinin (H) (304). Nipah, Hendra and respiratory syncytial virus (RSV) express a glycoprotein (G) polypeptide along with their F proteins (304). Engagement of the attachment protein with its receptors likely induces conformational changes that then activate the F protein which proceeds to mediate the close apposition, hemifusion, pore formation and expansion steps in a manner similar to HA or gp41. As with HIV, paramyxoviruses can also cause cell-cell fusion when their fusion machinery is expressed on the infected cell's surface (21, 227).

Paramyxovirus-mediated fusion is a good example of the need to define what constitutes a fusion protein. The HN/H/G component of the paramyxovirus fusion machinery has never been implicated in mediating lipid rearrangements, and its only function appears to be activating F in response to receptor binding. Under certain circumstances, such as natural mutation and experimental manipulation, F protein can cause fusion in the absence of HN/H/G, or in response to low pH, indicating that it should be classified as the fusion protein within the paramyxovirus fusion machine (304, 382, 461, 481, 482). It is suggested that F might bind cellular glycosaminoglycans and/or heparin sulphate to mediate attachment to cells (213, 382, 541). It should be noted however, that natural F protein-mediated fusion in the absence of HN/H/G is very inefficient. Thus, binding still serves to trigger fusion, which is a theme common among viral fusion protein.

1.9.4. Other Class I fusion proteins

Most other class I fusion proteins behave similarly to those described above. They share the same structural elements of a hydrophobic fusion peptide, buried in a trimeric structure that is exposed upon activation (low pH or receptor-binding) and inserts into the target membrane. Structural remodelling of α-helical bundles provides

sufficient energy, released from a metastable precursor state, to drive fusion peptide insertion and membrane fusion. Other class I fusion proteins include filovirus gp2, baculovirus gp46, arenavirus glycoprotein and the unusually long (~20 nm) cornavirus spike protein (41, 159, 550, 583, 590, 600).

1.9.5. Summary

All of the above examples require membrane binding as a prerequisite for triggered fusion. This is a common theme among viral fusion proteins (Table 1.1) because fusion must be tightly regulated. Since the conformational changes associated with fusion are irreversible, activating fusion at the wrong time or place could render the virion uninfectious. Thus, receptor-binding and membrane fusion functions need to be coupled to either the same multifunctional protein or to separate proteins that specifically interact with one another to regulate the fusion event.

1.10. Class II fusion proteins

Class II fusion proteins represent the other major class of viral fusion proteins and are encoded by the *Togaviridae* and *Flaviviridae* families (280). These small enveloped RNA viruses have a unique virion structure, since the ordered assembly of their envelope (E) proteins adopt an icosahedral structure reminiscent of non-enveloped virus capsids (108). Unlike other viral envelop proteins, the E proteins of togaviruses and flaviviruses do not protrude perpendicular to the membrane to form spikes, but lie parallel to the virion surface (Figure 1.4A) (196, 224, 378). Togaviruses encode two E proteins (E1 and E2) that are generated from one polyprotein whereas flaviviruses produce only one major envelope protein (E) that is synthesized and folded with its regulatory protein, prM (279). Like other viral fusion proteins, E proteins function as multimers. A togavirus particle contains 80 E1/E2 heterodimers whereas a flavivirus particle contains 90 E protein homodimers (279).

The E1 and E proteins are predominantly β-sheet in structure and contain three globular domains (224). Domain I contains the N terminus and is connected to domain II by a flexible hinge region (224). Doman II is formed by β-strands and contains an internal fusion peptide loop (Figure 1.4A, arrows) (8, 280). Domain II connects to

domain III, which houses the transmembrane anchor (224). E proteins are about 12 nm wide but extend only 5 nm from the virion surface (280). However, after the structural remodelling that promotes fusion, E proteins extend almost 10 nm from the membrane surface (Figure 1.4B) (280, 460).

Because these two families contain many members, their receptor usage for binding to target cells varies and is not always well characterized. However, domain III of the E protein is generally implicated as the receptor-binding domain and in the case of dengue virus it mediates binding to DC-SIGN (98, 379, 385, 425, 544). Depending on the length of the cellular molecule serving as the virus receptor, E proteins could anchor the viral membrane about 5 - 25 nm from the cell surface.

Receptor-mediated endocytosis internalizes the virus where low pH activates fusion (523). Entry of togaviruses and flaviviruses is dependent on cholesterol in the target membrane, as portions of the E protein interact directly with this sterol (195, 281, 524). This requirement is more stringent for the togaviruses, which also require low concentrations of sphingolipids (397). For both virus families, it does not appear that the ability of cholesterol to enhance fusion is related to its ability to form membrane microdomains, but rather is dependent on the presence of the 3β-hydroxyl headgroup of cholesterol (579).

For togaviruses, low-pH activation causes E1 to be released from the E1-E2 dimer, exposing the fusion peptide loop on domain II of E1 so it can insert into the target membrane (279). The E1 proteins also homotrimerize and 5-6 trimers are required to initiate membrane fusion (196). The hinge region between domains I and II allows the protein to form a hairpin, similar to the jackknifing step in Class I fusion (280). The E1 homotrimers adopt a rod-like conformation, extending perpendicular to the membrane, allowing bridging between the donor and target membrane at distances of about 10 nm (196). Flavivirus fusion proceeds in a similar manner, with low pH causing dimer-to-trimer rearrangements in the E protein resulting in hairpin formation and fusion peptide insertion in the target membrane (378). Similar to those for Class I fusion proteins, these triggered conformational rearrangements supply the energy required to execute the various steps of the fusion reaction (224, 280). Recent evidence suggests that the

Hantavirus (family *Bunyaviridae*) Gc glycoprotein may also function as a Class II fusion protein, based on sequence similarities and 3D modelling (545).

Class II fusion proteins differ greatly from Class I proteins in their structural organization, being composed of β-sheets rather than α-helices. However, both classes of fusion proteins rely on groups of mutimeric fusion proteins undergoing triggered conformational changes to promote membrane merger (Table 1.1). Other fusion proteins isolated from enveloped viruses are believed to retain similar dependence on structural plasticity to overcome the energy barriers preventing spontaneous membrane fusion.

1.11. Unclassified enveloped virus fusion proteins

1.11.1. Vesicular stomatitis virus G protein

One of the more interesting fusion proteins is the G glycoprotein encoded by the *Rhabdoviridae* family member, vesicular stomatitis virus (VSV). VSV-G is the only viral envelope protein and resembles Class I fusion proteins in that it is a trimeric spike protein ~500 aa long that extends perpendicular to the virion surface (118, 141). It is both the receptor-binding and fusion protein for the virus. The VSV-G protein is structurally distinct from other Class I fusion proteins because it lacks α-helical bundles and contains an internal fusion peptide motif that is not particularly hydrophobic (603).

Although the precise receptor for VSV-G is elusive, it likely binds to cellular PS through electrostatic interactions to anchor the viral membrane to the cell surface (66, 67, 107, 118, 472). Given that PS concentrations in the outer leaflet of the plasma membrane are generally quite low, researchers believe that other receptors must exist (106). Interestingly, the G protein from the related salmonid rhabdovirus can actually induce PS externalization upon binding (161). Thus, the rhabdoviruses may promote the expression of their own receptor (107). This process would have the added advantage of PS-induced negative curvature on the outer leaflet of the plasma membrane, which could promote fusion by lowering the energy required to form the highly curved hemifusion intermediate. Although unproven, this idea would indicate that lowering the thermodynamic barrier to fusion might be an equally viable option as overcoming it with mechanical force during protein-mediated fusion.

With regard to the fusion reaction, low pH (~6.3) causes histidine protonation and structural changes in VSV-G leading to stabilization of G protein trimers, insertion of the internal fusion peptide motif into the target membrane and ultimately membrane fusion (68, 141, 435, 603). Interestingly, in some circumstances VSV-G may acquire a fusion-active conformation during transport to the cell surface and can induce cell-cell fusion (447). The moderate hydrophobicity of the fusion peptide motif results in reversible insertion in the target membrane (175, 609). Once again, conformational rearrangements are believed to provide sufficient energy to drive fusion; however, as noted above, this energy barrier may be lowered by the potential PS-externalizing activities of G. Finally, unlike Class I and Class II fusion proteins, the conformational changes induced by low pH are completely reversible suggesting that the pre-fusion conformation of VSV-G is not in a metastable high-energy state (603). The lack of metastability might indicate that VSV-G releases less energy during its conformational changes, but perhaps more importantly, it suggests that fusion can proceed without the need for metastable fusion machinery (603). Ultimately, VSV-G research suffers from a lack of structural information about the pre-fusion and post-fusion protein conformations. In contrast, structural information has served to greatly advance our understanding of Class I and Class II fusion proteins.

1.11.2. Herpesvirus glycoproteins

The *Herpesviridae* family of viruses are large DNA viruses and encode about 11 surface glycoproteins and several non-glycosylated ones (361). For the prototype herpesvirus, Herpes simplex virus (HSV), attachment and membrane fusion requires the concerted efforts of four different proteins (gD, gB, gH and gL) (516). With the exception of gL, the surface glycoproteins are spike proteins anchored to the viral envelope with a single transmembrane domain (263, 361). *Herpesviridae* family members display a wide range of tissue tropism, and in the case of HSV attachment is mediated by the gD protein, which binds to nectins (a class of cellular adhesion molecules), herpesvirus entry mediator molecules (HVEMs), and heparin sulphate (102, 515). Receptor engagement by gD initiates the fusion process by triggering gD conformational changes and recruits gB, gH and gL (516). The HSV glycoproteins form

many homotypic and heterotypic interactions. For example, gH and gL form heterodimers (248) while gB forms homodimers as well as heteromultimers with gD and other herpesvirus glycoproteins (100, 309, 516). The gL protein lacks a transmembrane domain but its association with gH anchors it to the membrane (248). Fusion is believed to proceed through translation of conformational rearrangements by interacting gB, gD, gH and gL proteins although the exact mechanism is unclear (516). The gB and gL glycoproteins generally lack putative hydrophobic fusion peptides and do not appear to contain the α-helical or β-sheet structures found in Class I or Class II fusion proteins. However, gH contains a putative internal fusion peptide in a disulphide bond-stabilized loop and several potential α-helical regions (193, 194, 333). Similar to observations with the HIV gp160 protein, truncation of the cytoplasmic tail of gB causes enhancement of cell-cell fusion, suggesting that this region may regulate or inhibit syncytium formation (173). In addition, two other viral gene products, UL20p and gK, work with gB to induce the syncytial phenotype but are not required for virus entry (361), indicating that these two fusion reactions (virus-cell and cell-cell) may proceed through two different mechanisms. Herpesvirus-mediated fusion, whether it is cell-cell or virus-cell, is a complex, multi-factor process that requires significantly more experimentation to elucidate its precise mechanism(s).

1.11.3. Vaccinia virus A27L

The fusion protein for the pox family of viruses has not been conclusively identified. The task is complicated because of the complex structure and replication cycle of these viruses. They can exist in two forms, the extracellular enveloped virus (EEV) and the intracellular mature virus (IMV) (383). IMV particles contain a single envelope while EEV particles have a double membrane. IMV's enter cells by fusion with the plasma membrane, while EEV's enter by endocytosis (383). In both cases, the putative fusion protein is the 14 kDa A27L and is proposed to be functional at both neutral and acidic pH (204, 569). Although small in size, A27L contains a short coiled-coil domain and a potential fusion peptide; however, it lacks a transmembrane anchor, and so associates with the viral envelope through A17L (450, 568, 569, 580). Mechanistic data are lacking for A27L but it is unlikely to fit within the confines of Class I or Class II

fusion models. The large coding capacity of poxviruses makes it likely that several proteins may be involved in fusion, much like the herpesviruses.

1.11.4. Hepadnavirus L

Hepatitis B virus encodes many processing variants of its surface antigen (HBsAg), encoded by a single gene (109, 305). The long (L) variant is the putative fusion protein and contains as many as four transmembrane domains (109). In addition, and highly unusual, L protein places an N-terminal myristoylation moiety on the outside of the cell (419). L protein is activated by low pH to undergo conformational changes from a metastable pre-fusion state. Reminiscent of the Class I fusion proteins, the N terminus of L protein contains classical fusion motifs such as a hydrophobic fusion peptide and coiled-coil domains (109, 451-453).

1.11.5. Summary

Although these unclassified fusion proteins are poorly understood compared to Class I or Class II fusion proteins, enough is known to reveal that certain attributes are common amongst all the fusion systems described thus far. Many of the unclassified fusion proteins lack the structural elements common to the classical fusion proteins but experimental evidence suggests that they may still function similarly. In all of the above examples, fusion is a triggered event that induces structural reorganization in the fusion protein or fusion complex in a process that is predicted to supply sufficient energy to promote membrane merger. However, with the VSV-G protein we begin to see indications of another fusion strategy. It may employ a two-pronged approach whereby limited energy is supplied by conformational changes from a non-metastable state, but membrane remodelling (e.g. PS externalization) may compensate for the decrease energy release by lowering the activation barrier that prevents spontaneous fusion. Taking this path of reasoning to its logical conclusion, one is left with the question of whether protein-mediated fusion can be accomplished, not by forcing membranes to merge, but by making membrane merger more favourable. This would be a similar approach as divalent cation- or PEG-mediated fusion where causing electrostatic bridging and dehydration of the cell surface promotes lipid mixing.

At this point in our discussion of membrane fusion proteins it is important to introduce and define some terms that will reappear throughout this thesis. All of the fusion proteins identified thus far require a trigger to initiate fusion. In addition, all of the fusion reactions mediated by these proteins are spatially and temporally regulated to ensure fusion only occurs at the correct time and place. Regulation is achieved by the receptor-binding component of the fusion machinery. This binding element can be a domain on the fusion protein (e.g. VSV-G), an associated subunit of a cleaved precursor (e.g. HA_1) or a separate, but interacting protein (e.g. paramyxovirus G/HN/N proteins). Association with the binding component ensures that the fusion component is in proximity to a membrane when triggered. Fusion triggers are generally either receptor/co-receptor binding or low pH (Table 1.1) but always result in a conformational change in the fusion component that is responsible for lipid mixing. For example, HA is regulated by the specific binding of the HA_1 subunit to cell surface sialic acid, which promotes endocytosis of the virus where the low pH of the endosome triggers fusion to the target membrane. Similarly, the receptor-binding proteins of paramyxoviruses and herpesviruses regulate the fusion reaction through interactions with specific cell receptors. Conformational changes in the binding proteins trigger the fusion components, resulting in execution of the fusion reaction at the plasma membrane. Thus, regardless of the exact composition of the fusion machinery, virus-cell fusion is a triggered event regulated by receptor binding. This is an important point to remember since the FAST proteins may represent an exception to this rule.

1.12. Cellular SNARE proteins

The cellular proteins responsible for fusion of transport vesicles to their correct subcellular compartments share some notable similarities, and some fundamental differences, to the Class I fusion proteins. The SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins form the core of the cellular vesicular-transport fusion machinery (47). Like virus entry, vesicle fusion must be tightly regulated to ensure the correct vesicle is fusing at the right time to the proper target membrane. Coordination and execution of vesicle fusion involves the concerted efforts of four types of proteins: tethering factors to promote targeting, Rab-GTPases to establish

the fusion site, SNAREs to execute the fusion reaction and N-ethylmaleimide-sensitive factor (NSF) and cofactors to activate and recycle the fusion machinery (555). Thus, a more elaborate multi-component fusion complex mediates intracellular fusion than those described for paramyxovirus or herpesvirus fusion machinery.

Asssembly of the SNARE fusion complex begins with targeting and tethering of a vesicle to a target membrane, satisfying the initial binding step of the fusion reaction. Many different tethering proteins have been identified in vesicle trafficking with roles varying from tethering together organelles to recruiting transport vesicles (reviewed in (59, 199)). Tethers are generally long coiled-coil proteins which would anchor vesicles to membranes at distances between 50 nm and 300 nm (199, 528). This is far too great a distance for fusion initiation so, once tethered, a vesicle must be brought into closer contact through the activity of Rab GTPases (84). Different Rab proteins interact with a subset of molecular tethers and SNAREs to confer regulation and specificity to the fusion reaction, ensuring that vesicles are directed to the correct subcellular compartment. For example, ER - Golgi transport is regulated by the consecutive binding of COPII-coated vesicles to giantin (a tether), which binds to p115 (another tether), which interacts with Rab1-GTP, which binds to SNAREs (84, 199, 384). Other branches of the vesicular transport pathway would use a different subset of tethers, Rab GTPases and SNAREs. It should be noted as well that during the process of tethering and docking, but prior to interaction with Rab proteins, the vesicle coat must be disassembled to expose the highly curved and fusion prone ~50 nm vesicle membrane prior to SNARE engagement (47).

Once the two membranes are in close enough proximity, the SNARE proteins can begin to exert their effects. This is where another fundamental difference between the fusion reactions of the SNARE proteins and the Class I fusion proteins becomes evident. SNAREs function in pairs, with vesicle SNAREs (v-SNAREs) anchored in the vesicle membrane and a target SNAREs (t-SNAREs) attached to the target membrane (585). The variety of t-SNAREs and v-SNAREs helps to ensure that only the correct subtypes can pair in *trans* to further ensure the specificity of the fusion event (554). Structurally, SNAREs are similar to the Class I fusion proteins. They contain α-helical regions that, once paired, form coiled coils that very closely resemble those of the Class I fusion proteins (Figure 1.6A) (526). One main difference from class I fusion proteins is that

SNAREs form four-helix bundles instead of six-helix bindles, with one helix typically supplied by the v-SNARE and three helices contributed by the t-SNAREs (526). Recent reports suggest that 5-8 SNARE complexes may be required for fusion (216)

One of the best-studied examples of SNARE fusion is the process of synaptic vesicle exocytosis involving the v-SNARE synaptobrevin and two t-SNARES, syntaxin and SNAP-25 (Figure 1.6) (59, 360). Synaptobrevin and syntaxin are transmembrane anchored proteins that contribute one helix each to the SNARE complex (526). SNAP-25 is a 25 kDa protein that is anchored to the plasma membrane by palmitoylation and contributes the other two helices to the four-helix bundle (208, 229, 526, 577). When the opposing SNAREs are brought into proximity (~12 nm) they self-assemble into the four-helix bundle, pulling the vesicle and target membrane into close proximity (1-2 nm) and triggering fusion (Figure 1.6B) (441, 528, 554). Completion of the fusion reaction requires that at least one SNARE in the vesicle and plasma membrane have a membrane-spanning transmembrane domain, probably to promote pore formation and expansion through application of tension on the membrane (360). This is an interesting point, because it suggests that the various steps of the fusion reaction do not necessarily have to be linked.

After fusion, the SNARE proteins form a *cis* complex with all three components in the same membrane. Unlike Class I fusion proteins, SNARE proteins can be recycled and used in subsequent fusion reactions (47). Recycling requires the energy-dependent disassembly of the *cis* complex by NSF and SNAP (555).

In addition to the core machinery described above, several other cellular factors, often specific to the particular fusion event in question, can regulate and participate in the fusion reaction. The presence of these factors has sparked debate and generated confusion about what the core fusion machinery of vesicle trafficking actually is. Experiments using SNARE proteins reconstituted into liposomes demonstrate that the SNARE proteins alone are both necessary and sufficient to cause membrane fusion (335, 585). In addition, expression of SNAREs on the cell surface suffices to cause cell-cell fusion, although somewhat inefficiently (242). Thus, the SNARE proteins can be considered the minimal components necessary for fusion; however, whether these proteins function similarly during vesicle fusion is unclear (138). Two specific examples,

yeast vacuolar fusion and Ca^{2+}-triggered exocytosis, indicate that other proteins are required for SNARE protein-mediated fusion *in vivo*.

In yeast vacuolar transport, fusion cannot occur without the association of vacuolar ATPase V_0 subunits with the SNARE fusion complex (442, 612). It is suggested that V_0 may act as the actual fusion machinery, while SNAREs are only responsible for providing specificity and membrane apposition to the fusion reaction (25, 420). However, V_0 has not met the same burden of proof to be considered a fusion protein as have SNAREs.

With respect to Ca^{2+}-triggered exocytosis, it appears that SNAREs can form four-helix bundles without causing fusion, resulting in docking and possibly hemifusion of vesicles to plasma membranes (138, 441). Full fusion is instead triggered by calcium sensors (e.g. synaptotagmin 1) and associated molecules (e.g. complexins) that interact directly with the SNARE complex (35, 49, 90, 555). Although the presence of these SNARE-associated proteins is required for fusion, it does not necessarily follow that they are fusion proteins, or that SNAREs are not. What the identification of these associated factors reveals is that SNARE-mediated fusion is highly regulated. The presence of SNARE-associated factors may alter the normal fusion-inducing activity of the SNARE proteins in favour of tighter regulation, as with the critical event of neurotransmitter release. In other words, SNARE assembly into four-helix bundles is sufficient to cause fusion unless cellular factors alter this activity.

In many ways vesicle trafficking is much like virus entry. Both systems require tight control over when and where fusion occurs and use α-helical rearrangements to drive the fusion reaction. However, vesicle fusion requires the assembly of a multifactor fusion complex with different components working together to mediate the individual steps of the fusion reaction. The cellular system is more complex, but a cell has more types of fusion events to coordinate and the genome capacity to support a more complex system. To gain finer control of the fusion reaction, the cell may have introduced new gene products to halt fusion at different stages such as the docked, and possibly hemifused, synaptic vesicles. This concept is not unlike the use of competitive peptide inhibitors that bind to Class I fusion proteins and prevent the completion of conformational rearrangements necessary for fusion (363, 443, 457). One might predict

that the fusion machinery responsible for cell-cell fusion would be subject to similar regulation and complexity.

1.13. Cell-cell fusion

Finding the fusion proteins responsible for cell-cell fusion is a difficult undertaking. It seems that the larger the genome, the more complicated the fusion system. Large DNA viruses like herpesvirus encode multiple proteins required for fusion. Similarly, cellular vesicle trafficking machinery requires the assembly of multiprotein complexes with SNAREs at their core. Cell-cell fusion may also require multiple proteins, which may lack the structural elements common to Class I or Class II fusion proteins, further complicating the search. However, several candidate cell-cell fusion proteins have been identified and are discussed below.

1.13.1. Fertilization

Human life begins with membrane fusion when a sperm cell fuses to an egg. This is no easy task, as the sperm must penetrate the multiple protective layers surrounding the egg before it can even reach the membrane (537). Once a sperm successfully gains access to the egg plasma membrane, it must initiate membrane binding before fusion can proceed. This is accomplished through expression of fertilins, members of the ADAM (a disintegrin and metalloproteinases) family of proteins that bind to integrin targets on the egg plasma membrane (537). There was some early excitement over the potential for fertilin proteins to be the sperm-egg fusion proteins. Fertilin-α and fertilin-β form a heterodimer with fertilin-β implicated in the binding of sperm to egg surface $\alpha_6\beta_1$ integrins (96, 537). Fertilin-α has an amphipathic α-helical region that resembles a fusion peptide, so it was postulated that binding and fusion could be accomplished by the fertilin complex (375). However, subsequent work revealed that fertilin-α was not necessary for sperm-egg fusion (537).

More recently, CD9 has been implicated as the fusion protein required for fertilization (311, 372). CD9 is expressed on the egg surface and is a member of the tetraspanin protein family and thus contains four transmembrane domains and lacks α-helical bundles or a fusion peptide (86, 537). Mice deficient in CD9 are normal for

sperm-egg binding but fusion does not proceed (311, 375). The role of CD9 in fertilization is unclear but a recent report suggests that it may coordinate $\alpha_6\beta_1$ integrin and CD151 (another tetraspanin) organization on the egg surface as an essential step in the fusion reaction (615). Since $\alpha_6\beta_1$ is a receptor for ADAMs, and CD9 may be involved in fusion by regulating adhesion, the ADAM family of proteins, and the egg-associated integrins and tetraspanins, may have larger roles in the actual fusion reaction. In addition, a variety of poorly-characterized sperm-associated proteins including DE, equatorin and Izumo have also been implicated in the process of sperm-egg fusion (103, 252, 548).

Despite the number of proteins implicated in sperm-egg fusion, none can be positively identified as fusion proteins. The gene deletion studies that are offered to support claims of fusion activity could merely indicate the absence of a regulatory element responsible for activation of the actual fusion protein. Ideally, candidate fusion proteins should be able to cause fusion of pure lipid bilayers, or at least cause fusion in heterologous cell types. It appears that sperm-egg fusion results from a complex cell-cell adhesion system and involves the concerted efforts of many proteins. Dissecting out which molecules are performing the individual steps of the fusion reaction is a challenge awaiting future research.

1.13.2. The syncytiotrophoblast

In mammals, after fertilization and embedding of the embryo in the uterine wall, the maternal-foetal interface must be established in the placenta. This barrier is formed during implantation by the fusion of cytotrophoblasts and maintained through term by incorporating new cytotrophoblasts periodically (244, 431). In primates, the fusion process involves a protein called syncytin (368). Syncytin is a Class I fusion protein and is virtually identical to the human endogenous retrovirus *env* protein (203). While it is quite common for viruses to acquire host cell genes, it appears as though syncytin is a representative example of the rare occurrence of a host organism acquiring a viral gene (368). Other proteins must also be involved in fusion of placenta trophoblasts since syncytin is only found in primates, but all mammals must undergo this process. However, no other candidate fusion proteins have been identified.

1.13.2. Muscle development

Skeletal muscle is composed of long multi-nucleated syncytia formed when myoblasts fuse to form myotubes. There are two main areas of research that contribute to our understanding of this process, but as with other cell-cell fusion events, the proteins responsible for myotube formation are not well characterized.

The first area of research is in Drosophila development. One group of immunoglobulin domain-containing proteins that are implicated in the fusion reaction include dumbfounded (Duf), roughest (Rst), sticks and stones (Sns) and hibris (Hbs) (reviewed in (150)). These proteins all contain immunoglobin-like domains, lack hydrophobic fusion peptides and the structural components to form hairpins, and are most likely responsible for adhesion and/or priming of the fusion reaction (86, 150). In addition, several signalling molecules have been implicated in Drosophila fusion as well (150). Most of these proteins are involved in cytoskeleton remodelling, which may enhance fusion by facilitating membrane adhesion, or more directly influence it by promoting membrane curvature strain or altering lipid packing (345, 597). The second area of research that describes myotube formation comes from studies in mice and mouse cell lines. Several signalling factors are implicated in the fusion process, which include adhesion molecules (60, 83, 234), transcription factors (33), and GTPases (65, 73, 83, 184). None of the implicated proteins bare resemblance to Class I or Class II fusion proteins.

Although the actual fusion proteins for myotube formation have not been identified, research in this area has yielded some interesting observations. Myoblast fusion requires the transient activation of apoptotic signalling cascades to cause PS externalization (38, 245, 246, 431, 438, 557). This observation has been made for syncytiotrophoblast formation as well (123). The externalization of PS could facilitate membrane merger by promoting negative membrane curvature in the outer leaflet of the plasma membrane. Similar to the role suggested for VSV-G, a combination of PS externalization and energy-releasing fusion proteins might be able to successfully accomplish fusion. Combined with the possible role of cytoskeleton remodelling to alter membrane curvature or apply mechanical stress, cellular fusion proteins may not require hydrophobic fusion peptides or hairpin-forming structures to mediate fusion (86, 597).

Thus, the search for cell-cell fusion proteins is complicated because there is no basis for narrowing down the search parameters.

1.13.4. Lens development

The eye lens continually incorporates new cells at its outer edges by cell-cell fusion (313, 490). Interestingly, the lens "syncytium" is devoid of nuclei, organelles and *de novo* protein synthesis, but is maintained by gap junction communication channels and recruiting 'fresh' cells to the lens (313, 490). No proteins have been implicated in this fusion process.

1.13.5. Macrophage fusion

Macrophages are unusual in that they are capable of fusing to form different types of syncytia. Depending on the differentiation signals they receive, they can produce osteoclasts for bone resorption or giant cells in chronic inflammatory reactions (574). Fusion occurs through a process called cellocytosis, which involves one macrophage entirely engulfing the other, much like a large endocytic event (reviewed in (574)). Fusion between the engulfed cell and the surrounding "cellosome" releases the nuclear and cytoplasmic contents to the devouring cell producing a syncytium. Fusion involves the expression of macrophage fusion receptor (MFR), CD44 and CD47, three immunoglobulin-superfamily proteins, on opposing membranes (117, 131, 215, 465). Binding can generate intermembrane distances of between 5 nm and 10 nm (574), which is not likely to be close enough to allow membrane merger without the efforts of some other fusion factor. Once again, however, the proteins that execute the actual steps of the fusion reaction have not been identified.

Another interesting aspect of macrophage fusion is that it is preceded by extensive interdigitation of the plasma membranes (574). This is thought to enhance cell-cell adhesion to support fusion (574), but it is also possible that the interdigitations could be composed of actin-filled protrusions, which could promote fusion by altering membrane curvature and increasing lipid strain (597).

1.13.6. *C. elegans* and EFF-1

Over one third of the cells in the nematode *C. elegans* undergo cell-cell fusion during development (86, 598). There is compelling evidence that expression of the epithelial fusion failure-1 (EFF-1) protein is responsible for the majority of fusion events (133, 489). EFF-1 is a single-pass transmembrane protein that lacks coiled-coil motifs, but contains a hydrophobic domain that may be involved in assembly of the fusion complex rather than acting as a fusion peptide (133, 287, 488). In addition, the hydrophobic domain may have phospholipase (PLA_2) activity (488). Recent reports demonstrate that EFF-1 is both necessary and sufficient to induce fusion of nematode cells through a mechanism that requires the hydrophobic domain and may involve homotypic pairing of molecules on opposing cells, similar to SNAREs (133, 489). It has been postulated that the PLA_2 activity of EFF-1 might contribute to fusion by digesting membrane phospholipids (133). However, subsequent analysis revealed that the PLA_2 active site is not essential for EFF-1 function (133). It is doubtful that EFF-1 would employ such a strategy anyway, since the activity of PLA_2 would generate lysolipids (405) and promote positive membrane curvature, which should be inhibitory to fusion.

Although EFF-1 appears responsible for causing fusion in most nematode cells, it has not been tested for the ability to fuse cells from different organisms or pure lipid bilayers in a liposome reconstitution fusion assay. Thus, while EFF-1 clearly plays a role in fusion it cannot as yet be definitively classified as a fusion protein. It is still possible that some other fusion factor in nematode cells is activated by the over expression of EFF-1.

1.13.7. Summary

Essentially, very little is known about the cellular proteins that mediate cell-cell fusion. Most factors implicated in cell-cell fusion are adhesion molecules that more likely regulate fusion rather than execute actual lipid mixing. The lack of candidate fusion proteins with hydrophobic fusion peptides and hairpin-forming structures could indicate that the cellular fusion proteins have not been identified, or may suggest that cell-cell fusion proteins utilize a fundamentally different fusion mechanism than do viruses or SNAREs. The involvement of PS externalization and cytoskeleton remodelling

in certain cell-cell fusion systems supports the latter case and suggests that the cell may prime itself for a unique type of fusion reaction. Clearly, cell-cell fusion is a highly regulated and complex process involving many proteins, from adhesion and signalling molecules to the fusion proteins themselves. Dissecting these complex reactions and characterizing the proteins involved is currently a daunting task.

1.14 The fusion-associated small transmembrane (FAST) proteins

1.14.1. Overview

Recent years have witnessed the discovery and initial characterization of a novel class of membrane fusion proteins encoded by the non-enveloped reoviruses. While these proteins are efficient mediators of cell-cell fusion, they do not resemble the classical fusion proteins or candidate cell-cell fusion proteins. Instead, these fusion-associated small transmembrane (FAST) proteins appear to have developed unique ways to execute the various steps of the membrane fusion reaction and may significantly contribute to our understanding of these processes.

1.14.2. Reoviruses

The *Reoviridae* family is composed of non-enveloped viruses with double- or triple-layered capsids that house 10-12 segments of double-stranded RNA (262). Mammalian reovirus (MRV) is the prototype member of the *Orthoreovirus* genus (Figure 1.7) (262). MRV has been isolated from a wide variety of mammalian hosts but natural infections are associated with little to no disease symptoms (reviewed in (262)). MRV typically enters a host cell by receptor-mediated endocytosis, where the low pH of the endosome triggers the entry process (167). Conformational rearrangements in the viral coat proteins cause the exposure of amphipathic α-helical domains that are presumed to insert in the endosomal membrane (396, 500). The virus particle uses these components to penetrate the membrane, leaving the α-helical coat proteins behind and delivering a partially disassembled capsid to the cytosol (79, 395, 500). Thus, reoviruses are unusual in that they never fully uncoat. Because reoviruses are non-enveloped, their entry strategy does not include, or require, a membrane fusion protein.

1.14.3. Fusogenic reoviruses

Since reoviruses do not utilize membrane fusion during entry, it is unusual that members within several genera of the *Reoviridae* family induce cell-cell fusion and multinucleated-syncytium formation in infected cells. These are the only known examples of non-enveloped viruses that induce cell-cell fusion. In the case of rotaviruses and orbiviruses, this activity is attributed to the membrane-destabilizing properties of the outer capsid proteins (165, 172, 190, 197, 543, 578). These same capsid proteins are also involved in virus entry; thus fusion seems to be a consequence of membrane disruption during virus entry, rather than the primary function of these proteins. An example of this is rotavirus entry at the plasma membrane involving the activity of the capsid proteins VP4 and VP7 (137). These capsid proteins have membrane permeabilizing activity and induce syncytium formation by destabilizing the membrane at a point of cell-cell contact during the entry process (137, 143, 165, 197). This process is referred to as "fusion from without."

Another genus of the *Reoviridae* family also induces cell-cell fusion in infected animals, but this ability has been mapped to the expression of a viral gene encoding a non-structural membrane-anchored fusion protein that induces "fusion from within." Several species of *Orthoreoviruses*, isolated from a variety of host organisms, encode FAST proteins that possess the ability to cause syncytium formation (Figure 1.7) (111, 126, 494). The FAST proteins bear little resemblance to the much larger fusion proteins from enveloped viruses and represent a novel class of membrane fusion proteins.

1.14.4. Avian reovirus and Nelson Bay virus p10 FAST proteins

Avian reoviruses (ARV) were first identified over fifty years ago and have since been identified as the causative agents of cell-cell fusion in infected cells (164). Several different avian reoviruses have been isolated from numerous poultry species including geese, chickens, ducks and turkeys (18, 146, 237, 411, 480, 514, 610). These viruses can cause severe disease in infected animals resulting in respiratory and enteric problems, arthritis and death (132, 264, 411, 459, 514, 559). In addition, an unusual mammalian reovirus (Nelson Bay virus) isolated in the early 1970's was found to induce cell-cell

fusion (186, 187, 592). Although isolated from a flying fox, Nelson Bay virus (NBV) is genetically more related to the avian reoviruses rather than mammalian reovirus (146).

Fusogenicity was mapped to the S1 genome segments of ARV and NBV, which encode tricistronic mRNAs containing three partially overlapping open reading frames (ORF) (497). The first ORF encodes a small 10 kDa transmembrane protein referred to as ARV-p10 (98 aa) or NBV-p10 (95 aa) (494). Evidence that the p10 proteins are the viral fusion proteins comes from transfection studies that demonstrate p10 is the only viral gene product required to cause extensive cell-cell fusion (494). In addition, the p10 proteins can cause fusion in heterologous cell types, and mutations to the ARV-p10 protein abolish fusion activity, providing strong evidence that p10 proteins are the reovirus fusion proteins (493-496). Further, p10 expression is only required in the donor (i.e. fusion protein-containing) membrane of opposing cells, indicating that a SNARE-like *trans*-pairing is not a part of the p10 protein fusion mechanism (495).

The p10 proteins from the different ARV isolates are highly conserved and differ by only four amino acid substitutions, three of which are conservative (494). In contrast, ARV-p10 and NBV-p10 share many structural properties but are only 33% identical in amino acid sequence (494). The p10 proteins are single-pass transmembrane proteins that adopt a type I topology, placing their N terminus outside the cell and their C terminus in the cytoplasm (Figure 1.8) (494, 517). The ectodomains of ARV-p10 and NBV-p10 are 44 and 40 amino acids in length respectively, placing as much of the protein inside the cell as outside (494). This is in stark contrast to other viral fusion proteins that have large ectodomains and short cytoplasmic tails. The ARV-p10 ectodomain contains two cysteines at positions 9 and 21 that are necessary for function since cysteine-to-serine mutations at one or both sites abolishes fusion (495). These cysteines are not involved in intermolecular bonding or multimerization, but may intramolecularly bond to form a disulphide-stabilized ectodomain loop (495). The two cysteines flank a region of increased hydrophobicity from amino acid positions 9 to 24 which is reminiscent of the putative internal fusion peptide of herpesvirus gH (495). The features described below are explained in the context of ARV-p10, on which most experimentation has been conducted, but NBV-p10 contains the same domain organization and may function similarly.

The p10 hydrophobic patch (HP) is not as hydrophobic as a fusion peptide from a Class I or Class II fusion protein, and does not contain the same structural elements (e.g. glycine rich, amphipathic α-helix, bulky aromatics on one face) (494, 495, 506). However, even slight alterations to the hydrophobicity of this domain have dramatic effects on fusion activity (explained below). An ARV-p10 ectodomain peptide is able to induce lipid mixing in liposome-to-liposome fusion assays, supporting the hypothesis that the hydrophobic domain is directly involved in the fusion reaction (495). The p10 ectodomain also contains a region of nine amino acids absolutely conserved between ARV and NBV (494). Point mutations altering this area result in loss of fusion, but the function of this region is unknown (495). It could be a multimerization or protein-interaction motif, or it may be structurally relevant to the fusion reaction.

The ARV-p10 transmembrane domain spans amino acids 44 to 62 and contains three adjacent glycines at positions 49-51 that are also found in the NBV-p10 transmembrane domain (496). The presence of glycines in a transmembrane domain is unusual, but most viral fusion proteins contain a higher proportion of transmembrane domain glycines than do non-fusion proteins (101, 221, 377). In the case of VSV-G, transmembrane domain glycines are required for fusion activity (101). Because glycines are helix breakers, they are proposed to provide flexibility to the transmembrane domain to accommodate the torsional strains and highly curved membrane intermediates generated during fusion (101). Similar to VSV-G, point mutations affecting one or all of the ARV-p10 glycines do not affect protein expression or topology, but decrease or abolishe fusion activity, suggesting that this motif may play a role in the fusion reaction (496).

The endodomain of p10 contains two conserved cysteines adjacent to the transmembrane domain that are both modified with a 16-carbon palmitic acid acyl chain (496). This modification is necessary for function since changing one or both of the cysteines to serine prevents palmitoylation and membrane fusion (496). Similarly, palmitoylation of HA was reported to be necessary for progression from hemifusion to pore formation (467). In contrast, however, the fusion protein from MuLV mentioned earlier requires palmitoylation for microdomain association but not fusion activity (322). Other fusion proteins, like SNAP-25, are modified by acylation, but the role of acylation

in fusion is unclear (577). The endodomain also contains a membrane-proximal polybasic region with five positively charged residues between positions 67 and 79 (496). Elimination of just one of these charges ablates fusion activity without affecting protein expression or topology (496). The role of the basic domain is unknown, but it could be required for determining protein topology or raft association, or be mechanistically involved in the fusion reaction.

A final point worth noting about ARV-p10 is the sensitivity of the extracellular hydrophobic patch to mutation (493). Point mutations that decrease the hydrophobicity slow or eliminate fusion with a concomitant increase in protein stability (493). Combined with the observation that an ectodomain-deleted ARV-p10 is also quite stably expressed, it was concluded that the ARV-p10 ectodomain targets the protein for rapid degradation (493). In support of this idea, inhibition of proteasome-mediated protein degradation enhances ARV-p10-mediated fusion (493). However, the inability of the stable, but less hydrophobic, mutants to cause fusion suggest that ARV-p10 has struck a balance such that the protein is hydrophobic enough to cause fusion, but not so much that it is completely targeted for degradation. In addition, the ARV-p10 gene has a suboptimal translation initiation sequence; thus the rate of ARV-p10-mediated fusion might be regulated by poor translation and protein degradation and might indicate a need for the virus to control the rate of syncytium formation (497).

1.14.5. Baboon reovirus p15

Baboon reovirus (BRV) was isolated from baboon brain homogenates during an outbreak of meningoencephalomyelitis in a baboon colony in San Antonio, Texas (317). BRV is an orthoreovirus and is capable of inducing cell-cell fusion and syncytium formation in infected cells (146). A 15 kDa protein (BRV-p15) encoded by the S4 genome segment is the only viral protein required to promote cell-cell fusion in transfected cells and is a novel member of the FAST protein family (128). BRV-p15 shares no sequence and only limited structural similarities with the p10 proteins (128, 494).

BRV-p15 is a 140 aa type I transmembrane protein with a short ectodomain (20 aa) containing a polyproline region (PPAPPP) and an N-terminal myristoylation

sequence (Figure 1.8) (127). The addition of the 14-carbon myristic acid moiety is essential for the fusion activity of BRV-p15, although its absence does not alter expression or prevent the correct topological insertion of the protein in the membrane (127). It is highly unusual for the myristic acid modification to be located outside the cell, but interestingly, this is a feature shared with the hepatitis B surface antigen (419). The proline-rich domain of BRV-p15 is required for fusion activity, and it has been suggested that the flexibility of this region might allow the N-terminal myristate moiety to reversibly insert into both the donor and target membranes as part of the fusion reaction (125).

The transmembrane domain of BRV-p15 is the longest of all the FAST proteins and could influence its plasma membrane localization. The membrane is generally thicker at regions of raft formation, and transmembrane-domain length can be a determinant of raft association (355). Preliminary results suggest that BRV-p15 may associate with detergent-resistant membranes (E. Clancy, unpublished results). BRV-p15 also contains two glycines in its transmembrane domain separated by five amino acids, but the necessity of these residues for the fusion reaction has not been assessed.

Like those of the p10 proteins, the endodomain of BRV-p15 contains a membrane-proximal polybasic region. However, with BRV-p15 this region contains 8 basic residues over a 13-residue stretch (125). BRV-p15 tolerates mutations to its basic domain better than ARV-p10 does, with point mutations having limited or no effect on the fusion potential of BRV-p15 (125). However, gross disruption of the basic domain with large insertions or deletions abolishes fusion activity (125). Thus, there may be a threshold requirement for basic charges necessary for FAST protein-mediated fusion.

Unique to BRV-p15 is the presence of an intracellular hydrophobic region (127). This region is the most hydrophobic of all the FAST protein hydrophobic patches, is essential for fusion activity, but does not serve as a transmembrane domain (127). The C-terminal tail of BRV-p15 can tolerate truncations and retain function with deletions of up to 43 amino acids (125). However, intermediate levels of fusion are observed with progressive size of deletions, suggesting that the length of the C terminus can influence fusion efficiency (127).

1.14.6. Reptilian reovirus p14

Reptilian reoviruses (RRV) have been isolated from snakes, iguanas and lizards displaying respiratory or nervous system disorders (4, 39, 306, 573). RRV encodes a 14 kDa FAST protein on the bicistronic S1 genome segment. RRV-p14 is a 125 aa type I transmembrane protein with a 38 aa N-terminal ectodomain and a 61 aa endodomain (Figure 1.8) (111).

Like BRV-p15, RRV-p14 is N-terminally myristoylated and the modification is required for fusion activity (111, 112). The ectodomain also contains a hydrophobic patch (residues 7-21), slightly more hydrophobic than the equivalent p10 hydrophobic patches (111, 494). As with the other FAST proteins, the hydrophobic patch is necessary for function (112). Structural and mutational analysis has led to a greater understanding of the RRV-p14 ectodomain than any other FAST protein. NMR spectroscopy revealed that the hydrophobic patch forms the centre of a loop that may be anchored and stabilized by the transmembrane domain and myristic acid moiety (112). This structure might be functionally analogous to the ARV-p10 hydrophobic patch nested in a potentially disulfide-stabilized loop. Structural plasticity in the remainder of the RRV-p14 ectodomain may provide flexibility to the hydrophobic patch and myristic acid moiety, allowing them to mediate membrane interactions necessary to execute the fusion reaction (112). The presence of both the hydrophobic domain and myristic acid modification on a peptide containing the 30 N-terminal amino acids of RRV-p14 are required to induce lipid mixing when added exogenously to liposome preparations (112). These observations demonstrate the ability of the RRV-p14 ectodomain to interact with, and reorganize, lipid bilayers as part of the fusion process.

The transmembrane anchor of RRV-p14 ranges from amino acids 37 to 58 and contains two glycine residues at positions 42 and 53 (111). Thus, the presence of transmembrane domain glycines is a feature common to all of the FAST proteins. Mutational analysis has not been conducted to determine whether these residues are essential for fusion activity in RRV-p14.

Like those of other FAST proteins, the endodomain of RRV-p14 contains a characteristic polybasic region adjacent to the transmembrane domain consisting of 6 basic residues over a 7 residue stretch (111). Alternatively, this region could be

interpreted as including 10 basic residues over a 22 residue stretch, making it the largest FAST protein basic region (111). The importance of the basic region for fusion activity has not been directly assessed by mutagenic studies. However, C-terminal truncations that infiltrate the basic region result in a loss of fusion activity (110). It is unclear if this is due to the loss of positively charged amino acid side chains or to the decreased length of the protein, since similar C-terminal truncations that do not excise the basic domain are also fusion-deficient (111).

Also contained in the RRV-p14 endodomain is a polyproline domain that is not essential for fusion activity. C-terminal deletions that lack this portion of the protein retain fusion activity (111). It should be noted however, that these truncation mutants only fuse about 20-40% as well as the wild-type protein (111), perhaps indicating that the polyproline region may enhance RRV-p14-mediated fusion even if it is not absolutely required. This domain could potentially interact with cellular factors containing domains that bind proline-rich motifs (e.g. SH3 domains), many of which are involved in membrane dynamics, cell signalling and cytoskeleton alteration (238).

Studies using RRV-p14 provide the best evidence that the FAST proteins are *bona fide* fusion proteins. Liposomes containing purified RRV-p14 can fuse to target cells and to non-RRV-p14-containing liposomes, confirming that RRV-p14 is both necessary and sufficient to mediate membrane fusion and that this process only requires expression of the fusogen in the donor membrane (547). These experiments also exclude homotypic SNARE-like *trans* pairing as a model for RRV-p14-mediated fusion initiation. Few other fusion proteins can claim the same level of experimental proof to support their classification as such.

1.14.7. Commonalities among the FAST proteins

Although the FAST proteins vary dramatically in sequence, there are some common elements shared by each protein (Figure 1.8). FAST proteins are characterized by being small (10-15 kDa) single-pass transmembrane proteins that contain a membrane-proximal polybasic region, a hydrophobic patch and glycines in the transmembrane domain. The FAST proteins are also modified by acylation, either palmitoylation or myristoylation, and may contain a polyproline region. Each FAST

protein has a signature arrangement of these shared structural elements, suggesting that the FAST proteins may be modular fusion proteins. Structurally, the FAST proteins bear little resemblance to other identified fusion proteins and lack the structural complexity to undergo the dramatic triggered conformational rearrangements that drive membrane merger in other systems. While the mechanism of FAST protein-mediated fusion remains to be elucidated, it is likely to be quite different from the paradigms of SNAREs or the Class I and II fusion proteins.

The FAST protein ectodomains are predicted to form a loop that is stabilized by the myristic acid modification on RRV-p14 and BRV-p15, or by disulphide bonds in ARV-p10 and NBV-p10. Hydrophobic elements consisting of myristoylation and/or a stretch of hydrophobic amino acids may serve as membrane-interacting motifs that could alter lipid packing, promote lipid mixing, dehydrate the water layer or alter membrane curvature. Any or all of these activities could directly contribute to membrane fusion by decreasing the energy required for fusion initiation. The flexibility afforded by proline-rich domains (BRV-p15) or unstructured ectodomain elements (other FAST proteins) may accommodate the dynamic movement of the ectodomain hydrophobic elements during the fusion reaction. Thus, the FAST proteins may not have to force membranes together with energy-releasing conformational changes but rather coax membranes to fuse by creating disorder in the bilayer. This is in stark contrast to classical fusion proteins (HA, SNAREs) that depend on mechanical force to drive fusion. Thus, while the FAST proteins may be both necessary and sufficient to cause membrane fusion, they might be better classified as membrane-destabilizing proteins whereby fusion is merely a consequence of their membrane-disruptive properties. The question of whether fusion is the primary and specific function of the FAST proteins is addressed in the following chapters.

Another issue that cannot currently be resolved is how the FAST proteins can mediate contact with the target membrane. With ectodomains of 44 aa or less, containing little structure or sequence homology, it is difficult to see how the FAST proteins could extend far enough to specifically interact with a target membrane. Thus, the FAST proteins might be incapable of mediating membrane binding as the first step of the fusion reaction. Since most fusion proteins require binding as a prerequisite for fusion, this

seems a fundamental difference between the FAST proteins and other fusion proteins. Further, other fusion proteins use conformational changes to bring membranes to within 1-2 nm where hemifusion and pore formation can proceed. The FAST proteins also lack the structural elements to mediate membrane pulling, so even if they can bind a target membrane, it is unclear how they could get it to the 1-2 nm distance needed for membrane fusion to proceed.

1.14.8. Fusion correlates with pathogenicity

The previous sections have addressed how the FAST proteins might function to mediate membrane merger, but have ignored the question of why the non-enveloped reoviruses would encode proteins with fusion capability. As mentioned above, the non-fusogenic MRV causes little to no disease symptoms in its natural hosts. In contrast, the fusogenic reoviruses have been isolated because of the moderate to severe disease symptoms they cause in infected organisms. These symptoms range from severe flu-like symptoms to encephalopathies and death (317, 459, 514, 559). It should be noted that populations of reovirus hosts are seropositive for antibodies against fusogenic reoviruses but have no history of disease (264). This observation suggests that the periodic increased pathogenicity of the fusogenic reoviruses might be due to unfavourable conditions, such as population overcrowding in a zoo pen (BRV) or chicken barn (ARV), that tip the balance between infection and host defence in favour of the virus. However, the presence of severe disease in fusogenic reovirus-infected animals is the first indication that the ability to cause membrane fusion correlates with increased viral pathogenicity.

All of the research directed at addressing the role fusion plays in the reovirus replication cycle comes from work with the avian reoviruses. Two important observations arise from these studies. First, fusion activity is not required for the successful replication of the virus (145). This was demonstrated using the fungal metabolite brefeldin A (BFA) to prevent ER-Golgi transport, thus preventing surface expression of ARV-p10 during infection, resulting in fusion inhibition (145). At high multiplicities of infection (MOI = 10), BFA treatment had no effect on the production of progeny virions, but did significantly delay virus release, suggesting that syncytium

formation enhances virus release (145). BFA treatment did not influence the replication or release of the non-fusogenic MRV (145). These observations indicate that fusion may enhance virus release, and possibly pathogenicity as a consequence, but it is not essential for successful virus replication.

The second important observation to come from the study of ARV is that the rate of syncytium formation correlates with pathogenicity. Two ARV isolates, ARV-176 and ARV-138, display different abilities to cause syncytium formation in infected cell monolayers (147). ARV-176 induces fusion faster and more extensively than ARV-138, but both viruses replicate to equal titres at high MOI infections (147). Compared to MRV, ARV infections result in cell death and virus release by 20 h post-infection, while MRV remains cell-associated for four days. Further, ARV-176, which produces large syncytia, is more pathogenic to chicken embryos than ARV-138 (147). This pathogenicity is mapped to the p10-encoding S1 genome segment as ARV-138 reassortants containing the S1 genome segment from ARV-176 induce larger syncytia and exhibit increased pathogenicity (147). However, the S1 genome segment also encodes the σC cell-attachment protein, which could account for the difference in pathogenicity between ARV-176 and ARV-138 if different σC proteins affected infectivity (497). Two possible roles for how fusion can enhance pathogenicity include facilitating rapid cell-cell spread and/or promoting efficient virus release. Whether either or both of these options are the functions of p10 and the other FAST proteins is unclear.

1.14.9. Unanswered questions

The reovirus FAST proteins represent a novel and diverse group of membrane fusion proteins. These proteins appear to assist in viral pathogenesis, although a mechanism for this function has not been well characterized. Much has been learned in recent years about the structural organization of the FAST proteins and which elements play active roles in the fusion process. Given their diversity, it is unclear if the FAST proteins use these structural features to facilitate fusion through a shared mechanism or whether they employ individualized strategies. Further, it is unclear if the FAST proteins function as specific membrane fusion proteins, or if fusion is the consequence of membrane-disruptive activity. Also, could the diminutive size and minimalist nature of

the FAST proteins indicate a reliance on host cell factors to support efficient cell-cell fusion, or are the FAST proteins more self-sufficient? The following chapters describe the first comparative investigations into the role of host factors in FAST protein-mediated fusion and lead to a model for host-factor enhancement of FAST protein-mediated fusion and enhanced viral pathogenesis.

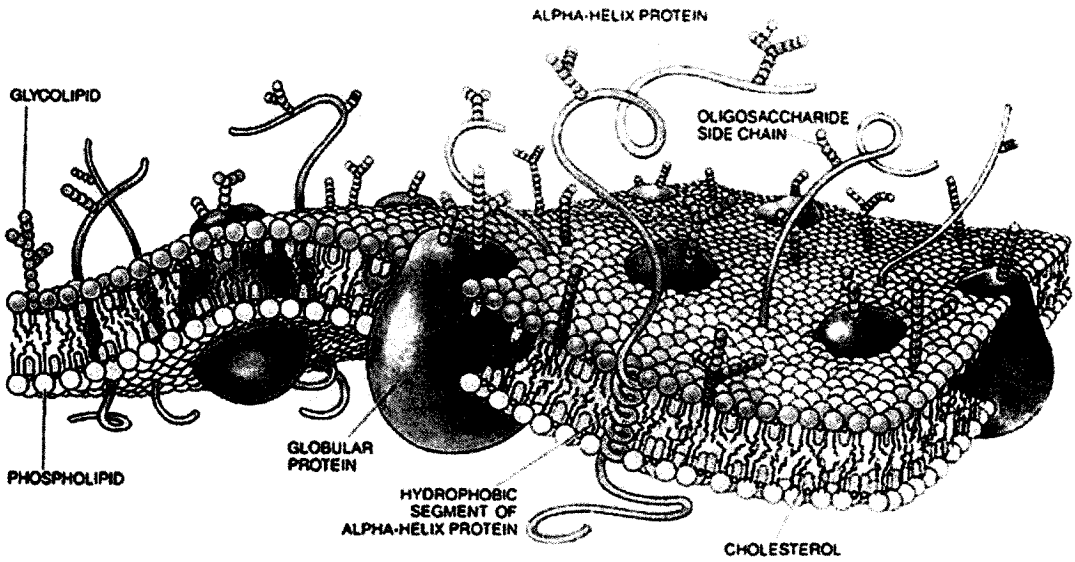

Figure 1.1 Biological membranes are complex structures with diverse carbohydrate, lipid and protein components. Omitted from this image are the proteins and proteoglycans that encase the interior and exterior surfaces of the membrane as described in Section 1.4. This drawing was made by Dana Burns and originally appeared in Scientific American, 1985, 253(4), pages 86-90, in the article *The molecules of the cell membrane* by M.S. Bretscher. It is reproduced here with permission.

Example	Shape	Membrane Curvature
PE **PS** **Unsaturated**	 Cone	Negative (-)
PIP **SM** **Cholesterol** **PC**	 Cylinder	Planar
LysoPC **Detergents**	 Inverted Cone	Positive (+)

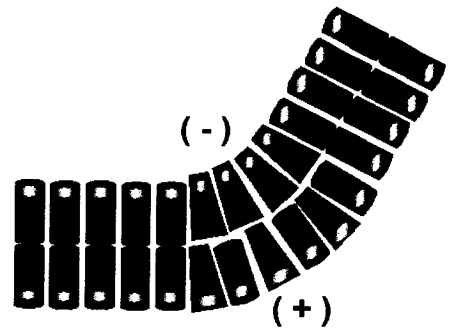

Figure 1.2 The molecular shape of a membrane lipid promotes membrane curvature. The table on the left illustrates, and provides examples of, the three lipid shapes (cone, cylinder and inverted cone). The model membrane on the right depicts how the shape of the lipid can induce positive (+, purple lipids) and negative (-, blue lipids) curvature in an otherwise planar membrane (green). Note how negative curvature (concave) of one leaflet results in positive curvature (convex) on the opposing leaflet.

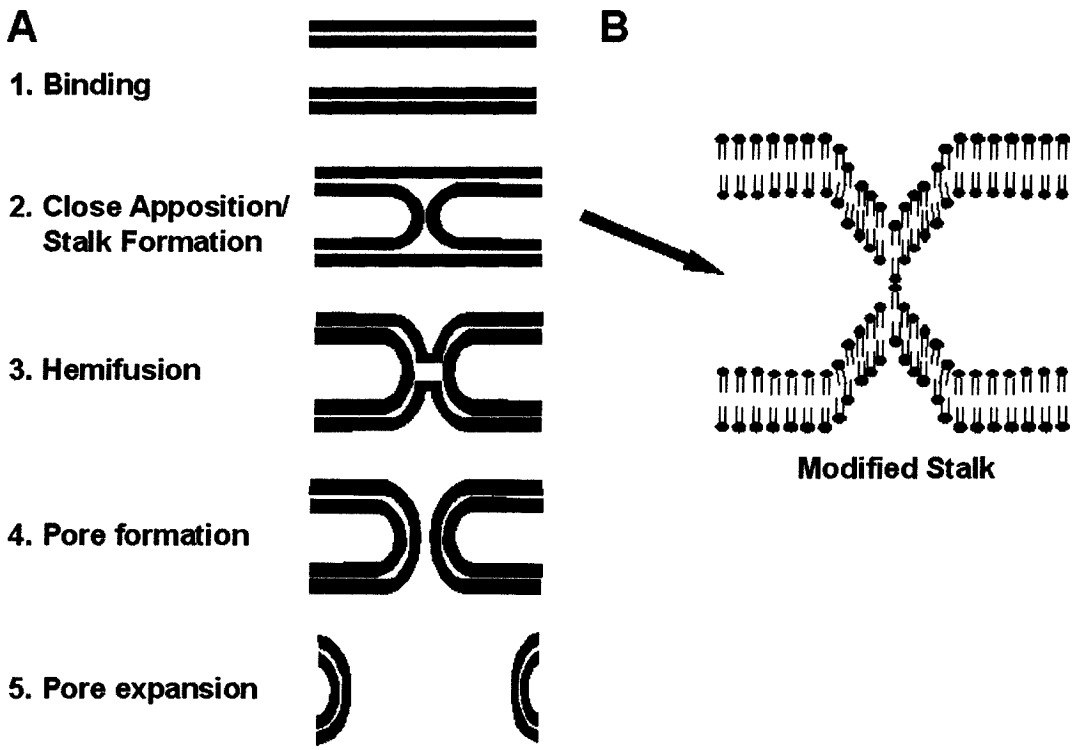

Figure 1.3 Fusion is a multistep process. **(A)** Fusion begins with binding (1) of opposing membranes (adhesion molecules have been omitted). Local dehydration of the water layer near membranes held in close apposition results in the formation of a fusion stalk (2). Mixing of the lipids in the outer membrane leaflets (red) of opposing membranes results in hemifusion (3). Mixing of the inner membrane leaflets (blue) results in the establishment of a fusion pore (4), which subsequently expands to complete the fusion process (5). Note the generation of negative curvature in the outer leaflet during the various fusion intermediates. **(B)** An enlargement of the fusion stalk in **(A)** showing the arrangement of individual lipids at a point of contact between opposing bilayers. This modified stalk model represents a low-energy membrane conformation that minimizes void spaces and membrane curvature.

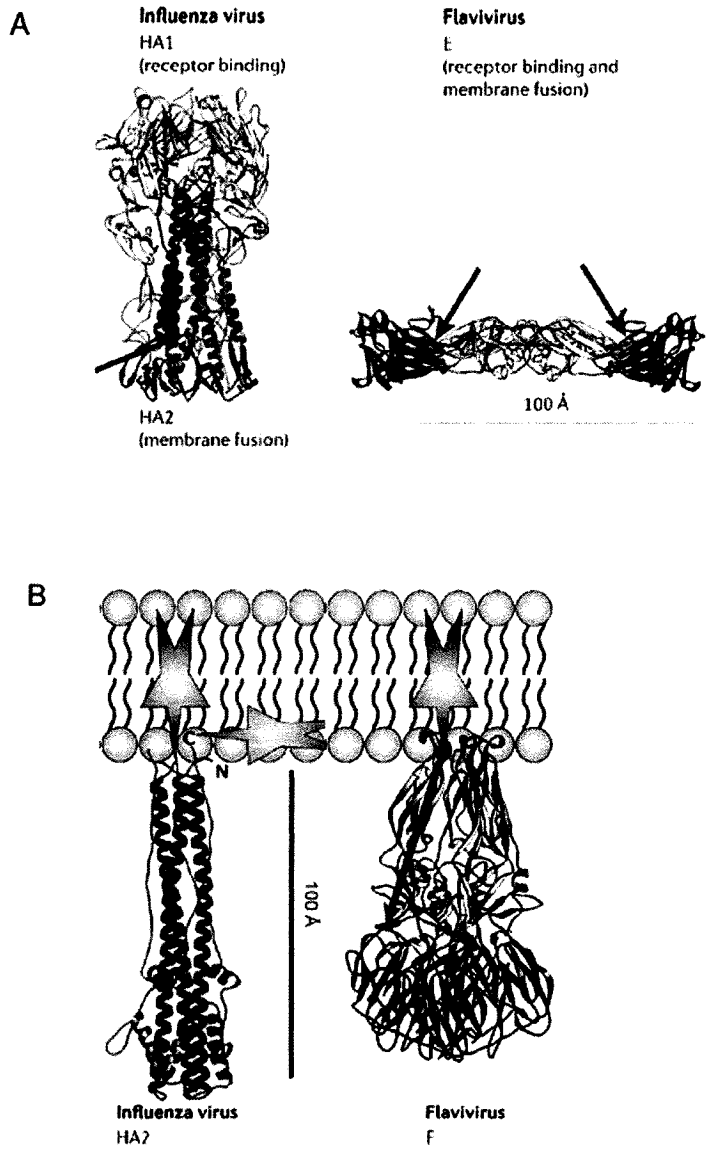

Figure 1.4 The prefusion and postfusion conformations of influenza HA and flavivirus E proteins. **(A)** Prefusion structure of an HA trimer (left) with the fusion peptides in yellow at the centre of the trimer (arrow). The HA_2 fusion subunits are in blue and red and the HA_1 receptor binding subunits are in white. Flavivirus E protein homodimers (right) in the prefusion conformation lie parallel to the membrane with the receptor-binding domain III shown in purple. The internal fusion peptides (one per monomer) are indicated by the arrows. **(B)** The post fusion structure of HA shows the six-helix bundle composed of one red and blue helix contributed by each monomer. The fusion peptide is embedded in the membrane indicated by the blue star. The red stars represent the transmembrane anchors. The post fusion structure of the E protein is now more extended than the prefusion structure and indicates the dramatic conformational rearrangements that drive the fusion reaction. Images are modified with permission from Macmillan Publishers Ltd: Kelian and Rey (2006), copyright (2006).

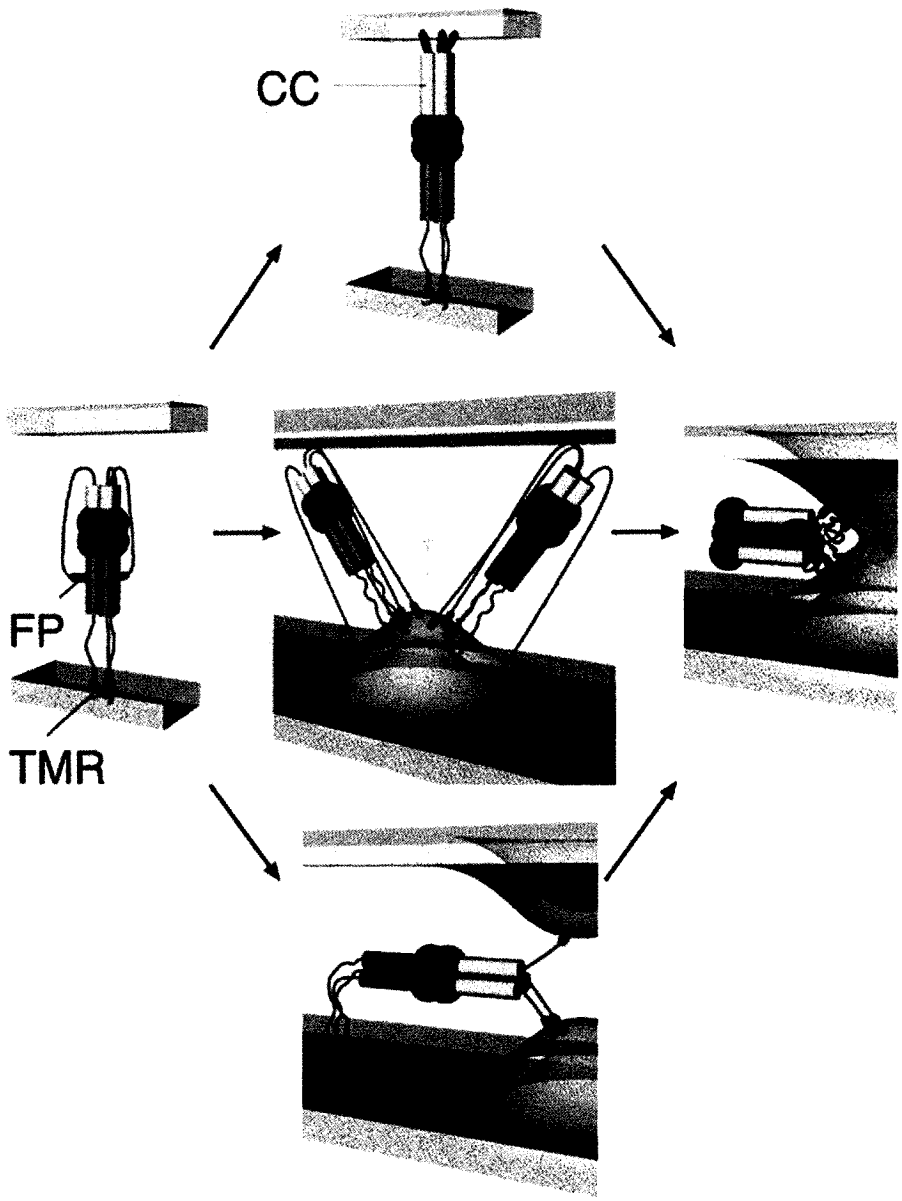

Figure 1.5 Possible models for HA-mediated fusion. The prefusion conformation of an HA_2 trimer (left) showing the transmembrane domain (TMR) in purple, the fusion peptide (FP) in green and α-helicies in yellow and orange. Upon low pH triggering, the FPs are exposed and might become buried in the target membrane (centre top), the donor membrane (centre) or both (centre bottom). Structural refolding to generate trimeric coiled-coils (CC) produces membrane bending and results in generation of six-helix bundles (right) and fusion. Reprinted from Jahn et al. (2003), copyright (2003), with permission from Elsevier.

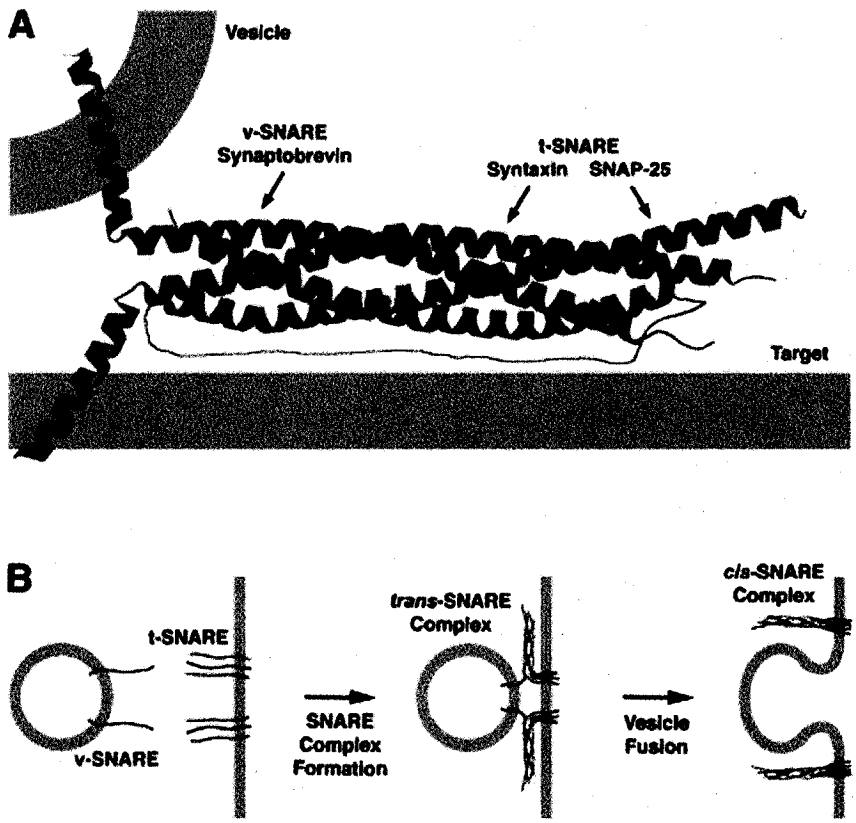

Figure 1.6 SNARE protein-mediated fusion. **(A)** The four-helix bundle formed by the v-SNARE synaptobrevin (red) and the t-SNAREs syntaxin (blue) and SNAP-25 (purple). **(B)** SNARE protein-mediated fusion begins with the *trans* pairing of v-SNAREs and t-SNAREs to form the four-helix bundle. Bundle formation provides sufficient energy to drive membrane merger at which point the SNARE complex can be disassembled and recycled. Not shown are the associated factors that coordinate vesicle docking, SNARE assembly and SNARE recycling. Adapted from Bonafacino and Glick (2004) with permission from Elsevier and copied under licence from Access Copyright. Further reproduction prohibited.

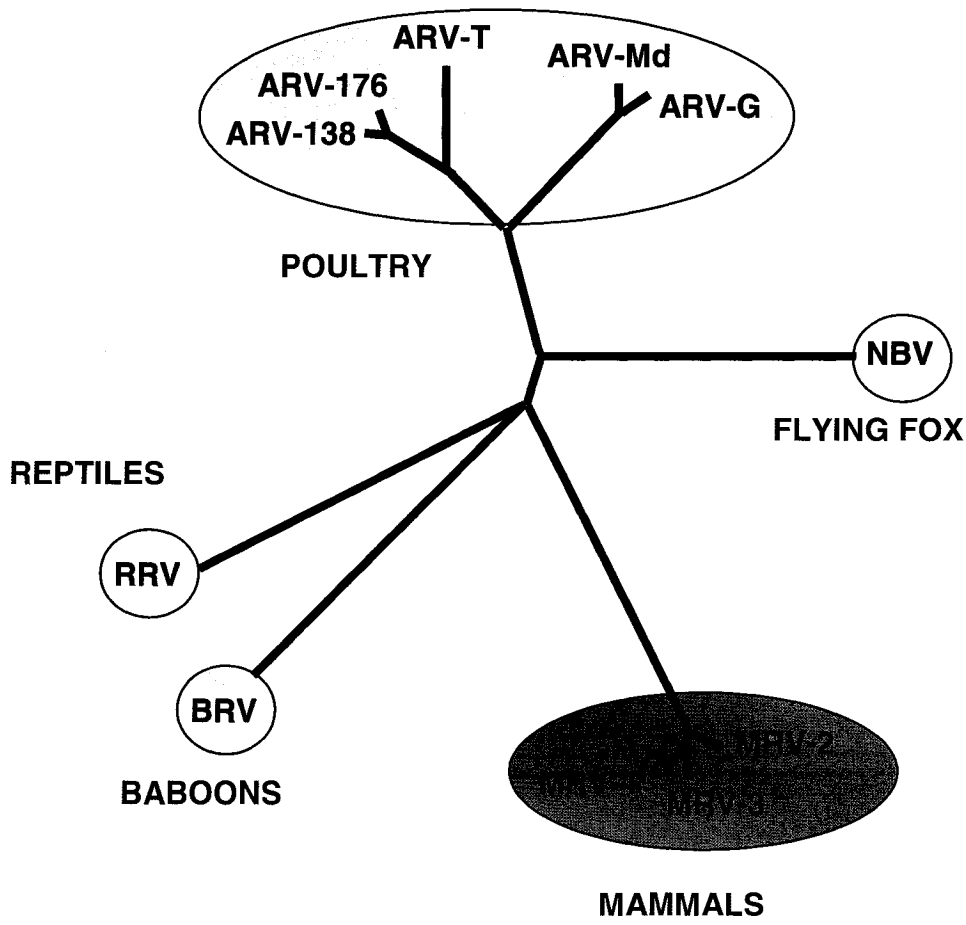

Figure 1.7 Phylogenetic relationship among the orthoreovirus species. Unrooted neighbour-joining tree shows the five different recognized orthoreovirus species (ovals) and their natural hosts. Fusogenic reovirus species are in peach-coloured ovals and non-fusogenic species are in purple ovals. The branch lengths are proportional to inferred evolutionary distances. Adapted from Duncan et al. (2004) with permission from Elsevier.

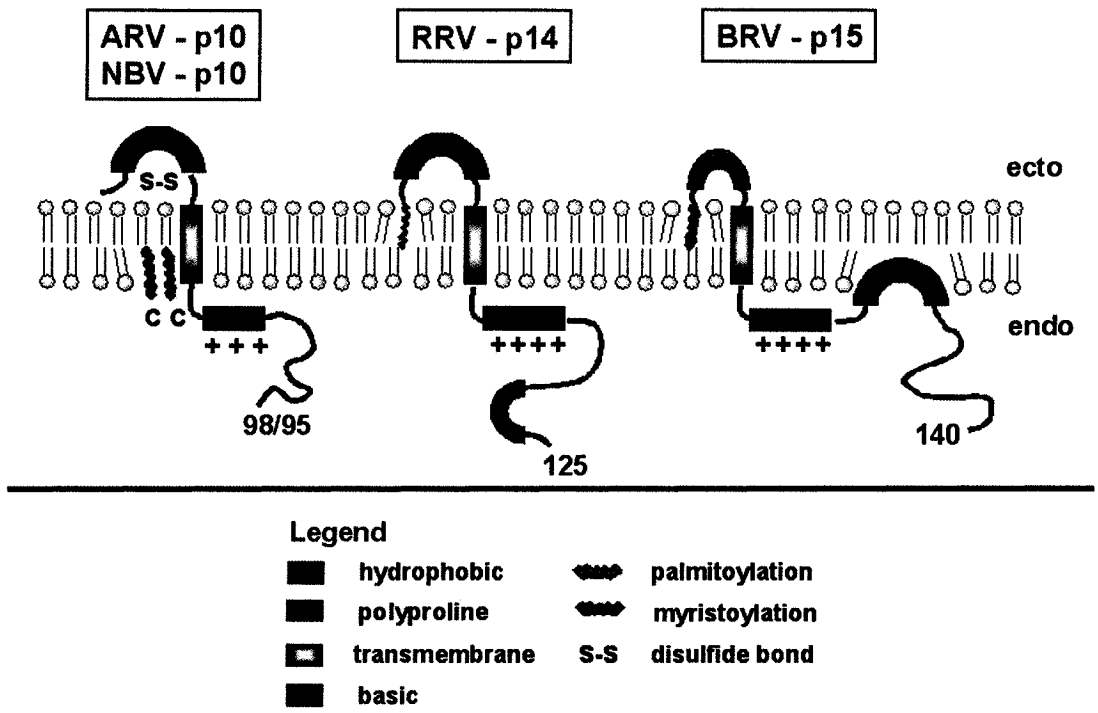

Figure 1.8 The diverse domain organizations of the reovirus FAST proteins. Cartoon schematic of FAST protein topology and structural organization. All are type I transmembrane proteins with N-terminal ectodomains (ecto) and C-terminal endodomains (endo). Each FAST protein has a signature arrangement of shared structural motifs including modification with acylation (palmitoylation or myristoylation), a hydrophobic patch (red), a polyproline region (green), a polybasic domain (blue) and a transmembrane domain (yellow). The numbers at the C-termini indicate the FAST protein sizes in amino acid number.

CHAPTER 2
Materials and Methods

2.1. Cells

2.1.1. Subculturing

All cell lines were maintained in 175-mm^2 flasks and subcultured 1:10 twice a week, unless otherwise stated. When subculturing cells, spent medium was removed and the cells were washed once with 10 mls PBS. Cells were then washed with 4 mls 0.1% trypsin (Gibco) for 30 s. The trypsin was removed and cells were further incubated at 37°C for 15-20 min until cells could be resuspended by tapping the flask. MDCK cells were incubated for 15 min in 2 mls trypsin:EDTA solution (Invitrogen) to facilitate disruption of cell-cell contacts. Cells were then resuspended in 10 mls of fresh growth medium and 1 ml was seeded back to the flask. The final volume of the flask was adjusted to 25 mls of growth medium and cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

2.1.2 Freezing and thawing cell stocks

Freshly subcultured cells were counted and resuspended to $2x10^6$ - $4x10^6$ cells per ml in growth medium containing 10% DMSO and 1 ml aliquots were added to 2 ml cryogenic tubes. These were placed at -80°C in an isopropanol bath overnight before transfer to liquid nitrogen. Fresh cultures were started by thawing frozen aliquots at 37°C and adding 10 ml of warm growth medium. The resuspended cells were pelleted (5 min, 700 x g) to remove the DMSO and the freeze medium, resuspended in 25 ml warm growth medium and transferred to a 175-mm^2 culture flask.

2.1.3. Cell Lines and Growth Media

Vero African Green monkey kidney epithelial cells were maintained in Medium 199 (Gibco) with Earle's salts supplemented with 5% FBS. QM5 quail fibroblasts, L-929 mouse fibroblasts, Hep-2 human epithelial, and HeLa human cervical epithelial cells (gift from Dr. Raphael Garduno) were maintained in Medium 199 with Earle's salts supplemented with 10% FBS. Madin-Darby Canine Kidney (MDCK) epithelial cells were a gift from Dr. Todd Hatchette and were maintained in MEM (Gibco) or Medium 199 with Earle's salts supplemented with 10% FBS. Mouse L-cell fibroblasts and L-cells

stably transfected with mouse E-cadherin from F9 cells (EL cells) were a gift from Dr. Masatoshi Takeichi and maintained in MEM supplemented with 5% FBS (389). EL cells were also grown in the presence of 500 μg/ml G418 (Gibco) to maintain selective pressure. CHO-K1 Chinese hamster ovary epithelial cells were a gift from Dr. Neale Ridgway and were maintained in αMEM (Gibco) supplemented with 10% FBS. N-BP-2 cells, CHO cell cholesterol auxotrophs with a defect in the site-2 protease necessary for activation of the SREBP-responsive genes (409), were provided in 35-mm dishes ready for transfection by Dr. Ridgway and maintained by his laboratory in DMEM (Gibco) with 5% FBS, oleate, mevalonate and cholesterol (445). CHO cells expressing influenza A HA X-31 (H3N2) were a gift from Dr. Steven Wharton and were maintained in αMEM with 0.5 mg/ml G418 to maintain selection pressure (564). NIH-3T3 mouse embryo fibroblasts were a gift from Dr. Patrick Lee and maintained in DMEM supplemented with 10% FBS. C2C12 mouse myoblasts were obtained from Dr. Victor Rafuse and grown on gelatin- (Sigma) coated flasks in DMEM supplemented with 10% FBS and never grown to confluency to prevent cell differentiation into myotubes. Jurkat human T-cell leukemia cells were a gift from Dr. David Hoskin and maintained in RPMI-1640 medium (Gibco) supplemented with 10% FBS.

2.2. Clones and Plasmids

All plasmids were transformed into *E. coli* DH5-α cells and plasmid DNA was purified using the DNA midi plasmid purification kit (Qiagen). Bacterial stocks were frozen in LB medium with 10% glycerol at -80°C. The cDNAs of RRV-p14, BRV-p15, NBV-p10 and ARV-p10 cloned into the pcDNA3 mammalian expression vector were obtained and produced as previously described (111, 128, 494). The RRV-p14 and BRV-p15 mutants RRV-p14-C88 (C-terminal truncation at aa 88, fusogenic), RRV-p14-G2A (non-myristoylated, non-fusogenic), RRV-p14-2HAN (2 HA epitope tags in ectodomain, non-fusogenic) and BRV-p15-2HAN (2 HA epitope tags in ectodomain, non-fusogenic) were obtained and generated in pcDNA3 as previously described (111, 128). An ARV-p10 construct containing two HA epitope tags at the N terminus (ARV-p10-2HAN) and which displays slower fusion ability was obtained and was generated as previously described (494). Several ARV-p10 mutants were also obtained that were generated as

previously described from the ARV-p10-2HAN construct (494). These ARV-p10 mutants are all non fusogenic and include ARV-p10-Basic (endodomain mutation, K67M), ARV-p10-TM (transmembrane domain mutation G49/50A) and ARV-p10-C9S (ectodomain mutation) (494). ARV-p10del (ectodomain deletion, non-fusogenic) was obtained and generated as previously described from ARV-p10 and lacks HA epitope tags (494). RRV-p14 was also obtained C-terminally tagged with GFP (p14-GFP) by insertion into the eGFP vector (Clontech) (110). Influenza hemagglutinin (HA) from influenza A virus A/WSN/33 strain (H1N1) in a eukaryotic expression plasmid with the pCAGGS backbone was provided as a gift from Dr. Earl Brown (393, 398). Dr. Michael Way provided various GTPases and their effector-domain mutants C-terminally tagged with GFP in a pCB6-GFP eukaryotic expression vector (354, 381). These included cDNAs of wild type human RhoA, Cdc42 and Rac1 and constitutively active point mutants of each (Table 6.1). The cDNA for human placental alkaline phosphatase (PLAP) in a eukaryotic expression vector was donated by Dr. Deborah Brown (32). The cDNA for the vesicular stomatitis virus G-protein (VSV-G) in a eukaryotic expression vector was donated by Dr. Patrick Lee.

2.3. Virus, infections and plaque assay

Laboratory stocks of avian reovirus strain SK138a (ARV-138) were obtained that were originally isolated from a hock joint extract from an infected chicken in New Brunswick, Canada, as previously described (147). QM5 cells grown to approximately 70% confluence in 12-well cluster plates were infected at a multiplicity of infection (MOI) of 5. Virus adsorption occurred for 1 h at 37°C in 100 µl of serum-free growth medium per well with shaking every 10 min. After adsorption, virus was aspirated from the cells and fresh growth medium (1 ml) with 2% FBS was added to cells for the duration of the infection. Plaque assays were used to determine viral titres. Serial dilutions of cell-free supernatants and cell lysates were used to infect 90%-confluent QM5 monolayers in duplicate as above. Infected cells were overlaid with growth medium (1% FBS) containing 1% agar and incubated inverted at 37°C. At 3 d post-infection wells were bathed with 10% formaldehyde in PBS for 10 min to fix cells. The agar plugs were removed and the cell monolayer was stained with crystal violet (1% w/v,

50% EtOH) to expose plaques. Plaques were counted and the viral titre was calculated using the following formula:

Viral titre = average # of plaques X dilution factor X volume added = pfu/ml

2.4. Transfections

Cells grown to 60-80% confluency, or 0.5 ml of suspension cells at a density of 5 x 10^5 - 1 x 10^6 cells/ml were transfected with 1 μg of plasmid DNA and 3 μl of Lipofectamine transfection reagent (Invitrogen) per well of a 12-well cluster plate following the manufacturer's instructions. Briefly, DNA and lipofectamine were mixed in serum-free medium for 45 min at room temperature then added drop-wise to cells in 0.4 ml of serum-free medium. Depending on the DNA transfected, 5-20 h post-transfection cells were washed with PBS and the transfection mix was replaced with growth medium to enhance protein expression. Suspension cells were also treated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) at 5 h post-transfection to enhance CMV promoter activity and promote suspension cell homotypic aggregation. Co-transfections were conducted similarly using 0.5 μg of each plasmid DNA (1 μg total) and 3 μl of Lipofectamine per well of a 12-well plate.

2.5. Syncytial indexing assay

Transfected cells were fixed with cold methanol for 2 min at various times as dictated by the given experiment. The methanol was aspirated off and cells were stained with Wright-Giemsa to visualize nuclei. The extent of fusion was determined using either a qualitative or quantitative syncytial-indexing assay. For qualitative analysis, cells were visualized on a Nikon Diaphot inverted microscope at 200x magnification. Ten random fields were observed and assigned a number between 0 and 4. The average was rounded to the closest whole number and represents the syncytial index (SI). A score of 0 indicates the complete absence of fusion. A value of 1 indicates a limited number of small syncytia containing less than 10 nuclei. A value of 2 represents an increase in both number and size of syncytia with some syncytia containing 20-30 nuclei. Progression to SI = 3 indicates extensive fusion with 5 or more syncytia per field and individual syncytia beginning to fuse with each other. A value of 4 represents complete fusion with

>90% of the monolayer fused and beginning to detach from the culture dish. Examples of the different fusion indices are shown in Figure 3.2.

Quantitative fusion analysis was conducted by counting the number of syncytial nuclei in 5-10 random fields at 200x magnification on a Nikon Diaphot inverted microscope and determining the average. This was found to be a more reproducible and sensitive method for quantifying fusion than counting the number of syncytia or the size of syncytia. Also, this assay was determined to be most accurate and sensitive when fusion progressed to an average of 50-200 syncytial nuclei per field. For samples with less than 50 syncytial nuclei per field, 10-20 random fields were quantified to enhance accuracy.

2.6. Uridine release assay

Cell membrane integrity was assessed using a standard [^{3}H]-uridine release assay commonly employed to analyze the membrane-lytic properties of viroporins (6, 43, 57, 71, 303). QM5 cells seeded in 12-well cluster plates were incubated for 18 h in the presence of 2 μCi/ml of [^{3}H]-uridine (Sigma). After incubation, the radiolabeled medium was removed, cells were washed three times with HBSS and fresh growth medium was added until the cells were ready for infection or transfection. At appropriate times post-transfection, the cell monolayer and culture medium were harvested to determine the percent release of [^{3}H]-uridine. The cell medium was collected and the volume was adjusted to 1 ml with PBS and centrifuged at 1500 x g for 5 min to pellet detached cells. The supernatant was transferred to a fresh microfuge tube and the pellet resuspended in 20 μl lysis buffer containing protease inhibitors (50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Igepal (Sigma), 0.5% (w/v) NaDOC, 0.1% (w/v) SDS, 200 nM aprotinin, 1 μM leupeptin, and 1 μM pepstatin). The remaining cell monolayer was washed with PBS, incubated on ice in 200 μl of lysis buffer for 5 min, and then the volume was adjusted to 1 ml with PBS and added to the cell pellet from the previously removed medium. Samples (200 μl) were added to 1 ml of scintillation cocktail for aqueous samples (Beckman Coulter) in duplicate and radioactivity was quantified on a liquid scintillation counter (Wallac 1410). Control experiments, involving addition of lysis buffer or unlabeled cell lysates to culture medium containing [^{3}H]-uridine,

determined that the efficiency of scintillation counting was the same under all conditions. Percent uridine release was determined by dividing the average counts per min present in the culture medium by the total counts in the culture medium plus cell lysates. The labeling efficiency (total counts incorporated into cells) was approximately $1x10^5$ cpm and maximal release from syncytial cells corresponded to ~80% (~80,000 cpm). TCA precipitation of the cell supernatant fractions confirmed that ~90% of the [^{3}H]-uridine released from mock-transfected cells was not in macromolecules. Between 30% and 50% [^{3}H]-uridine released from extensively fused FAST-transfected cells was in macromolecules.

2.7. Antibody inhibition

RRV-p14-transfected QM5 cells were incubated in the presence of 1:20 dilution of complement-fixed (56°C, 30 min) rabbit polyclonal anti-p14 antibody to prevent syncytium formation (110, 111). Antibody was added at 3 h post-transfection, prior to the onset of fusion, and medium was replaced with fresh antibody-containing medium every 5 h to minimize the effects of endocytic internalization of antibody and ensure neutralizing levels of extracellular antibody persisted. Complement-fixed normal rabbit serum (1:20 dilution) was added to control wells.

2.8. Hygromycin-B incorporation assay

At the appropriate times, FAST protein-transfected Vero cells were incubated for 45 min in methionine-free MEM with or without 1.5 mM hygromycin B (Sigma), a membrane-impermeable translation inhibitor that inhibits EF-2-dependent translocation by stabilizing peptidyl-tRNA bound to the ribosomal acceptor site (205). Cells were then labeled with 75 μCi/ml of [^{35}S]-methionine for 45 min in the presence or absence of 1.5 mM hygromycin B. Labeled cells were washed three times with cold PBS and lysed for 2 min in 400 μl of cold lysis buffer. Nuclei and particulate cell debris were pelleted by centrifugation at 16,000 x g for 5 min. Lysates were collected and added to 100 μl of 5X protein sample buffer (0.2 M Tris pH 8.0, 5% w/v SDS, 50% glycerol, 10% β-mercaptoethanol, 0.005% bromophenol blue) (300). Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% acrylamide) and

visualized using dimethyl sulfoxide-2,5-diphenyloxazole (DMSO-PPO) fluorography to detect decreases in methionine incorporation indicative of altered membrane permeability (43, 48, 71, 310).

2.9. DNA fragmentation assay

FAST-protein transfected QM5 cells, grown in 6-well cluster plates, were harvested at various times post-transfection with 10 mM EDTA in PBS by gentle pipetting. In some experiments, cells were treated prior to and during transfection with 50 μM of the irreversible general caspase inhibitor Z-VAD-fmk (Calbiochem) to prevent caspase cleavage and propagation of potential apoptotic signaling. Collected cells were pelleted at 1500 x g for 5 min and resuspended in 200 μl of PBS. DNA was isolated using the DNeasy DNA extraction kit (Qiagen) as per the manufacturer's instructions. Samples were subjected to 1% w/v agarose gel electrophoresis and visualized with 1 % w/v ethidium bromide to detect DNA degradation in the form of nucleosomal laddering, a hallmark feature of apoptosis.

2.10. Flow cytometry

Transfected cells expressing GFP or GFP-tagged proteins were analyzed by flow cytometry to quantify expression and transfection efficiencies. QM5 cells transfected with p14-GFP or one of the GTPases were harvested at various times post-transfection with 450 μl 10 mM EDTA in PBS and gentle pipetting. Resuspended cells were transferred to plastic test tubes containing 50 μl 37% formaldehyde (3.7% final concentration) for fixation and stored at 4°C until analysis by flow cytometry. Transfected Jurkat cells were pelleted (1500 x g, 5 min), washed once with PBS and analyzed live in PBS. In both types of experiments untransfected cells, GFP vector-transfected and mock-transfected cells served as controls. GFP fluorescence was detected by excitement at 488 nm and detection using channel 1 (500-560 nm) on the FACSCalibur fluorescent cell sorter (Becton Dickinson), and results were analyzed using Cell Quest software. Calculation of overton subtractions and other analyses were conducted using FSC Express 2.0 software (De Novo Software).

2.11. Calcium Switch

Disruption of cadherin-mediated cell-cell contacts was accomplished by depletion of extracellular calcium (2, 241, 566). Cells were washed once with PBS and incubated for 1-2 min with PBS containing 0.5 mM EDTA until cell-cell contact disruption was visually confirmed by light microscopy. Cells were then washed twice with PBS to remove residual EDTA. Either MEM or S-MEM (calcium-free MEM) containing 5% FBS dialysed against PBS was added for the duration of the experiment.

2.12. Immunofluorescence microscopy

2.12.1. Permeabilized cells

For detection of protein expression and localization, cells grown on glass coverslips (No. 1 thickness) in 6-well cluster plates were fixed for 20 min with 3.7% formaldehyde and permeabilized for 20 min with 0.1% Triton X-100 in PBS. For staining of DNA, 50 nM DAPI (4',6-diamidino-2-phenylindole, dihydrochloride; Molecular Probes), with an excitation peak at 358 nm and an emission peak at 461 nm (ex/em 358/461), was added for 30 min and cells were washed extensively. DNA was also visualized with 1 μg/ml propidium iodide (20 min, ex/em 535/617; Molecular Probes) after RNaseA treatment (100 μg/ml, 20 min; Qiagen) to degrade cellular RNA. Actin was visualized with AlexaFluor488- or AlexaFluor555-conjugated phalloidin (Molecular Probes) using a 1:200 dilution of methanolic stock for 30 min. Cells expressing transfected and endogenous proteins were blocked with purified goat IgG (Jackson ImmunoResearch) diluted 1:1000 in HBSS (blocking buffer) for 30 min. The appropriate primary antibody diluted 1:800 in blocking buffer was added for 1 h with shaking. Cadherins were visualized using either mouse anti-human E-cadherin cytoplasmic domain (Transduction Labs) or mouse anti-N-cadherin monoclonal antibodies (Transduction Labs). Beta-catenin was detected with mouse monoclonal anti-beta-catenin antibodies (Transduction Labs). RRV-p14 and RRV-p14G2A were visualized with rabbit polyclonal anti-p14 antiserum. RRV-p142HAN was detected using mouse monoclonal anti-HA epitope tag produced from 12CA5 mouse hybridoma cells (obtained from Dr. Mark Nachtigal). Influenza HA, H1 strain, was detected with rabbit polyclonal anti-H1N2 antiserum (gift from Dr. Earl Brown). Primary antibody-labelled

cells were washed extensively (3x15 min) with HBSS before addition of appropriate secondary antibodies diluted in blocking buffer for 45 min. The secondary antibody was removed with extensive washing in HBSS (3 x 15 min). Secondary antibody labelling was conducted in the dark with purified goat anti-mouse or goat anti-rabbit $F(Ab)_2$ H+L chain polyclonal antibody fragments conjugated with either AlexaFluor488 or AlexaFluor555 (Molecular Probes). All secondary antibodies were used at a 1:800 dilution for intracellular staining. Immunostained cells were mounted on glass slides using DakoCytomation fluorescent mounting medium (DakoCytomation). Slides were stored in the dark overnight to allow the mounting medium to harden before microscopic examination. GFP and GFP-tagged proteins were detected in formaldehyde-fixed and permeabilized cells using AlexaFluor488 capture parameters detailed below.

2.12.2. Surface staining

Surface staining of unpermeabilized live cells was conducted at 4°C to prevent antibody internalization. Detection of the endogenous raft marker ganglioside GM1 was accomplished with purified AlexaFluor488- or AlexaFluor555-conjugated cholera toxin B subunit (0.1 μg/ml in blocking buffer) for 30 min. After washing, goat anti-cholera toxin B polyclonal antiserum (Calbiochem) was added (1:200 dilution) in blocking buffer for 30 min to promote patching of lipid rafts. PLAP was detected with mouse anti-human PLAP monoclonal antibodies (1:50 dilution, DakoCytomation) and purified goat anti-mouse AlexaFluor488- or AlexaFluor555-conjugated $F(Ab)_2$ H+L chain polyclonal antibodies (1:200 dilution). RRV-p14 and RRV-p14G2A were detected with rabbit anti-p14 antiserum (1:200 dilution) and goat anti-rabbit AlexaFluor488-conjugated $F(Ab)_2$ H+L chain polyclonal antibodies (1:200 dilution). BRV-p152HAN and RRV-p142HAN were detected with mouse anti-HA monoclonal antibodies (1:100 dilution) and goat anti-mouse AlexaFluor555-conjugated $F(Ab)_2$ H+L chain polyclonal antibodies (1:200 dilution). Influenza HA, H1 strain, was detected with rabbit polyclonal anti-H1N2 antiserum (1:400 dilution) and goat anti-rabbit AlexaFluor555-conjugated $F(Ab)_2$ H+L chain polyclonal antibodies (1:200). After staining, cells were fixed with 3.7% formaldehyde and mounted as described above. For co-immunofluorescence both appropriate primary or secondary antibodies were added simultaneously. However, when

co-labelling for actin the phalloidin stain was added after immunodetection of the other protein. Co-labelling of RRV-p14G2A and N-cadherin was accomplished by surface staining for RRV-p14-G2A followed by fixation, permeabilization and intracellular staining for the cytoplasmic N-cadherin epitope.

2.12.3. Image acquisition and processing

Images were acquired on a Zeiss LSM510 laser scanning confocal microscope using Zeiss LSM Image Examiner software. Images were acquired using the 63X or 100X oil-immersion objective lenses. AlexaFluor488 (ex/em 495/519) and GFP (ex/em 488/507) were excited with the 488 nm line of the argon laser and emissions were detected with an AxioCam HR camera using a 505-550 nm bandpass filter. AlexaFluor555 (ex/em 555/565) was excited with the HeNe laser (543 nm) and detected using a 560 nm longpass filter. Differential interference contrast (DIC) images were simultaneously captured for each image as well. The image acquisition software was used to ensure captured images had pixel intensities within the linear detection range, minimizing pixel saturation. For co-localization and cytoskeleton experiments, capture parameters (i.e. pinhole, laser power and detector gain) were adjusted to ensure optical slices ranged from 0.5-1.0 μm thick. Images were processed with Zeiss LSM 5 Image Browser and Adobe Photoshop 6.0 software. Aside from the addition of scale bars and cropping, image processing involved only linear adjustments to each colour channel (e.g. brightness and contrast) and was applied to the entire image area.

2.13. Influenza hemagluttinin cell lines and fusion assay

QM5 cells were co-transfected with 0.5 μg HA (H1 strain) plasmid and 0.5 μg pcDNA3, which contains a neomycin-resistance cassette. Twenty-four h post-transfection, cells were treated with growth medium containing 0.8 mg/ml Genetecin (Gibco) for 5 d to select for neomycin-resistant cells (398). After 5 d cells were cultured in growth medium containing 0.4 mg/ml Genetecin to maintain selection pressure. By 14 d post-transfection all cells were expressing HA as determined by immunostaining. Immunostains were conducted by fixing cells with methanol, blocking for 30 min with goat IgG in HBSS, binding rabbit polyclonal anti-H1N2 antiserum (1:800 dilution) for 1

h, labelling with alkaline phosphatase-conjugated goat anti-rabbit antibody (1:800 dilution; Jackson ImmunoResearch) for 45 min and developing with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) alkaline phosphatase substrate kit IV (Vector Laboratories) as per the manufacturer's instructions. To ensure high HA expression and consistency, these QM5-HA cells were frozen in liquid nitrogen and thawed aliquots were passaged fewer than 10 times, and then discarded.

HA-mediated fusion was triggered in QM5-HA and CHO-HA cells by washing confluent cell monolayers once with HBSS followed by treatment with 10 μg/ml trypsin for 5 min in HBSS to cleave the HA_0 precursor to the fusion-active form. Cells were washed once with HBSS and incubated in growth medium containing 10% FBS for 10 min to inhibit residual trypsin activity (116). Calcium was depleted as described below followed by incubation for 20 min in MEM or S-MEM to allow HA to bind to its sialic acid receptor in low-calcium conditions. Fusion was triggered with MEM or S-MEM (pH 4.8) buffered with 10 mM citrate for 60 s. Cells were then transferred to MEM or S-MEM (pH 7.4) containing 10% FBS to allow syncytia to progress before fixation with methanol and Giemsa staining to quantify syncytial nuclei. All fusion steps were carried out with reagents at 37°C as fusion activation was found to be highly temperature sensitive. In some experiments horse serum (Sigma) was used to block HA binding to its sialic acid receptor.

2.14. Generating different adhesion phenotypes

The three different adhesion phenotypes (active, passive, no adhesion) were generated in QM5 cells using a combination of calcium depletion to disrupt cell-cell contact and low-dose cytochalasin D to prevent actin remodelling (566, 567). Control cells were maintained in growth medium containing normal calcium levels with or without 0.1 μg/ml cytochalasin D (Sigma). Both conditions maintain the active adhesion phenotype although cytochalasin D causes some disruption of cell junctions. Active adhesion also occurred in cells that underwent calcium depletion, followed by incubation in growth medium with normal calcium. The 'no adhesion' phenotype was generated by calcium depletion followed by incubation in calcium-free medium (S-MEM) in the presence and absence of 0.1 μg/ml cytochalasin D. Passive adhesion was generated by

first subjecting cells to calcium depletion followed by incubation in normal calcium-containing medium containing 0.1 ug/ml cytochalasin D. These conditions allow cadherin-mediated contacts to form, but resist junction formation because of the actin inhibition. Cells under these conditions were fixed as for immunofluorescence microscopy or transfected prior to treatment and subsequently fixed for quantitative syncytial indexing.

2.15. Western Blotting

MDCK, L and EL cells grown to confluency in 10-cm dishes were harvested by scraping cells into 400 µl of cold lysis buffer and incubating on ice for 30 min. Cells debris was pelleted at 13,000 x g for 5 min and the supernatants were transferred to fresh eppendorf tubes. Protein concentrations were quantified using the Bio-Rad Protein Assay kit (Bradford method; Bio-Rad) and remaining cell lysates were mixed with 100 µl of 5x protein sample buffer (0.2 M Tris pH 8.0, 5% w/v SDS, 50% glycerol, 10% β-mercaptoethanol, 0.005% bromophenol blue). Samples were boiled for 5 min and immediately frozen at -20°C, or 5 µg of protein was subjected to SDS-PAGE (10% acrylamide). Samples were transferred on a semidry western blotting apparatus (Bio-Rad) onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was blocked overnight in TBST (0.1 M Tris pH 8.0, 0.5 M NaCl, 0.1% Tween-20) containing 4% dry milk and 1:1000 dilution of purified goat IgG. Primary antibody was bound for 1 h in blocking buffer. Actin was detected with rabbit anti-actin polyclonal antiserum (1:15,000 dilution; Sigma), E-cadherin was detected with mouse anti-E-cadherin monoclonal antibodies (1:5000 dilution) and β-catenin was detected with mouse anti-β-catenin monoclonal antibodies (1:5000 dilution). After extensive washing, horseradish peroxidase (HPO)-conjugated secondary antibodies (goat anti-rabbit or goat anti-mouse polyclonal antisera; Jackson Immunoresearch) were diluted 1:10,000 in blocking buffer and added for 45 min. After extensive washing, membranes were developed using ECLplus chemiluminescent western blot detection system (Amersham Biosciences) according to the manufacturer's instructions and analyzed using a Typhoon 9410 variable mode imager (Amersham Biosciences) and Image Quant software (Molecular Dynamics).

2.16. Cholesterol alteration

Rapid cholesterol depletion using methyl-β-cyclodextrin (MβCD) was performed on cell monolayers using 2 mM, 10 mM or 20 mM cyclodextrin for 30 min at 37°C in serum-free medium (97). For cells requiring longer incubation times where drug toxicity was a concern, cells were depleted as above followed by incubation in the presence of 2 mM MβCD in medium containing 10% FBS or 5% lipoprotein-deficient serum (cLPDS). For FAST protein-expressing cells, cholesterol depletion was performed prior to the onset of fusion.

The effectiveness of cholesterol depletion with MβCD was assessed by labeling QM5 cells with 5 μCi/ml [^{3}H]-cholesterol (ICN) for 4 hours. Excess label was removed with 4 washes of HBSS and cholesterol extracted with 0, 2 or 20 mM MβCD in serum-free medium or medium containing 10% FBS or 5% lipoprotein-deficient (cLPDS) for 1 h at 37°C. Cell supernatants (0.5 ml) were removed and the cells washed with 0.5 ml HBSS and the wash was pooled with the supernatant. Cells were then harvested by lysis in 200 μl RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Igepal/NP-40 (Sigma) 0.5% (w/v) NaDOC, 0.1% (w/v) SDS) and the volume increased to 1 ml with HBSS. The amount of the label in the supernatant or cell lysate was determined by analyzing 200 μl of each sample on a Wallace Liquid Scintillation Counter (Beckman). The results were expressed as the percent of total [^{3}H]-cholesterol that was extracellular (cpm supernatant/[cpm supernatant + cpm cell lysate] x 100) and are expressed as the mean ± the SE (n=3).

For experiments requiring the restoration of depleted cholesterol, QM5 cells were first treated with 10-20 mM MβCD in serum-free medium for 30 min at 37 °C. Cholesterol (6 mg/ml, Sigma) was dissolved in 20 mM MβCD (in serum-free medium) by vigorous vortexing and heating (37 °C for 30 min). Undissolved cholesterol was removed by filtering the solution before addition to depleted cells for 30 min at 37 °C.

Growing QM5 cells in the presence of 25 μM of the HMG-CoA reductase inhibitor lovastatin (Sigma) for 24 h in 10% FBS prior transfection also decreased membrane cholesterol by preventing *de novo* cholesterol synthesis (5, 273, 582). Transfected cells were incubated in the presence of lovastatin for the duration of the

experiment. Culturing QM5 cells for 24 h in the presence of 2 μg/ml U18666A (3β-(2-(diethylamino)-ethoxy)-androst-5-en-17-one) was also used to decrease plasma membrane cholesterol. U18666A (gift from Dr. Neale Ridgway) induces a Niemann-Pick disease type-C phenotype where cholesterol cannot be correctly recycled back to the cell surface and may also inhibit cholesterol synthesis (105, 171, 270, 326, 353). U18666A levels were maintained throughout the experiment. Experiments using lovastatin and U18666A were conducted in growth medium containing 10% FBS. Using 5% cLPDS during drug incubations was determined to have minimal effects on fusion inhibition (data not shown).

CHO cells deficient in the site-2 protease necessary for induction of *de novo* cholesterol synthesis (N-BP-2 cells) were depleted of exogenous cholesterol using DMEM with oleate and 5% cLPDS for 24 or 48 h prior to and throughout transfection with RRV-p14. Cells were fixed at 12 h post-transfection, Giemsa-stained and assessed for fusion ability relative to cells grown in the presence of cholesterol.

2.17. Heterotypic fusion assay

QM5 cells were labelled with 5 μM CellTracker Orange - CMTMR (5-(and-6) - (((4-chloromethyl)benzoyl)amino)-tetramethylrhodamine, ex/em 541/565) or 2 μM CellTracker Blue - CMAC (7-amino-4-chloromethylcoumarin, ex/em 353/456) for 45 min at 37°C in HBSS as per the manufacturer's instructions (Molecular Probes) to track nuclei from different cell populations. CMTMR-treated cells were transfected with RRV-p14 (donor cells) and at 2.5 h post-transfection, cells were treated with 0, 10 or 20 mM MβCD for 30 min at 37°C and washed with twice with HBSS. Target cells (labelled with CMAC) were also treated with 0, 10 or 20 mM MβCD at this time. Donor or target cells not treated with MβCD were lifted by a 30 s wash in 10 mM EDTA in PBS and resuspended in 500 μl PBS with gentle pipetting. These cells were then transferred to a 1.5-ml eppindorf tube containing 200 μl FBS and gently mixed. Cells were pelleted by centrifugation at 700 x g for 5 min and the supernatant was aspirated. Cells were resuspended by pipeting in 100 μl of PBS followed by the addition of 400 μl 5% cLPDS in medium 199 with Earle's salts. Suspended donor cells were added to an equal number of MβCD treated target cells grown on coverslips and suspended target cells were added

to MβCD treated donor cells. Cells were incubated for 2-4 hrs, washed with twice with HBSS, fixed with methanol and mounted on slides for fluorescence microscopy.

Images were acquired on a Zeiss Axiovert 200 inverted microscope attached to a Zeiss AxioCam HRc digital camera using Axiovision 3.1 software. Samples were excited with an AttoArc 2 HBO 100 w lamp using the UV filter (365 nm) to excite CMAC and the green filter (546 +/-12 nm bandpass) to excite CMTMR. CMAC was detected with a 420 nm longpass filter and CMTMR was detected with a longpass 590 nm filter. The relative number of syncytia per field was determined by counting the number of syncytia in 20-50 random fields at 400 X magnification. The ratio of donor (red) to target (blue) nuclei in syncytia was determined by photographing 10-15 random syncytia at 400 X magnification and examining them in Adobe Photoshop 6.0.

2.18. Live cell imaging

QM5 cells were grown on 35-mm ΔT dishes (Bioptechs) and co-transfected with RRV-p14 and GFP. At the first signs of syncytia formation (~4 h post-transfection) culture dishes were removed from the incubator and placed on the Zeiss Axiovert 200 inverted microscope equipped with a heated stage, objective (40x) and ΔT dish lid (Bioptechs). The culture medium was changed to pre-warmed (37°C) medium 199 with Hank's salts to maintain the culture pH under atmospheric CO_2 conditions. Transfected cells were identified by GFP fluorescence or syncytium formation and the field of view was centred on transfected cells. DIC images were captured every 20 s for 2 h and compiled into an avi video format.

2.19 Electron microscopy

2.19.1. Scanning electron microscopy

QM5 cells grown on glass coverslips were transfected with constitutively active RhoA and fixed at 12 h post-transfection with 2.5% gluteraldehyde in cacodylate buffer (0.1 M sodium cacodylate) for 2 h. Gluteraldehyde was removed with three 10 min washes with cacodylate buffer before impregnation with 1% osmium tetroxide for 2 h. Samples were then rinsed with distilled water and washed with cacodylate buffer before dehydration with ethanol and HMDS (hexamethyldisilazane; Sigma). Samples were

incubated for 10min each in 0, 25, 50, 75 and 100% ethanol in cacodylate buffer. Cells were washed three times in 100% ethanol before incubation for 10 min each in 0, 25, 50, 75 and 100% HMDS in ethanol. Samples were rinsed three times in 100% HMDS and then just enough HMDS was added to barely cover the sample. This was placed in a fume hood until the HMDS was completely evaporated. Dehydrated samples were adhered to metal studs and sputtercoated with gold/palladium before analysis using a Hitachi S-4700 scanning electron microscope. Digital images of the sample were acquired and processed in Adobe Photoshope 6.0.

2.19.2. Transmission electron microscopy

QM5 cells transfected with RRV-p14 and fixed at 8 h post-transfection with 2.5% gluteraldehyde in cacodylate buffer (0.1 M sodium cacodylate) for 2 h. Gluteraldehyde was removed with three 10 min washes with 0.1 M sodium cacodylate buffer before impregnation with 1% osmium tetroxide for 2 h. Samples were rinsed quickly with distilled water and placed in 0.25% uranyl acetate at 4 °C overnight. Dehydration of samples was accomplished with a graded series of acetone washes beginning with 50% acetone for 10 min. This was followed by two 10 min washes in 75% acetone, two 10 min washes in 95% acetone, two 10 min washes in 100% acetone and a final 10 min wash in dried 100% acetone. Samples were then infiltrated with epon araldite resin in a graded series beginning with a 3:1 ratio of dried 100% acetone to resin for 3 h. This was followed by a 1:3 ratio of acetone to resin and left overnight. Finally, samples were incubated in two successive treatments with 100% resin for 3 h each. Samples in 100% epon araldite resin were then cured at 60°C for 48 h. Thin sections (~100 nm) were prepared using a LKB Huxley Ultramicrotome with a diamond knife and placed on 300-mesh copper grids. Samples were stained with 2% aqueous uranyl acetate for 10 min, rinsed with distilled water and incubated in lead citrate for 4 min. After staining, samples were washed and air-dried. Samples were viewed using a JEOL JEM 1230 transmission electron microscope at 80 kV and images were captured on a Hamamatsu ORCA-HR digital camera and processed in Adobe Photoshope 6.0.

2.20. Cytoskeleton alterations

QM5 cells were transfected with RRV-p14, NBV-p10, BRV-p15 or ARV-p10 and, just prior to the onset of fusion, were treated with 0, 0.1, 0.5, 1 or 2 μg/ml of the actin-depolymerizing drug cytochalasin D for the duration of the experiment (473). RRV-p14-transfected QM5 cells were treated with 0, 50, 250, 1000 nM concentrations of the actin stabilizing agent jasplakinolide (Sigma), at 3 h post-transfection to prevent actin remodelling. The microtubule network was disrupted in FAST protein-transfected QM5 cells by addition of the tubulin-binding drug nocodazole (Sigma) at 0, 1.25, 2.5, 5 or 10 μM concentrations just prior to and during syncytium formation. Cell motility was disrupted in FAST protein-transfected QM5 by addition of the PI3K inhibitor wortmannin (Sigma) at 0, 25, 100, 200 or 800 nM concentrations just prior to and during syncytium formation.

2.21. Annexin V inhibition

C2C12 mouse myoblasts were transfected with RRV-p14, NBV-p10, BRV-p15 or ARV-p10 and, just prior to the onset of fusion, were treated with 100 μg/ml annexin V from human placenta (Sigma) for the duration of the experiment. At 12-24 h post-transfection, cells were fixed with methanol, Giemsa-stained and fusion was quantified.

2.22. Statistical analysis

Statistical analysis was conducted using Microsoft Excel 2000 (Microsoft) for calculation of means, standard deviations and standard error of the mean. Graphs were generated using Slide Write Plus version 5.0 (Advanced Graphics Software). Analysis of variance (ANOVA), Tukey-Kramer multiple comparisons test and student's t-tests were performed using Instat software. For data presented from a representative experiment, error bars represent the standard deviation. The standard error of the mean was graphed when the mean was calculated from multiple independent experiments.

CHAPTER 3

FAST Protein-Mediated Syncytium Formation Induces Apoptosis and Loss of Membrane Integrity, Suggesting Possible Roles for Fusion in the Reovirus Replication Cycle

3.1. Introduction

The fusogenic reoviruses are the only non-enveloped viruses known to encode membrane fusion proteins. There are, therefore, no precedents to define the role of fusion proteins in the replication cycles of non-enveloped viruses. Studies conducted using ARV infections revealed that syncytium formation correlates with efficient virus release and increased pathogenicity, indicating that the FAST proteins may act as virulence factors (145, 147). These same studies hint at two advantages cell-cell fusion may confer on a replicating virus. First, fusion would facilitate the rapid transmission of the virus to neighbouring cells. Second, syncytium formation is correlated with rapid virus release, and a concomitant loss of membrane integrity (145). The ability of the FAST proteins to mediate cell-cell fusion has since been well documented but their ability to alter membrane integrity has not been assessed (110, 128, 494). A dual role for the FAST proteins as mediators of cell-cell fusion and membrane disruption might help explain their unusual structure. It is possible, for example, that the FAST proteins evolved as membrane permeabilizing molecules and that fusion is a mere consequence of this lytic activity.

Previous studies (43, 494) noted the similarity between the FAST proteins and a diverse group of viral membrane-interactive proteins, collectively referred to as viroporins (70). Viroporins share several features with the FAST proteins: they are small (60-120 amino acids), often nonstructural, membrane-disruptive proteins with one or more hydrophobic motifs, frequently with an adjacent polybasic region (206). The membrane-destabilizing activity of viroporins may contribute to virus-induced cytopathic effects, and to facilitating the release of both enveloped and nonenveloped viruses (57, 206, 330, 560). Examples of viroporins include the HIV vpu, influenza M2, and picornavirus 3A and 2B proteins, as well as two viroporins encoded by genera within the family *Reoviridae*, the rotavirus NSP4 and the orbivirus NS3 proteins (57, 206, 217, 560). In addition to their structural features, viroporins share the functional property of

altering membrane permeability to ions and small molecules such as uridine and hygromycin B (70, 71, 206, 330). To date, membrane-fusion activity has not been ascribed to any viroporin.

As with viroporins, ARV-p10 has been implicated in supporting avian reovirus release from infected cells. Blocking ARV-p10 surface localization prevents syncytium formation and delays cell lysis and virus release (145). More recently, expression of ARV-p10 in transfected cells was shown to alter membrane permeability (43), leading to the proposal that ARV-p10 functions as a membrane-destabilizing viroporin. The related NBV-p10 protein and the more recently discovered BRV-p15 and RRV-p14 have not been analyzed for the membrane-lytic properties ascribed to the ARV-p10 protein. The ability of ARV-p10 to cause membrane disruption helps explain the correlation between ARV-induced syncytium formation and viral pathogenesis (147), and suggests that the FAST proteins may function to promote cell lysis and virus release, thereby contributing to the natural pathogenicity of the fusogenic reoviruses (317, 558, 573)

An interesting report demonstrated that deletion of the ARV-p10 ectodomain abolishes fusion activity while the ability to alter plasma membrane integrity remains intact (43). The authors suggest that the membrane-destabilizing and membrane-fusion activities of the ARV-p10 protein represent distinct phenomena mediated by different protein domains (43). It was also suggested that enhanced virus release from ARV-infected cells reflects the membrane-destabilizing viroporin-like activity of the ARV-p10 FAST protein. These data make it unclear whether FAST protein-mediated fusion is the consequence of viroporin-like membrane destabilizing activity, or whether membrane fusion proceeds through a series of specific lipid rearrangements similar to what is envisioned during fusion mediated by enveloped virus fusion proteins (258). In the case of RRV-p14, it is known that it is both necessary and sufficient to cause membrane fusion (547), making it a *bona fide* fusion protein however; it is not known if membrane disruption is part of the fusion process. This newly attributed viroporin-like activity of ARV-p10 raises the question of what function, if any, cell-cell fusion serves in the replication cycle of the fusogenic reoviruses.

To better understand the connection between FAST protein-induced syncytium formation, altered membrane integrity, and the virus replication cycle, the syncytium-

inducing and membrane-lytic properties of RRV-p14, NBV-p10, ARV-p10 and BRV-p15 were analyzed. In contrast to published results, all FAST proteins studied here induce altered membrane permeability as a consequence of prior extensive syncytium formation. Further, pharmacological inhibition of apoptosis prevented membrane disruption while having no effect on fusion. Thus, the FAST proteins are not viroporins, but rather dedicated cell-cell fusion proteins. I propose that the FAST proteins serve dual roles in the virus replication cycle by mediating non-leaky membrane fusion and early cell-cell transmission of the infection followed by apoptotis-mediated disruption of syncytia and dissemination of the infection.

3.2. Results

3.2.1. Avian reovirus induces late-stage alterations in membrane permeability

Previous studies with ARV-176 demonstrated a relationship between membrane fusion, membrane disruption and virus release, implicating cell-cell fusion as a virulence factor (145, 147). To expand our understanding of the relationship between membrane fusion and membrane disruption in the reovirus replication cycle, another strain of ARV (ARV-138) was examined which induces smaller syncytia and is less pathogenic that ARV-176 (147). ARV-138 reassortants that contain the p10-encoding S1 genome segment from the highly fusogenic and highly pathogenic ARV-176 display increased fusion ability and pathogenicity (145, 147). This result suggests that the ARV-138-encoded p10 is responsible for the poor fusion activity and limited pathogenicity of the wild type ARV-138 virus. A recent report using ARV-S1133, a close relative of ARV-176 with a p10 protein identical to that of ARV-176, demonstrated the ability of this virus to induce membrane disruption at late infection times as detected by small molecule influx or efflux (43). Similar studies were conducted here with ARV-138 to determine if the slower fusion kinetics of this virus correlate with decreased or delayed membrane permeabilization in an attempt to better understand how fusion and membrane disruption promote viral pathogenesis.

Alterations to membrane permeability were detected and quantified using a standard [^{3}H]-uridine release assay used extensively to characterize small molecule release caused by viroporin-induced changes in membrane permeability (6, 43, 57, 71, 303, 418). Briefly, this method involves incubating cells with isotopically labeled uridine

prior to infection or transfection. Alterations to membrane integrity facilitate the release of uridine to the culture medium where it can be detected and compared to the intracellular amount. QM5 cells were infected with ARV-138 at an MOI of 5, and cells and supernatants were collected at various times post-infection and assayed for [^{3}H]-uridine release and infectious progeny virus (Figure 3.1A). Cells infected in parallel were fixed and Giemsa-stained to identify the extent of syncytium formation at each time point (Fig 3.1B). By 9 hpi ARV-138-induced syncytia were visible but no extracellular virus or changes to membrane permeability were observed (Figure 3.1). Significant levels of uridine release occurred between 20 and 36 h post-infection concomitant with extensive virus release and destruction of the cell monolayer (Fig 3.1). In contrast, ARV-176 and ARV-S1133 induce extensive fusion, decreased cell viability and uridine and virus release by 9-12 hpi (43, 145). Therefore, there is a correlation between the rate of polykaryon formation, membrane permeabilization and virus release, suggesting that FAST protein-mediated fusion induces membrane alterations and enhances a lytic response.

3.2.2. The extent of p10-mediated syncytium formation correlates with the rate of small molecule efflux

In order to directly examine the relationship between FAST protein-mediated syncytium formation and membrane alterations, the kinetics of syncytium formation and increased membrane permeability were examined in transfected cells expressing the related ARV-p10 or NBV-p10 proteins. Syncytium formation was qualitatively assessed by microscopic observation of Giemsa-stained monolayers, and a syncytial index (SI) was assigned by scoring the relative extent of cell fusion on a scale from 0 to 4 (see Figure 3.2). ARV-p10 and NBV-p10 displayed dramatically different fusion kinetics despite sharing 33% amino acid identity and containing a similar arrangement of structural motifs (Figure 3.2). NBV-p10 was the more robust fusogen, causing extensive syncytium formation (SI=3) by 12-14 hpt and by 16-18 hpt the entire monolayer was fused and beginning to detach. In contrast, ARV-p10 transfected cells took 24-36 h to reach a SI= 3, and 48 h before the entire monolayer was fused. The basis for the distinct fusion kinetics of these related FAST proteins is unknown, but may reflect anything from

protein expression, stability and trafficking to differences in the specific activity of these proteins.

The kinetics of uridine release correlated with the relative rates of syncytium formation induced by these p10 homologues, with altered membrane permeability only evident well after extensive syncytium formation. For example, ARV-p10-induced uridine release barely exceeded the level of spontaneous release from mock-transfected cells by 36 hpt, at which point the monolayer had already been extensively fused for approximately 12 h, and uridine release was not statistically significant ($p<0.01$) until 48 hpt (Figure 3.2A). The same trend (i.e. no significant uridine release until well after syncytium formation was extensive) was observed in NBV-p10-transfected cells, where uridine leakage was minimal, though statistically significant ($p<0.005$), at 16 hpt even though the entire monolayer had already fused (Figure 3.2A and B). Uridine leakage from NBV-p10-transfected cells reached maximal levels by 20-24 hpt, coinciding with the destruction of the cell monolayer. Therefore, as with ARV-infected cells, there existed a correlation between the rate and extent of syncytium formation induced by the p10 FAST proteins and membrane leakage. These results were also the first indication that the fusion reaction itself is relatively non-leaky, since many fusion events occur to generate large syncytia without concomitant uridine release.

3.2.3. Altered membrane integrity is a generalized feature of FAST protein-mediated syncytium formation

To determine whether the rate of syncytium formation mediated by other members of the FAST protein family also correlated with altered membrane permeability, RRV-p14 and BRV-p15 were examined using the same assays. RRV-p14-transfected cells displayed remarkably fast fusion kinetics, with syncytia appearing as early as 4-6 hpt (Figure 3.3A). Uridine release, however, was not detected until 16 hpt, approximately 4 h after the majority of cells in the monolayer were fused and syncytia began detaching from the substratum (Figure 3.3A). In contrast, BRV-p15-induced syncytium formation at a very slow rate, with polykaryons not appearing until 13-16 hpt (Figure 3.3A and B). As a result of the slow rate of cell fusion, BRV-p15-induced syncytium formation did not exceed a SI=3 by conclusion of the experiment at 48 hpt.

Uridine leakage from BRV-p15-transfected cells did not reach statistically significant levels above spontaneous release from mock-transfected cells, although a trend of increased uridine release was noted between 36-48 hpt (Figure 3.3A).

Therefore, the extent of altered membrane permeability induced by all members of the FAST protein family, as inferred from increased small molecule release from syncytial cells, correlated with the respective ability of each FAST protein to mediate multinucleated syncytium formation in cell culture. These data also suggested that the fusion reaction is not membrane disruptive to the point of causing loss of membrane integrity.

3.2.4. Extensive syncytium formation is a requirement for membrane leakage

To determine whether the observed correlation between FAST protein-induced syncytium formation and subsequent membrane leakage reflected a causal relationship, two experiments were conducted to test whether altered membrane permeability was due to cell-cell fusion or to some syncytial-independent influence of the FAST proteins. In other words, are the syncytia breaking down and becoming leaky or is it the mere presence of the FAST proteins in the cell membrane that is disruptive, as is the case with viroporins? In the first study, the ability of a polyclonal anti-p14 antiserum to inhibit RRV-p14-mediated syncytium formation was exploited (111). The effect of antibody-mediated fusion inhibition on membrane permeability was assessed using the uridine release assay. Treatment of RRV-p14-transfected cells with anti-p14 virtually abolished syncytium formation (SI=1) and uridine release at 20 hpt (Figure 3.4). Antibody inhibition of syncytium formation did not affect the expression of RRV-p14 on the plasma membrane as determined by flow cytometry (C. Barry, unpublished results). Control cells, transfected with RRV-p14 and treated with normal rabbit serum (NRS) displayed both extensive syncytium formation and uridine leakage indicating that extensive fusion, and not the presence of RRV-p14 in the plasma membrane, is responsible for membrane alteration (Figure 3.4).

In the second approach, non-fusogenic ARV-p10 mutants were used that are based on the fusogenic ARV-p10-2HAN (2HAN, two HA epitope tags at the N terminus) construct with point mutations in the ectodomain (C9S, cysteine 9 to serine), transmembrane domain (TM, glycines 49 and 50 to alanine) or the polybasic region of

the endodomain (Basic, lysine 67 to methionine). An ARV-p10 construct was also used that contains a deletion of the N-terminal 24 residues (p10del) that retains the integral membrane nature of authentic p10 but is devoid of membrane fusion activity (494). Authentic ARV-p10 induced both syncytium formation and uridine release while the p10del mutant did neither (Figure 3.5). This was contrary to a previous report stating that although p10del is non-fusogenic, it retains membrane permeabilizing activity (43). ARV-p10-2HAN caused cell-cell fusion at a slower rate than ARV-p10 (SI=2) and never displayed uridine release (Figure 3.5). Further, none of the non-fusogenic ARV-p10 point mutants was able to alter membrane integrity (Figure 3.5).

The use of both antisera and fusion-minus FAST protein constructs to prevent syncytium formation provides compelling evidence that the observed alterations in membrane stability result from extensive syncytium formation and not the mere presence of the FAST proteins in the plasma membrane.

3.2.5. Syncytial-induced membrane leakage is bidirectional

The analysis of membrane leakage from FAST protein-induced syncytia by monitoring uridine efflux was confirmed by examining the sensitivity of syncytial cells to influx of another small molecule, hygromycin B. Hygromycin B is a translation inhibitor, similar in size to uridine, and normally membrane impermeable (70, 71). When membrane stability is altered, hygromycin B entry into cells can be detected by a global decrease in cellular translation. This assay is commonly employed to analyze viroporin activity (70, 206). As with mock-transfected cells, NBV-p10-transfected cells were insensitive to hygromycin B at early times post-transfection (12 h) when syncytia formation had progressed to a SI=2-3, confirming the membrane impermeant nature of the inhibitor (Figure 3.6A). By 20 h post-transfection, when the NBV-p10-transfected cells reached a SI=3-4, cells had become sensitive to hygromycin B as evidenced by a decrease in translation levels. These results were in close agreement with the uridine leakage data, indicating that the permeability of syncytial cell membranes to both efflux and influx of small molecules was not compromised until syncytium formation was extensive.

The same situation applied to RRV-p14-transfected cells, although with different kinetics, where a SI=4 was reached by 12 hpt by which time cells were sensitive to

hygromycin B treatment (Figure 3.6B). A decrease in cellular translation in extensively fused, RRV-p14-transfected cells that were not treated with hygromycin B was also observed and was likely due to cell death or detachment associated with extensive syncytium formation. As with the uridine release assay, the addition of anti-p14 to prevent RRV-p14-mediated fusion also eliminated the sensitivity of cells to hygromycin B (Figure 3.6B). Moreover, these observations were not cell-specific, as RRV-p14-mediated syncytium formation resulted in hygromycin B sensitivity that correlated with extensive fusion when the assay was performed in Vero cells (Figure 3.6C), while similar analysis of NBV p10-transfected cells at 19 hpt when the SI=3 revealed no effect of hygromycin B treatment. Thus, the altered bi-directional membrane permeability associated with FAST protein expression is dependent on extensive FAST-mediated syncytium formation.

3.2.6. Extensive syncytium formation results in an apoptotic response

It was apparent that one consequence of FAST protein-induced cell-cell fusion is the ultimate detachment and death of the syncytial cells. To better understand the nature of the loss of syncytial cell viability, the large syncytia induced by RRV-p14 were analyzed for indicators of apoptosis. RRV-p14-transfected cells were fixed at 24 hpt (SI=4) and nuclei were stained with DAPI, a fluorescent dye used to detect the chromatin condensation associated with cells undergoing apoptosis (44, 201, 260). While mock-transfected cells displayed the occasional cell with fluorescent nuclei suggestive of condensed chromatin and apoptosis, syncytia present in RRV-p14-transfected cells demonstrated numerous nuclei with condensed chromatin (Figure 3.7). Interestingly, some syncytia contained almost entirely nuclei with condensed chromatin while few of the nuclei in other syncytia were fluorescently labeled with DAPI (data not shown), suggesting that an apoptotic response, once triggered in a syncytium, rapidly proceeds to affect all nuclei in that syncytium.

To confirm the DAPI results implicating an apoptotic response in the destruction of syncytial cells, FAST protein-transfected cells were analyzed for oligonucleosomal DNA fragmentation, a hallmark feature of apoptosis (233, 332, 390). Chromosomal DNA was isolated from RRV-p14- and NBV-p10-transfected cells at various times post-

transfection and analyzed for DNA fragmentation by agarose gel electrophoresis and ethidium bromide-staining (Figure 3.8). In both cases, no DNA degradation was apparent by 12 hpt a SI=3, but became detectable by 18 hpt and obvious by 24 hpt as indicated by the appearance of oligonucleosomal laddering. DNA fragmentation occurred at approximately the same time as uridine release (see Figures 3.2 and 3.3) and appeared to be dependent on extensive cell-cell fusion, as antibody-mediated inhibition of p14-mediated syncytium formation eliminated DNA fragmentation (Figure 3.8). ARV-p10- and BRV-p15-transfected cells, which in this experiment had only reached a SI=3 by 48 hpt, failed to induce detectable DNA fragmentation. Therefore, the FAST protein-mediated induction of extensive syncytium coincides with a loss of membrane integrity and an apoptotic response.

3.2.7. The apoptotic response is responsible for altered membrane integrity

The kinetics of membrane leakage and chromatin condensation and degradation did not permit an assessment of whether membrane leakage triggers an apoptotic response, or whether syncytial-induced activation of apoptotic pathways results in alterations to membrane integrity. To distinguish between these two possibilities, the effects of the cell permeant broad-spectrum caspase inhibitor Z-VAD-fmk on membrane leakage were examined. Addition of the caspase inhibitor to RRV-p14-transfected cells had no effect on RRV-p14-induced syncytium formation (Figure 3.9C), but had a dramatic effect on [^{3}H]-uridine leakage (Figure 3.9A). By 18 hpt, monolayers that were not treated with the apoptosis inhibitor, and which had already been at a SI=4 for 4 h, displayed the oligonucleosomal laddering characteristic of apoptotic cells (Figure 3.9B). Coincident with the appearance of chromatin degradation, extensive uridine leakage occurred from untreated p14-transfected cells (Figure 3.9A). The addition of Z-VAD-fmk to transfected cells prevented chromatin degradation (Figure 3.9B), consistent with the ability of this inhibitor to prevent caspase-activated, DNase-mediated oligonucleosomal DNA cleavage (510). The apoptotic inhibitor also eliminated uridine leakage from cells (Figure 3.9A) in spite of the fact that the entire monolayer had fused into one giant syncytium (Figure 3.9C). Therefore, it can be concluded that extensive

syncytium formation triggers apoptotic pathways that contribute to altered membrane integrity and cell death.

3.3. Discussion

The fusogenic reoviruses are the only non-enveloped viruses capable of inducing cell-cell fusion. This ability is the result of expression of a single gene encoding one of the FAST protein family members (43, 111, 128, 494). In the case of reptilian reovirus, its p14 protein is both necessary and sufficient to induce membrane fusion in pure lipid bilayers (547). Exactly how these unusual proteins cause cell-cell fusion and the role of cell-cell fusion in the reovirus replication cycle are poorly understood. The results presented here demonstrate that the FAST proteins are dedicated cell-cell fusion machines that initiate membrane merger in a relatively non-leaky manner. Furthermore, alterations to membrane stability caused by FAST protein expression are not the result of a porin or lytic activity associated with the FAST proteins, but instead reflect a cellular response to FAST-induced syncytium formation that results in apoptotis-mediated effects on membrane stability. Together these observations help to define a role for the FAST proteins as virulence factors in the reovirus replication cycle.

3.3.1. The FAST proteins function as membrane fusion proteins, not viroporins

The FAST proteins bear no resemblance to the much larger, multimeric fusion proteins of enveloped viruses that have complex ectodomain structures needed for mediating membrane merger (224, 507). Instead, the FAST proteins share similarity with a diverse group of viral proteins that perturb host membranes, the viroporins. Like the FAST proteins, viroporins are typically small, integral membrane proteins with membrane-proximal polybasic domains. The ability of viroporins to alter membrane stability and increase permeability to small molecules such as uridine and hygromycin B implicates these proteins in virus-induced cytopathic effects and in facilitating virus release (70, 71, 206, 330).

ARV-p10 was recently reported to be a viroporin based on its ability to cause membrane destabilization (43). Further, partial deletion of the ARV-p10 ectodomain rendered the protein unable to cause cell-cell fusion but it retained the ability to induce

membrane leakage from transfected BSC-40 cells, suggesting that p10-induced membrane leakage is not dependent on membrane fusion (43). The authors proposed that the fusion and membrane permeabilizing functions of ARV-p10 represent the actions of distinct and unrelated domains of the protein and report that leakage always precedes syncytium formation (43). These observations suggest that the primary function of ARV-p10 is to destabilize cell membranes. These results also suggest the interesting hypothesis that the fusogenic activity of the FAST proteins evolved from a viroporin ancestor common to members of the *Reoviridae* family. Historically the presence of a viroporin may have helped these non-enveloped viruses escape from infected cells by causing membrane disruption late in infection. The fusogenic reoviruses could have gained the ability to destabilize both a donor and target membrane sufficiently to mediate membrane merger. This speculation is not unreasonable, as evidenced by the recent demonstration that the VP5 protein of Bluetongue virus, a nonenveloped virus capsid protein involved in endosomal membrane permeabilization, is capable of causing cell-cell fusion when a modified version of VP5 that includes a signal peptide and transmembrane domain is expressed inside transfected cells (172). However, the evidence presented here does not support the view that FAST protein-mediated fusion is a consequence of membrane perturbation. Instead, the data suggest that the FAST proteins are dedicated membrane-fusion proteins and are mediators of non-leaky fusion.

All four FAST proteins examined (RRV-p14, NBV-p10, ARV-p10 and BRV-p15) induced substantial syncytium formation with no alterations in membrane permeability to efflux or influx of small molecules. Such changes in membrane stability occurred only after extensive fusion in both QM5 fibroblast and Vero epithelial cells. Further, membrane alterations were not observed when fusion was inhibited using either fusion-minus ARV-p10 constructs or by antibody-mediated inhibition of RRV-p14-induced syncytium formation. Also, the slowly fusogenic ARV-p10-2HAN failed to induce membrane alterations despite the presence of syncytia, similar to the effects of the weakly fusogenic BRV-p15, lending further support to the notion that extensive syncytium formation is required for alterations to membrane permeability.

Contrary to a previous report by Bodelon *et al.* (43), the partial ectodomain deletion of ARV-p10 did not cause fusion or membrane alterations in the experiments

described here. The only apparent difference between these experiments is the cell lines used. However, BSC-40 (used by Bodelon *et. al.*) and Vero cells (used here) are both monkey kidney epithelial cells, so the chance of cell-line specific differences accounting for the different results is minimal. Regardless, it is possible that the ARV-p10 deletion mutant can induce fusion-independent membrane permeability in BSC-40 cells, but this may be an isolated incident. The data acquired here using multiple FAST proteins in several cell lines supports the contention that the FAST proteins are not inherently membrane destabilizing, that they mediate membrane fusion in a non-leaky manner and that extensive syncytium formation is required to induce membrane permeability.

Bodelon *et. al.* also suggest that the ARV-p10 ectodomain is responsible for fusion while the endodomain mediates membrane disruption. The use of several non-fusogenic point mutants of ARV-p10 does not support this theory. Since point mutations in the transmembrane and endodomains of ARV-p10 abrogate fusion, it seems clear that those domains are also essential for the fusion reaction. Conversely, ectodomain mutants failed to cause membrane alterations, despite the presence of wild type transmembrane and endodomains. It is possible that the presence of two HA epitope tags in these mutants is responsible for loss of membrane disruption in a fusion-independent manner. However, this explanation is in conflict with the proposal that the ectodomain mediates fusion while the transmembrane and endodomains are responsible for membrane disruption.

In further support of membrane permeability being the result of extensive syncytium formation, FAST protein-transfected cells failed to display altered membrane integrity in the absence of syncytium formation when other permeability assays based on DAPI or trypan-blue staining, or on [^{51}Cr] release, were employed (data not shown).

Therefore, results obtained using four different FAST proteins, two different cell lines and five different leakage assays indicated that FAST protein-induced syncytium formation was a prerequisite for, and always preceded, membrane leakage. This observation argues against FAST protein-induced syncytium formation being the consequence of viroporin-like membrane disruption. It would appear that the FAST proteins are capable of orchestrating the reorganization of lipid bilayers in such a manner

so as not to alter the membrane permeability barrier, suggesting that the FAST proteins have evolved specifically as membrane fusion proteins.

3.3.2. FAST protein-induced syncytium formation triggers an apoptotic response that contributes to altered membrane integrity

Cell-cell fusion is a very uncommon cytopathic effect to be induced by non-enveloped viruses. Syncytium formation is generally associated with enveloped-virus infections, where infected cells express the viral fusion proteins on their plasma membranes as a natural consequence of the assembly and release strategies of these viruses. The role of syncytium formation in the replication cycle of enveloped viruses is unclear, but it is becoming increasingly apparent that cell-cell fusion may confer certain replication advantages (63, 74, 269, 449). The ultimate fate of virus-induced syncytium formation is the death of infected cells as progeny virions assemble and are released from infected cells. The mechanism of cell death, however, is a matter of debate. For example, numerous studies are in conflict as to whether syncytia induced by measles virus or HIV undergo apoptotic or necrotic cell death (23, 72, 160, 168, 169, 178, 414, 471).

Based on the data presented here, it is now apparent that syncytia generated by FAST protein-mediated fusion die by a mechanism characteristic of apoptosis. The morphology of syncytial nuclei at late transfection times was indicative of apoptosis (Figure 3.7) and contained signs of chromatin condensation and marginalization (44, 260). Exclusive to apoptotic death is the generation of oligonucleosomal DNA fragments which were observed in FAST protein-transfected cells only at late transfection times when syncytia formation was extensive (Figure 3.8). DNA degradation was abrogated by inhibition of effector caspases using the pancaspase inhibitor Z-VAD-fmk (Figure 3.9). Similar observations have been reported for lentivirus-induced syncytium formation where apoptotic cell death is inhibited by Z-VAD-fmk (72, 148, 471), while the necrotic cell death attributed to syncytia induced by the Gibbon Ape leukemia virus fusion protein shows no such inhibition (232). Thus, the data indicate that extensive FAST-induced cell-cell fusion triggers an apoptotic response in syncytial cells.

Furthermore, apoptotic signaling serves as the trigger for alterations to membrane stability in FAST protein-transfected cells, since prevention of apoptosis with a caspase

inhibitor prevented both DNA degradation and uridine release (Figure 3.9). RRV-p14-mediated syncytium formation was unaffected by caspase inhibition and the entire monolayer (>10^6 cells) fused without a concomitant loss of membrane integrity. This observation strongly supports the hypothesis that the FAST proteins are mediators of non-leaky fusion and do not have inherent membrane-permeabilizing properties. Further, it is the extent of syncytium formation that triggers the apoptotic response and subsequent loss of membrane integrity. The exact trigger for syncytium-induced apoptosis is unknown, but others propose that the inability to maintain homeostasis because of alterations to the surface area to volume ratio of syncytial cells, or incorporation of apoptotic cells from the monolayer could be contributing factors (259, 471). The results presented here argue against the latter since apoptotic signaling or subsequent membrane permeability is never observed when syncytia are smaller. The presence of individual apoptotic cells in untransfected monolayers is not uncommon (data not shown). If syncytial cells were incorporating already apoptotic cells from the surrounding monolayer, then one would expect small syncytia to become apoptotic and leaky, and they do not. Instead, the data suggest that anti-apoptotic signals from the non-apoptotic syncytium may be dominant. Alterations to the surface area to volume ratio is more likely to affect syncytia formed with suspension cells since this ratio quickly changes in these spherical syncytia. This ratio is easily maintained in relatively two-dimensional cell monolayers, which likely explains why physiological syncytia consist of structures such as long thin myotubes and the sheet-like syncytiotrophoblast barrier (367, 431). However, very large syncytia might succumb to such changes or any number of other challenges to maintaining homeostasis in a cell with tens or hundreds of nuclei.

The fate of FAST protein-mediated syncytia lies in stark contrast to physiological syncytia formed during such cellular processes such as the formation of syncytiotrophoblasts, myotubes, and osteoclasts (246, 431, 438, 557). In these cases, cell-cell fusion is the result of specific differentiation pathways that are actually dependent on triggering early stages of the apoptotic cascade through activation of the initiator caspase 8 and externalization of phosphatidylserine (38, 245, 557). Unlike FAST protein-induced syncytia, these physiological examples are able to regulate and control the apoptotic signaling cascade in mature syncytia through expression of the anti-

apoptotic Bcl-2 protein throughout the syncytiotrophoblast, or through localized expression of the pro-apoptotic Bak protein to regulate turnover and maintenance of the syncytium (245, 246, 431, 438). Whatever the signal for apoptosis activation is in the experiments described here, kinetic analysis indicates that there is a significant lag between FAST protein-induced polykaryon formation and the effects of triggering the apoptotic response. As a result, syncytial cells remain viable and metabolically active until late in the infection cycle.

3.3.3. A potential dual role for syncytium formation in the virus replication cycle

Although many enveloped viruses cause cell-cell fusion during the course of infection, the role of this process in the virus replication cycle is poorly defined. The traditional interpretation is that syncytium formation is merely a consequence of plasma membrane expression of the viral fusion protein, but it is becoming increasingly clear that cell-cell fusion may confer certain replication advantages to the infecting virus. In the case of HIV, transition from a nonsyncytial- to syncytium-inducing phenotype correlates with advanced disease progression (50, 63, 269, 449). However, since this transition is due to changes in the gp120 receptor-binding protein, it is difficult to separate the effects of altered coreceptor utilization and cell tropism from the influence of syncytium formation. The contribution of syncytium formation to the pathogenicity of measles virus is more clear, since increased pathogenicity correlates with the presence of the syncytial phenotype, even in the presence of reduced viral titers (74). The ability to cause extensive cell-cell fusion also increases the pathogenic potential of herpes simplex virus type 1 (155, 207, 255). The obvious advantage of syncytium formation for a virus is the ability to gain access to the replication machinery of neighboring cells without exposing virus particles to the hostile extracellular environment.

The replication advantages of cell-cell fusion are equally applicable to non-enveloped viruses and several lines of evidence suggest that the FAST proteins serve as virulence factors. Natural infections with the fusogenic reoviruses are associated with a variety of clinical disease symptoms that are not associated with their relatively benign cousins the non-fusogenic mammalian reoviruses (317, 394, 512, 573). Histopathological examination of tissues from animals infected with fusogenic reoviruses reveals syncytia

in a variety of tissues and organs suggesting that cell-cell fusion may directly contribute to the pathology of these viruses (230, 277, 278, 306, 573). Finally, increased pathogenicity is correlated with the genome segment encoding the FAST protein and a comparative study of two different strains of ARV revealed a correlation between the extent of syncytium formation and viral pathogenesis (147). It is interesting to note that highly pathogenic non-fusogenic reoviruses have not been isolated, and that all fusogenic reovirus isolates identified to date display increased pathology. However, it remains possible that other fusogenic species go undetected because they fail to induce appreciable pathological symptoms. Regardless, the body of evidence thus far strongly suggests that reovirus-induced syncytium formation functions as a virulence factor.

Building on a previous report that enhanced virus release due to syncytial-lysis might contribute to the pathogenic potential of ARV (147), the data presented here suggest that syncytium formation may serve additional roles in the reovirus replication cycle. First, cell-cell fusion allows the infecting virus to access the replication machinery of adjacent cells without having to first expose progeny virions into the host immune system in the extracellular milieu (Figure 3.10). Maintenance of syncytial membrane integrity at early infection times ensures the viability of the infected-cell translation machinery and establishment of a productive localized infection. Late in infection, when syncytia become much larger, induction of apoptosis produces alterations in membrane integrity that can facilitate efficient virus release and systemic dissemination of the infection (Figure 3.10). It is critical that the virus establish a balance between the rate of syncytium formation and virus replication in order to ensure that syncytial death coincides with completed assembly of progeny virions. In the case of ARV-p10, it has been suggested that the rate of syncytium formation may be controlled by the rate of protein degradation (493). This unusual regulatory mechanism may have evolved to ensure the infecting virus has sufficient time to replicate before syncytia become leaky. The ability to cause rapid virus release from syncytia is shown here for ARV-138 (Figure 3.1) and was previously demonstrated for ARV-176 (145). Interestingly, other viruses use apoptosis as a means to spread to adjacent cells, providing the process is not triggered too early in the replication cycle (404).

It would be ideal to further validate these studies with analysis of a fusion-deficient avian reovirus isolate, but such studies require, and await, the development of a reverse-genetics system for the fusogenic reoviruses. Fortunately, several recent reports present encouraging data that suggest reverse genetics may finally be a possibility for reoviruses (454-456). This system could be employed to confirm our hypotheses about the role of the FAST proteins in the reovirus replication cycle by generating non-fusogenic versions of ARV, NBV, RRV and BRV. Conducting low-MOI infections with these new viruses in parallel with wild-type virus and assessing the viral titre over time would allow one to monitor how syncytium formation affects the rate of virus replication. These same viruses could also be introduced into host organisms to determine the contribution of fusion to viral pathogenesis.

3.4. Figures

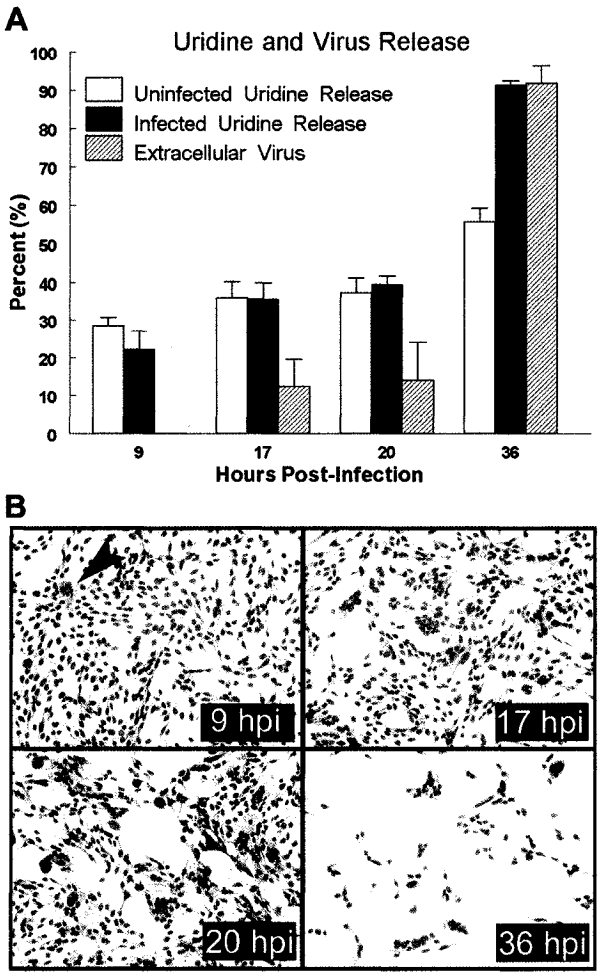

Figure 3.1 Membrane permeability of late-stage avian reovirus infection correlates with virus release. **(A)** QM5 cells loaded with [^{3}H]-uridine were infected with ARV-138 (MOI = 5) and harvested at the indicated times post-infection. Percent uridine release and extracellular virus from mock- and ARV-138-infected cells are expressed as the mean ± the SD of a representative experiment conducted in triplicate. **(B)** Infected cells were fixed and Giemsa-stained at the indicated times post-infection. The arrow indicates early syncytia. Infections and plaque assays were performed by J. Boutilier (collaborator, Dalhousie University).

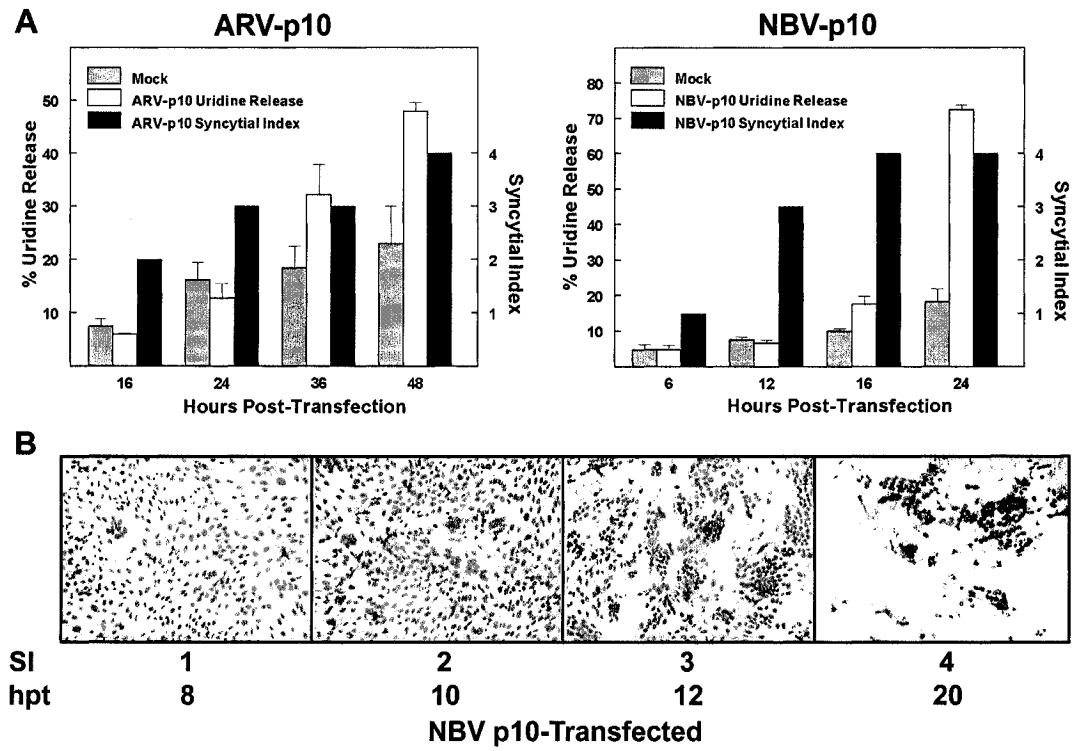

Figure 3.2 p10-mediated syncytium formation results in late-stage membrane permeability. **(A)** QM5 cells were labeled with [^{3}H]-uridine prior to transfection with NBV-p10, ARV-p10 or empty vector (mock). At the indicated times, cells were harvested, the extent of syncytia formation was rated and the percent of uridine in the supernatant was determined. Data are presented as mean ± the SE (n=3). **(B)** Wright-Giemsa stains of NBV-p10-transfected cells, fixed at the indicated hours post-transfection (hpt), and scored for the syncytial index (SI) on a scale of 1-4.

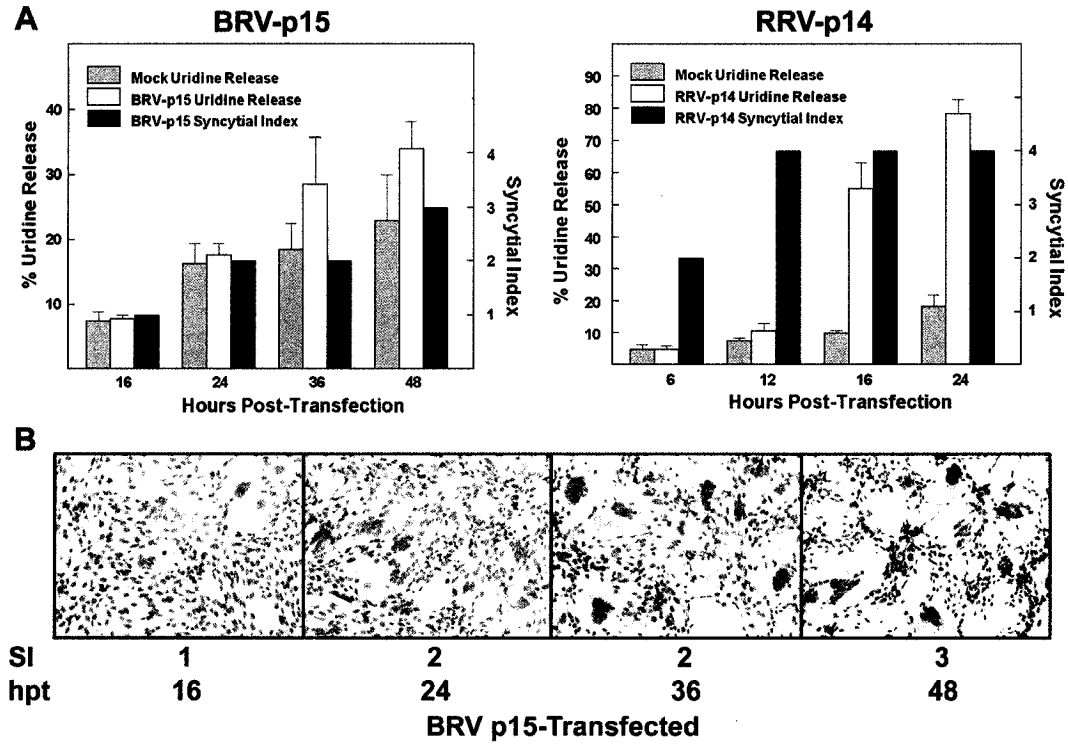

Figure 3.3 BRV-p15 and RRV-p14 induce late-stage membrane permeabilization. **(A)** QM5 cells were labeled with [^{3}H]-uridine prior to transfection with BRV-p15, RRV-p14 or empty vector (mock). At the indicated times, cells were harvested and the extent of syncytia formation and uridine in the supernatant was determined. The values are presented as mean ± the SE (n=3). **(B)** Giemsa stains of BRV-p15-transfected cells fixed at the indicated hours post-transfection (hpt) and indicating the syncytial index (SI).

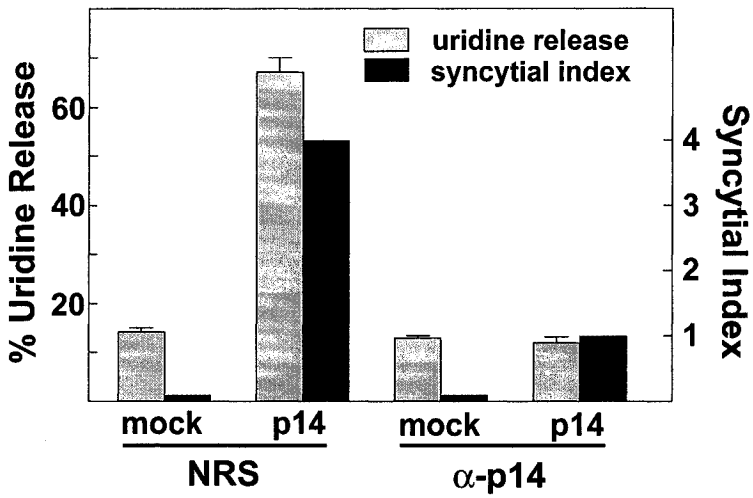

Figure 3.4 Inhibition of syncytia formation prevents membrane permeabilization. QM5 cells were labeled with [^{3}H]-uridine for 18 hours prior to transfection with RRV-p14 or empty vector. At 3 hpt, anti-p14 polyclonal antiserum, or normal rabbit serum (NRS) was added to cells (1:20 dilution). Cells and supernatants were harvested at 20 hpt and the extent of syncytia formation and the percent of uridine release were determined. Values represent the mean ± the SD of a representative experiment conducted in triplicate.

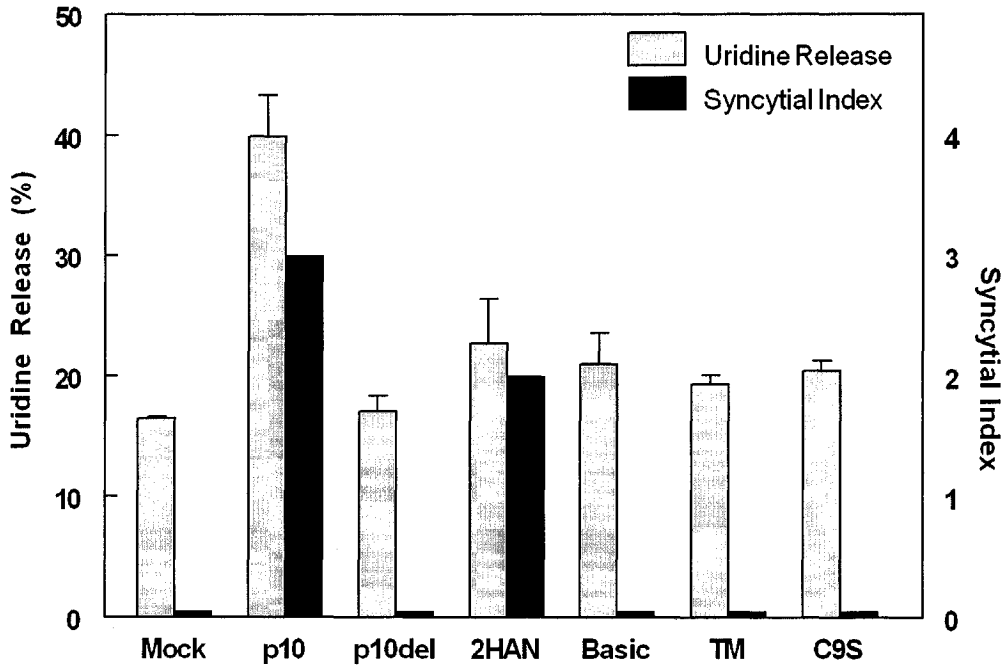

Figure 3.5 ARV-p10-induced membrane permeability correlates with extensive syncytium formation. QM5 cells were labeled with [^{3}H]-uridine for 18 hours prior to transfection with empty vector (Mock), ARV-p10 (p10), or the indicated ARV-p10 fusion-deficient mutants. Cells and supernatants were harvested at 48 hours post-transfection and the extent of syncytia formation and the percent of uridine release were determined. Values represent the mean ± the SD of a representative experiment conducted in triplicate

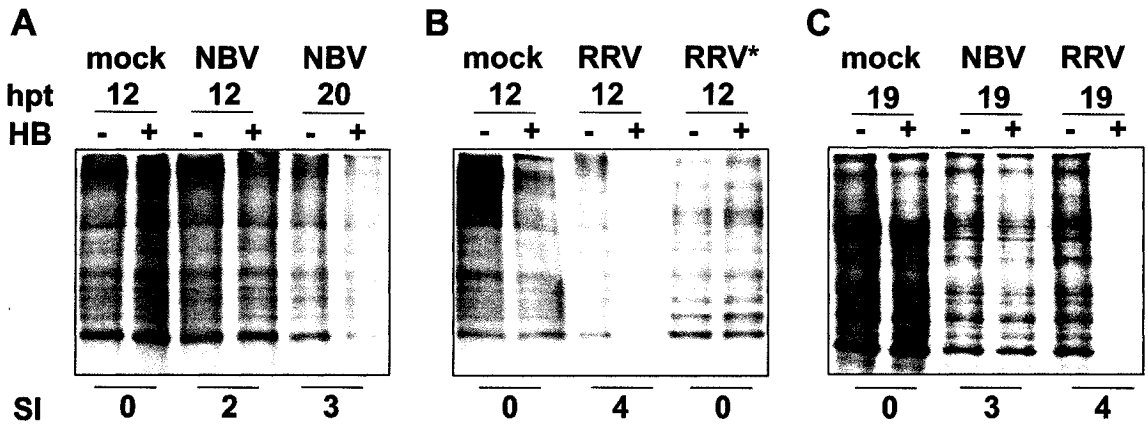

Figure 3.6 Membrane permeabilization is bidirectional and not cell specific. QM5 (**A** and **B**) or Vero (**C**) cells were transfected with empty vector (mock), RRV-p14 or NBV-p10 and incubated in methionine-free medium in the presence or absence of 1.5 mM hygromycin B for 45 min at the indicated times post-transfection. Cells were then labeled with [^{35}S]-methionine in the presence or absence of hygromycin B for 45 min, lysed in RIPA buffer and subjected to 15% SDS-PAGE. The extent of syncytia formation at the time of labeling was noted (SI). RRV* denotes p14-transfected cells treated with anti-p14 antibody to inhibit fusion.

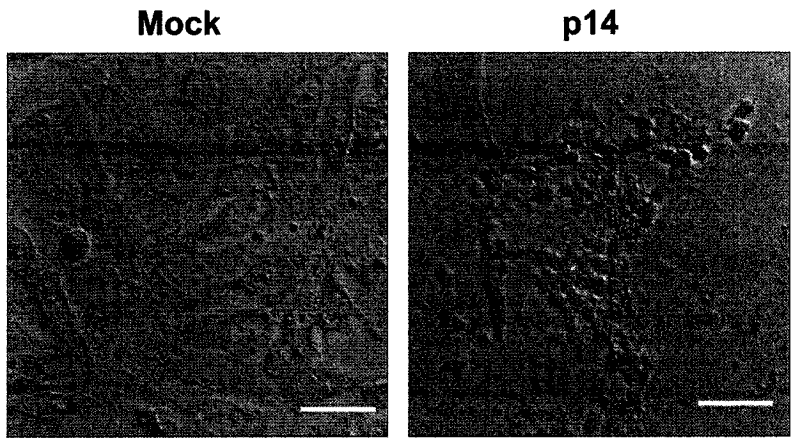

Figure 3.7 FAST-protein induced syncytia undergo apoptosis. QM5 cells transfected with empty vector (mock) or RRV-p14 were fixed at 24 hpt and stained with DAPI. The clustered pyknotic nuclei of RRV-p14-transfected cells are indicative of a syncytium undergoing apoptosis. Scale bar = 50μm

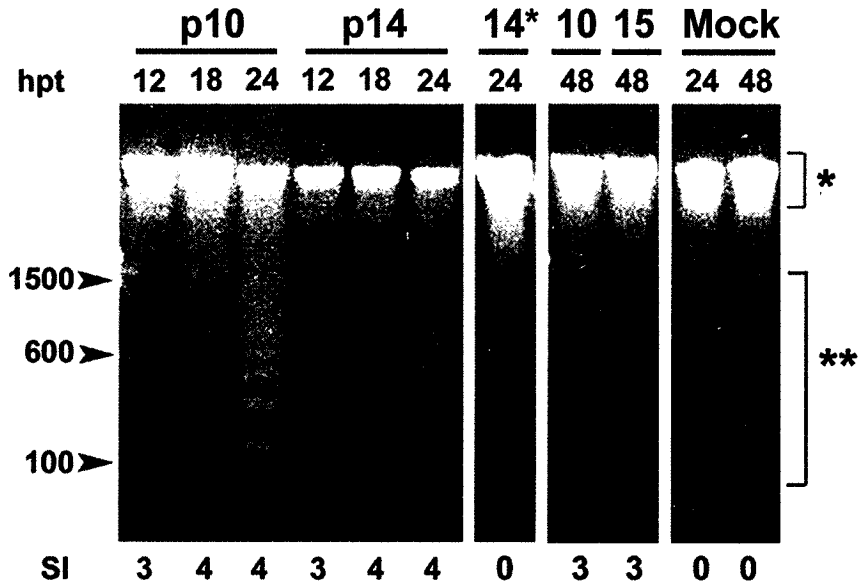

Figure 3.8 Extensively fused FAST protein-transfected cells undergo apoptosis. DNA was isolated from FAST protein-transfected cells at the indicated times post-transfection and intact (*) and fragmented DNA (**) was resolved on 1% agarose gels. The syncytial index (SI) was also determined at the time of DNA isolation. The lane labelled 14* denotes RRV-p14-transfected cells treated with anti-p14 antibody to inhibit fusion. The data for this figure were generated and compiled by D. Top (collaborator, Dalhousie University).

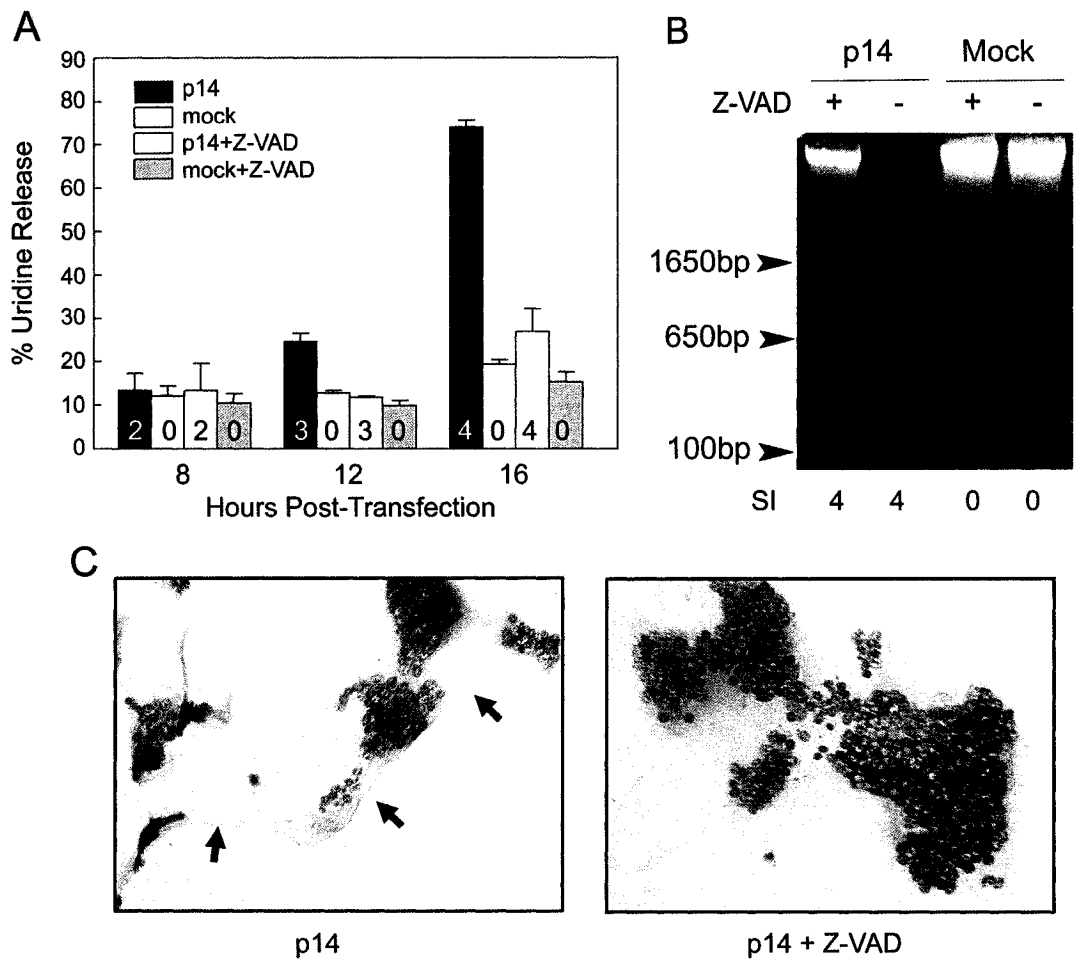

Figure 3.9 Apoptosis triggers membrane permeability. **(A)** [^{3}H]-uridine labelled QM5 cells were transfected with RRV-p14 or empty vector (mock) and incubated in the presence or absence of the caspase inhibitor Z-VAD-fmk. At the indicated times post-transfection, the percent uridine release and syncytial index (inset numbers) were determined. Values represent the mean ± the SD of a representative experiment conducted in triplicate. **(B)** DNA was isolated at 16 h post-transfection from RRV-p14- or empty vector (mock)-transfected QM5 cells incubated with or without caspase inhibitor and resolved on a 1% agarose gel. The syncytial index (SI) of each sample at the time of DNA isolation is indicated. **(C)** Giemsa stains of completely fused QM5 cell monolayers 16 hours after transfection with RRVp14 in the presence or absence of caspase inhibitor (Z-VAD). Arrows indicate the borders of the syncytium detaching from the culture dish in the absence of Z-VAD-fmk.

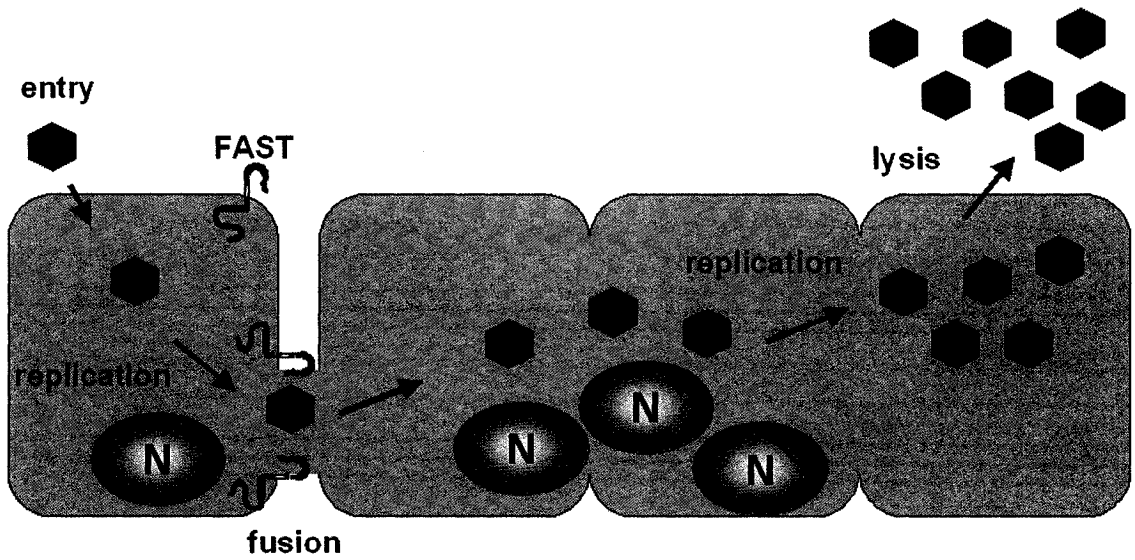

Figure 3.10 The fusogenic reovirus replication cycle. The fusogenic reovirus enters the cell without the need for membrane fusion. Translation of viral genes supports viral replication and expression of the FAST protein. The FAST proteins exploit cellular adhesion structures to cause cell-cell fusion and multinucleated syncytium formation. The replicating virus now has access the nucleotide pool and translation machinery of neighbouring cells without having exposed itself to the extracelluar environment. Extensive syncytium formation induces apoptosis, resulting in loss of membrane integrity and assisting in virus release.

CHAPTER 4
The Reovirus FAST Proteins use Surrogate Adhesion Proteins in an Uncoupled Membrane Fusion Reaction

4.1. Introduction

Membrane fusion is an energetically unfavourable process, requiring protein catalysts to orchestrate the complex lipid rearrangements that occur during membrane merger. The proteins involved in membrane fusion function as homotypic multimers or heterotypic multi-protein complexes to execute the binding, close membrane apposition, hemifusion, pore formation and pore expansion of the fusion reaction. Most viral fusion proteins, such as influenza virus HA, HIV gp160, VSV-G and flavivirus E proteins, are receptor-binding proteins as well as fusion proteins, and thus contain all of the components necessary to regulate and execute membrane merger (118, 258, 280).

Other fusion systems are more complex. Paramyxoviruses, for example, express two different glycoproteins on their surfaces, both of which are required for fusion. Different receptor-binding proteins specifically interact with the F protein, the fusion protein, to activate F and trigger membrane fusion (304). Like other fusion proteins, F protein-mediated fusion uses conformational rearrangements to pull membranes into close apposition and initiate hemifusion and pore formation (304). Thus, paramyxovirus fusion proteins are an example of a multi-component fusion machine that uses one protein for binding and regulation and another for mediating the last four steps of the fusion reaction.

Cellular SNARE proteins function as part of a more complex fusion machine. Many different tethering and binding proteins promote the targeting and docking of vesicle membranes to the correct subcellular membrane (47). These interactions promote the assembly of the SNARE proteins, as well as the recruitment of other associated factors, that contribute to the fusion reaction (59). It is generally believed that the SNARE proteins act as the core fusion machinery since pairing of opposing SNAREs is required for fusion. Thus, an individual t-SNARE or v-SNARE cannot be classified here as a fusion protein (Section 1.8), but together they comprise the minimal unit necessary and sufficient for fusion (335, 585).

From the above discussion, there are many ways to assemble a fusion machine and different steps of the fusion reaction can be assigned to different parts of the same protein (e.g. HA, VSV-G) or to different proteins altogether (e.g. SNAREs, paramyxovirus). Generally, in muti-component fusion complexes, receptor binding is the step most often assigned to an individual protein, which then specifically interacts with the protein(s) responsible for lipid merger to induce structural changes in the protein complex and drive the fusion reaction. Targeting and docking of the two membranes, therefore, usually precedes, and is a trigger for, activation of the fusion reaction. The coupling of binding to fusion in these diverse fusion machines reflects the need for temporal and spatial regulation of fusion to prevent inappropriate content mixing.

This ability to assign binding function to another component in a fusion complex may be part of the problem when trying to identify cell-cell fusion proteins. It is likely, and becoming increasingly evident, that cell-cell fusion is the result of multi-component fusion complexes that are regulated by the activity of cell adhesion molecules (86). Most of the cellular fusion proteins implicated in cell-cell fusion reactions are actually receptor-binding proteins and lack classical fusion-promoting domains such as fusion peptides and α-helical bundles (e.g EFF-1, CD9, ADAMs) (86). Thus, the core fusion machinery of these cell-cell fusion complexes remains elusive.

As specific mediators of cell-cell rather than virus-cell fusion, the FAST proteins are better classified as "cellular" fusion proteins and as such are the only positively identified proteins with the primary function of causing cell-cell fusion. In contrast to candidate cell-cell fusion proteins, the adhesive properties of the FAST proteins is highly questionable while the fusion activity is confirmed (547). The classification of the FAST proteins as fusion proteins results from their conforming to several strict criteria. First, the FAST proteins are the only viral proteins necessary to cause cell-cell fusion between several heterologous cell types demonstrating their ability as cell-cell fusion proteins. Reconstituted RRV-p14 in liposomes is necessary and sufficient to mediate liposome-cell and liposome-liposome fusion confirming that no other protein factors are necessary in the donor or target membrane for fusion to proceed (547). Only a few other fusion proteins (e.g. HA, VSV-G, SNAREs) have met these stringent requirements for

classification as the necessary and sufficient machinery to cause fusion (86, 152, 308, 371).

What remains unclear is whether the FAST proteins function as autonomous fusion machines. With ectodomains of less than 45 aa, the FAST proteins apparently lack the structural complexity necessary to mediate membrane binding or generate the force required for close membrane apposition. If the FAST proteins require other co-factors to mediate either of these steps in the fusion reaction, then their definition as fusion machines may need to be re-evaluated. The fact that RRV-p14-containing liposomes fuse to target lipid bilayers confirms that RRV-p14 is a fusion protein capable of inducing membrane merger (547). These results do not, however, imply that RRV-p14 is a fusion machine capable of executing all five fusion steps. For instance, liposome-liposome fusion experiments required calcium to aggregate liposomes and allow fusion to proceed while liposome-cell assays used excess quantities of liposomes, of which only a small percent bound to cells (547). Thus, calcium and liposome concentration can serve as aggregating forces that provide membrane proximity, suggesting the possibility that the FAST proteins may not be responsible for the binding and close apposition steps of the fusion reaction.

These observations suggest three possible models for FAST protein-mediated fusion. First, the FAST proteins may be fusion machines capable of mediating all five of the fusion steps. This would require an unexpected and unusual mechanism for FAST protein-mediated receptor binding and close membrane apposition. Second, the FAST proteins might directly interact with cellular adhesion machinery that supplies the binding and apposition steps of the fusion reaction. In this example, the FAST proteins would be fusion proteins that are the core fusion machinery of a multi-component cell-cell fusion machine. The caveat for this model is that the FAST proteins would have to directly interact with cellular machinery that triggers FAST protein-mediated fusion.

The third model is that the FAST proteins are unregulated fusion proteins that rely on surrogate adhesion machinery to provide membrane closeness, but do not specifically interact with this adhesion machinery for an activation signal. In this scenario, the binding and fusion steps are uncoupled and are mediated by separate and non-interacting proteins.

The data presented here demonstrate that the FAST proteins have uncoupled adhesion and fusion and rely on the host cell to provide membrane proximity as a prerequisite to untriggered membrane fusion. As a result, the FAST proteins do not rely on triggered structural rearrangements to promote close membrane contact in a manner characteristic of the enveloped virus and SNARE fusion machines. Instead, the small ectodomains of the FAST proteins are devoid of the structural elements found in other fusion systems, which forces the FAST proteins to adopt a novel mechanism for promoting hemifusion and pore formation in the absence of the mechanical energy provided by dramatic conformational rearrangements.

4.2. Results

4.2.1. The FAST proteins are not receptor-binding proteins

To determine whether the FAST proteins might utilize cellular cofactors for early steps in the fusion reaction (i.e. receptor binding and/or close membrane apposition), the FAST proteins were expressed in multiple cell lines of various species and tissue origins to determine if a specific cellular factor might be acting as a FAST protein receptor. RRV-p14 was the most robust fusogen and was able to cause fusion within 16 h of transfection in every adherent cell line tested from a wide range of species and tissue origin (Figure 4.1). Similarly, the other FAST proteins were able to fuse a variety of adherent cell lines, although with varied rates of fusion depending on the FAST protein and the cell type (Figure 4.1). In general, RRV-p14 and NBV-p10 caused fusion much faster than BRV-p15 and ARV-p10 (Figure 4.1). In some cases, the level of fusion in cells transfected with the slower fusogens was difficult to distinguish from background fusion in control cells transfected with empty vector leading to inconclusive results (Figure 4.1). An interesting outlier to the general trend of ARV-p10 being the weakest fusogen was when this protein was expressed in L-929 cells where it caused fusion at a rate similar to that produced by RRV-p14, perhaps indicating that some unique attribute of L-929 cells might enhance ARV-p10-mediated fusion (Figure 4.1). Finally, none of the FAST proteins was able to initiate cell-cell fusion in the non-adherent Jurkat cells (Figure 4.1). Other non-adherent cell lines not listed in Figure 4.1, including EL-4 murine B-cells and Sf21 insect cells, did not fuse when RRV-p14 was expressed in them.

One explanation for the promiscuous fusion activity of the FAST proteins is that they do not bind to a specific cellular molecule as a prerequisite for fusion. In support of this observation, during the course of these studies RRV-p14 was shown to mediate fusion of pure lipid bilayers (547). However, RRV-p14-containing liposomes do not specifically adhere to QM5 target cells in liposome-cell fusion studies, suggesting that RRV-p14 does not possess receptor-binding activity (D. Top, unpublished data). However, the small size of the FAST proteins suggests that some mechanism for bringing membranes together must be required.

Since Jurkat cells grow in suspension, they do not form the number and type of contacts found in adherent cell lines. Unless activated, they form very few stable cell-cell contacts, which could prevent the FAST proteins from being able to initiate fusion in these cells. T-cells can be induced to homotypically aggregate *via* the cell adhesion molecules leukocyte function antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1) when stimulated to proliferate with phorbol 12-myristate 13-acetate (PMA) (94, 130). If the FAST proteins exploit the closeness provided by cell adhesion molecules, activated T-cells expressing one of the FAST proteins may be susceptible to fusion. To test this hypothesis, Jurkat cells were transfected with plasmids encoding GFP or RRV-p14-GFP and assessed by FACS analysis for protein expression in the presence or absence of PMA (Figure 4.2A). About 10-15% of all cells showed increased fluorescence above that of mock-transfected cells (Figure 4.2A) with some cells fluorescing 10,000 times more intensely than background, indicating strong expression in these cells (data not shown). Activation of Jurkat cells with PMA promoted strong homotypic aggregation (Figure 4.2B) but had no effect on the susceptibility of these cells to FAST protein-mediated fusion as determined by Giemsa-staining (Figure 4.2C). Thus, despite protein expression and aggregation, Jurkat cells did not fuse.

These results led to the hypothesis that the FAST proteins are dependent on specific types of cell-cell contacts that do not exist in suspension cells. Adherent cells generate many specialized cell-cell adhesive structures and any, or all, of these might be important for promoting FAST protein-mediated fusion (254, 302, 388, 474). In view of the ability of the FAST proteins to fuse numerous different types of adherent cells, the FAST proteins may have evolved to rely on ubiquitous cellular adhesion machinery,

common to all adherent cell types, to supply the binding and membrane apposition steps of the fusion reaction. To investigate the contributions of cellular adhesion molecules to FAST protein-mediated fusion, a model system was needed. QM5 cells were chosen as a model adherent cell line because of their high transfection efficiency and resistance to spontaneous fusion (10). RRV-p14 was chosen as the model FAST protein because of its robust and promiscuous fusion activity.

4.2.2. Cadherins are important for FAST protein-mediated fusion

If the FAST proteins rely on cellular machinery to bridge opposing membranes, then that machinery should be common to the epithelial and fibroblast cells tested. There are only a few types of adhesion molecules and cellular junctions common to all adherent cell types including cadherins, adherens junctions and gap junctions (254, 302, 388). Adherent cell types initiate cell-cell junction formation through calcium-dependent adhesion molecules called cadherins (Figure 4.3) (52). There are several different cadherin family members with epithelial cells generally expressing E-cadherin and fibroblasts expressing N-cadherin (285, 535). Sequence differences between different cadherins ensure that they only homotypically pair in *cis* and in *trans* between opposing cells in a calcium-dependent manner to mediate cell-cell contact (285, 401). The highly conserved cadherin cytoplasmic domains recruit cytoskeleton adaptor proteins, actin and other adhesion molecules to the contact site (Figure 4.3) (427, 567), leading to the formation of cell-cell junctions that include, depending on the cell type, adherens junctions, tight junctions, desmosomes and gap junctions (52, 474, 491). Thus, inhibition of calcium-dependent cadherin engagement results in loss of virtually all cell-cell contacts.

The calcium dependence of cadherins was exploited to examine the role of cadherin-mediated contacts and junction formation in the FAST protein fusion reaction. Cadherin interactions for fibroblast and epithelial cells were disrupted by removing extracelluar calcium and incubating cells in calcium-free medium (566, 567) (Figure 4.4A). The role of cadherin-dependent contacts on FAST protein-mediated fusion was investigated by comparing the extent of fusion of FAST protein-transfected cells under normal and low-extracellular-calcium conditions. The extent of syncytium formation in

RRV-p14-transfected QM5 fibroblasts and MDCK epithelial cells incubated in calcium-deficient medium was dramatically inhibited compared to cells incubated with normal calcium levels (Figure 4.4B). Quantification of fusion under each condition using the syncytial indexing assay determined that for all FAST proteins, fusion was inhibited by 80-90% under low-calcium conditions relative to normal calcium levels in QM5 cells (Figure 4.5A). Similarly, RRV-p14-mediated fusion in MDCK cells was inhibited by ~70% under low-calcium conditions (Figure 4.5A). The observed fusion inhibition in the absence of extracellular calcium was consistent with a possible role for cadherin-mediated junctions.

If the FAST proteins are relying on cadherins to generate fusion sites, then at least some of the surface-localized RRV-p14 should co-localize with surface cadherins. In QM5 cells, N-cadherin and RRV-p14 could be found together at regions of cell-cell contact (Figure 4.5C, arrows). While RRV-p14 was predominantly randomly distributed on the cell surface, it did concentrate near regions of intense N-cadherin labelling, supporting the idea that cadherins could compensate for the inability of the FAST proteins to mediate membrane adhesion.

To demonstrate the specificity of cadherin involvement in FAST protein-mediated fusion, the fusion activity of VSV-G and influenza virus HA was assessed under low-calcium conditions. Both of these enveloped-virus fusion proteins bind to their cellular receptors (PS and sialic acid, respectively) in a calcium-independent manner and were unaffected by calcium depletion (Figure 4.5B). These observations support the idea that the FAST proteins have an atypical dependence on cadherin-mediated contacts for fusion. Further, depletion of extracellular calcium and loss of cell-cell adhesion could have a number of effects on cell signalling or actin remodelling that could render a cell incapable of fusion or syncytium formation (181, 284, 567). However, the ability of HA and VSV-G to cause fusion in the absence of extracellular calcium confirmed that these effects did not influence the general ability of treated cells to fuse or generate syncytia under low-calcium conditions. Thus, the inability to mediate fusion in low-calcium conditions was a unique property of the FAST proteins.

To further implicate cadherins and cadherin-mediated junctions in the FAST protein fusion reaction, L cells, which are deficient in cadherin expression, and the same

cells stably transfected with E-cadherin (EL cells), were used. L-cells transfected with RRV-p14 displayed limited cell-cell contact and, although fusion occurred, it was slow to initiate (~20 hpt) and progress in these cells compared to other fibroblast cells, such as QM5 cells (4 hpt), despite similar transfection efficiencies (50-70%) (Figure 4.1A). Introduction of E-cadherin into L cell fibroblasts, which do not normally form close cell-cell contacts, resulted in increased contact and conferred a more epithelial-like morphology (Figure 4.6A). Given the role of cadherins in FAST protein-mediated fusion, it was expected that the increase in cadherin-mediated cell-cell contacts in EL cells would dramatically increase the susceptibility of these cells to fusion. Unexpectedly, RRV-p14-transfected EL cells did not appear, by visual analysis, to fuse much better than RRV-p14-transfected L cells (Figure 4.6B). However, when transfected in parallel under normal and low-calcium conditions, EL cells under normal calcium levels did fuse slightly, but significantly ($p < 0.05$), better than L cells under normal calcium conditions (Figure 4.6C).

As expected, since L cells lack cadherin-based contacts, RRV-p14-mediated fusion in the cadherin-deficient L cells was insensitive to calcium depletion (Figure 4.6C). Conversely, the moderate increase in RRV-p14-mediated fusion in EL cells over L-cells was lost in the absence of calcium, suggesting that the increase in fusion in EL cells was due to the presence of E-cadherin, and that this advantage was lost when calcium was removed and cadherins could not function. This experiment also confirmed that the inhibition of fusion under low-calcium conditions was due to cadherin disruption and not a pleiotropic effect of calcium disruption such as signalling or cytoskeleton alterations. These results suggested that cadherins can enhance FAST protein-mediated fusion, but cadherins alone do not promote efficient cell-cell fusion mediated by the FAST proteins.

4.2.3. Active adhesion facilitates efficient FAST-mediated fusion

While FAST-mediated fusion of L-cells was enhanced by the presence of E-cadherin, the increase in fusion was only about 35% (Figure 4.6C). However, in QM5 and MDCK cells, fusion under high-calcium conditions was generally 4-6 fold higher than when calcium was removed (Figure 4.5A). This discrepancy could be explained if

cellular factors downstream of cadherin contacts contribute to the efficiency of FAST protein-mediated fusion. Cadherins are critical for the formation of adherens junctions, which in turn help regulate the establishment and maintenance of other cell-cell junctions including tight junctions, desmosomes and gap junctions (52, 474, 491). The maturation of a cadherin-based contact into an adherens junction requires the recruitment of actin to the cytoplasmic domain of the cadherin *via* adaptor proteins such as β-catenin, α-actinin and α-catenin (427, 567). This remodelling increases the stability of the contact allowing other adhesion structures to form (254, 427).

To determine whether interactions that occur downstream of calcium-mediated cadherin-pairing influence FAST protein-mediated fusion, MDCK cells were used as an epithelial cell model and positive control. MDCK, L and EL cells were examined for the expression and localization of E-cadherin, β-catenin and actin to determine if EL cells were able to form stable adherens junctions (Figure 4.7). As expected, actin, β-catenin and E-cadherin in MDCK cells were found associated with regions of cell-cell contact, indicating the presence of adherens junctions and the ability of actin to remodel in response to E-cadherin engagement (Figure 4.7A and 4.3C) (427, 566). This phenotype is referred to as "active adhesion". Conversely, L cells do not express E-cadherin and expression of β-catenin is restricted to a small percentage of L cells as indicated by immunofluorescence microscopy and western blot analysis (Figure 4.7). Further, extensive actin structures were generally not found at cell-cell contacts in L cells (Figure 4.7A). This phenotype, which lacks cadherin contact and actin remodelling, is referred to as "no adhesion", meaning no cadherin-mediated adhesion.

As expected, EL cells strongly express E-cadherin, which could be found at regions of cell-cell contact (Figure 4.7). Similarly, β-catenin was localized to sites of cell-cell contact, indicative of its responsiveness to E-cadherin *trans*-pairing (Figure 4.7A). However, actin did not ring the cell periphery as in MDCK cells. Instead, it appeared more disorganized, forming long fibres which could even be detected by light microscopy (Figures 4.6 and 4.7). This phenotype, where cadherins were engaged but actin was not remodelled to form cellular junctions, is referred to as "passive adhesion". It is not clear why the EL cells do not form proper junctions, but they may lack an adaptor protein to recruit actin or contain disregulated signalling pathways that prevent

correct actin responses to cadherin engagement. Regardless of the cause, the lack of active adhesion may explain why supplying receptor-binding proteins alone (*i.e.* E-cadherin) resulted in only a moderate increase in RRV-p14-mediated fusion. Thus, active adhesion may better support FAST protein-mediated fusion than passive adhesion.

To directly test the influence of active adhesion on FAST protein-mediated fusion, a method was devised to generate the three different adhesion phenotypes in the same cell line. QM5 cells were chosen because of their sensitivity to calcium depletion and high transfection efficiency. The active-adhesion, passive-adhesion and no-adhesion phenotypes were generated using a combination of low-calcium treatment to disrupt cadherin contacts and cytochalasin D treatment to prevent actin remodelling, as described in Materials and Methods. Figure 4.8A and B shows the different adhesion phenotypes in QM5 cells. Typical of fibroblast morphology under active adhesion conditions, N-cadherin was localized to regions where cells contact and overlap. Under no-adhesion scenarios (i.e. calcium depletion), both actin and cadherin were dispersed and no cell-cell contacts were formed. Passive-adhesion conditions were generated by first disrupting cadherins and cell-cell junctions with low-calcium, then adding calcium back in the presence of actin inhibition (i.e. cytochalasin D) to allow cadherin-mediated contacts to form between cells but prevent actin remodelling and active adhesion. Under passive-adhesion conditions, cells were able to mediate contact but not remodel actin (Figure 4.8A and B).

The effect of the different adhesion scenarios on RRV-p14-mediated fusion was determined by quantifying the extent of syncytium formation under the various treatment conditions (Figure 4.8C). Since cytochalasin D has an inhibitory effect on FAST protein-mediated fusion (see Figure 6.5C), a very low dose of the drug was used where fusion was minimally affected. Addition of 0.1 μg/ml of cytochalasin D to cells where cadherins were not disrupted was sufficient to partially disrupt actin while maintaining active adhesion conditions, and caused only a ~35% inhibition of RRV-p14-mediated fusion (Figure 4.8C, grey bars, and Figure 6.5A). At these low concentrations of cytochalasin D, the actin-capping activity of the drug is sufficient to decrease the ability of cells to generate new actin filaments at cell-cell adhesion sites, but is not sufficient to disrupt junctions that have already formed. Fusion under low-calcium conditions, with or

without cytochalasin D, was inhibited by ~80% (Figure 4.8C, black bars). Cells that received low-calcium to disrupt junctions, followed by incubation in normal calcium medium without cytochalasin D, restored their cell-cell contacts and fused as well as did control cells (Figure 4.8C, white bar). However, cells treated with low-calcium, then incubated in normal calcium with cytochalasin D fused only slightly better than cells in low-calcium (Figure 4.8C white bar). These cells were only able to form passive adhesions, with cadherins mediating cell-cell contact without actin remodelling to form junctions (Figure 4.8A and B). Therefore, cells forming active adhesions consistently support RRV-p14-mediated fusion better than cells forming passive adhesions. This suggests that the FAST proteins require intact cellular adhesion and junction-forming machinery for efficient cell-cell fusion.

4.2.4. Surrogate receptors can increase FAST protein-mediated fusion efficiency

The FAST proteins appear to rely on cellular adhesion machinery to provide close membrane apposition as a prerequisite for fusion. The fact that homotypic aggregation of Jurkat cells did not support FAST protein-mediated fusion might indicate that the FAST proteins specifically require cadherin-mediated contacts to increase fusion efficiency. To test this hypothesis, the ability of influenza HA to bind cellular sialic acid in a calcium-independent manner was employed. Stably transfected QM5 cells expressing influenza HA (H1 strain) were created and HA expression was confirmed by immunostaining (Figure 4.9A) and a syncytium-forming assay based on low-pH triggering of HA-mediated fusion (Figure 4.5B). Fusion in RRV-p14-transfected QM5 cells without HA was inhibited by 85% in low-calcium conditions (Figures 4.5A and 4.10A). However, RRV-p14-mediated fusion in QM5 cells stably expressing influenza HA (QM5-HA) was only inhibited by about 45% under low-calcium conditions, demonstrating the ability of HA to partially compensate for the loss of cadherin-mediated contacts (Figures 4.9B and 4.10A).

Similar results were obtained with CHO cells stably expressing the influenza HA X-31 strain (H3N2) (Figure 4.10B). RRV-p14-mediated fusion of normal CHO cells was inhibited by almost 70% in low-calcium conditions while fusion in the CHO-HA cells was only inhibited by about 30% (Fig 4.10B). That HA expression did not result in a full

restoration of RRV-p14-mediated fusion is consistent with the idea that active adhesion is required for efficient fusion. QM5-HA and CHO-HA cells would not be expected to form active adhesion under low-calcium conditions since HA molecules lack the conserved cadherin cytoplasmic domain required for actin remodelling in response to *trans*-cadherin binding.

Because the pH was not decreased during the experiment, and HA requires low pH as a fusion trigger, it is unlikely that the increase in fusion in the HA-expressing cells was due to the fusion activity of HA. However, to further confirm that HA fusion was not being triggered during the experiment, cells transfected with vector alone or the non-fusogenic mutant RRV-p14-G2A were analyzed for signs of fusion. Under similar treatment conditions, in the absence of functional RRV-p14, there was absolutely no indication of fusion in the HA-expressing cells, indicating that only RRV-p14 is contributing to the fusion signal in these experiments (Figure 4.10).

Further, horse serum (HS) was used to inhibit HA binding in QM5 cells. The α_2-macroglobulin and other components in horse serum are known inhibitors of HA binding to sialic acid (351, 463). The appropriate concentration of horse serum to inhibit ~90% of HA-mediated fusion in QM5-HA cells was experimentally determined to be 20% (Figure 4.11A). The addition of horse serum on QM5-HA cells restored the sensitivity of RRV-p14 to calcium-depletion resulting in significant fusion inhibition similar to non-HA QM5 cells (Figures 4.9B and 4.10A). To ensure that the changes in fusion were due to inhibited binding of HA to sialic acid and not the effect of increased serum percentage or the change from FBS to HS, QM5-HA cells were transfected in parallel and treated with 10% or 20% FBS or HS. All treatment conditions yielded similar amounts of fusion, supporting the contention that blocking HA receptor binding renders RRV-p14 transfected QM5-HA cells more susceptible to fusion inhibition in low-calcium conditions (Figure 4.11B).

If the receptor-binding ability of HA is able to enhance FAST protein-mediated fusion then, as with cadherins, RRV-p14 and HA should be found at regions of cell-cell contact even under low-calcium conditions. Immunofluorescence microscopy confirmed that RRV-p14 and HA are at least partially co-localized to regions of cell-cell contact in normal and low-calcium conditions, supporting the hypothesis that HA can act as a

surrogate receptor for RRV-p14 (Figure 4.12). Thus, the FAST proteins are able to exploit the membrane proximity provided by proteins other than cellular adhesion machinery, indicating that the FAST proteins have a general requirement for surrogate adhesion machinery to enhance their fusion potential.

4.3. Discussion

4.3.1. The FAST proteins uncouple binding and fusion by using cellular adhesion machinery

Models for protein-mediated membrane fusion based on viral envelope or cellular SNARE proteins involve five fusion steps (22, 104, 318, 509). First, opposing membranes are tethered and docked by a receptor-binding protein that also confers specificity to the fusion reaction (280, 528). Second, triggered structural rearrangements pull the donor and target membranes into close proximity (1-2 nm) (22). This facilitates lipid mixing (hemifusion), pore formation and pore expansion as the final fusion steps (22). The need for regulation has forced fusion proteins and fusion complexes to rely on binding to regulate and trigger fusion.

In all systems other than the FAST proteins, binding and fusion are coupled, with the former regulating and often triggering the latter through specific protein-protein interactions. Such is not the case with the FAST proteins where, prior to these studies, it was unclear if the FAST protein are complete fusion machines, like influenza HA, and capable of mediating the five steps of the fusion reaction. Alternatively, the FAST proteins could be the fusion component of a multiprotein fusion complex with cellular adhesion machinery providing membrane proximity that triggers FAST protein-mediated fusion. This latter option would be consistent with their apparent role as "cellular" fusion proteins.

The data presented here demonstrate that the reovirus FAST proteins are somewhere in between these two options, in that they are dedicated cell-cell fusion proteins that have completely uncoupled binding from fusion by relying on surrogate adhesion machinery to bring two membranes together while retaining the ability to mediate membrane merger. Thus the FAST proteins do not require a specific membrane-binding event as a fusion trigger, but are able to exploit the closeness provided by generic adhesion machinery to execute the latter steps of membrane fusion involving lipid

reorganization. These results are in contrast to molecules like HA that are able to execute all five steps of fusion, or SNAREs and parmyxovirus fusion systems where fusion is accomplished by the concerted efforts of individual proteins directly interacting as a fusion complex.

4.3.2. FAST proteins are not receptor-binding proteins and are not fusion machines

Several lines of evidence indicate that RRV-p14 and the other FAST proteins are not receptor-binding proteins. Every adherent cell line tested was capable of supporting RRV-p14-medited fusion regardless of species or tissue origin (Figure 4.1), and RRV-p14-containing liposomes bind non-specifically and with low affinity to target cells (547) (D. Top, unpublished data). The other FAST proteins also showed an ability to fuse a variety of cell types, suggesting the lack of specific receptor-binding capability. The small sizes and sequence diversity of the FAST protein ectodomains further argue against specific receptor-binding activity, as the potential cellular ligands would have to be ubiquitously expressed and conserved enough among cell types of various species and tissue origin to be recognizable by all the different FAST proteins. In an equally improbable scenario, it seems unlikely that several ubiquitous cellular factors are expressed on the variety of cell types analyzed, and that each could serve as specific ligands for individual FAST proteins.

Rather than interacting specifically with surface receptors, the FAST proteins, as shown here, can initiate cell-cell fusion by exploiting the closeness provided by cellular adhesion machinery or foreign receptor-binding proteins. Disruption of cadherin-based cell-cell junctions by depletion of extracellular calcium or with cytoskeleton-altering drugs caused significant inhibition of FAST protein-mediated fusion (Figures 4.5 and 4.8). Further, the binding activity of influenza HA was sufficient to partially restore FAST protein-mediated fusion when cellular junctions were disrupted (Figure 4.9). Similarly, the introduction of E-cadherin into E-cadherin-deficient L cells was able to increase fusion efficiency (Figure 4.6). Thus, the requirement for receptor-binding proteins is a general one and not specific for any particular adhesion molecule. However, different surrogate receptors (HA and cadherins) can influence the efficiency of FAST protein-mediated fusion.

These differences might be attributed to the closeness provided by surrogate adhesion molecules. When the largest FAST protein ectodomain (ARV-p10, 44 aa) is modelled in an extended conformation the maximum distance it could protrude from a cell surface is about 15.4 nm. Modelled as an α-helix, this number is reduced to 6.6 nm. Either of these distances might be sufficient to span the distance between cells held together by certain adhesion molecules, but this would involve the exposure of either myristic acid (BRV-p15 and RRV-p14) or a hydrophobic patch (ARV-p10, NBV-p10 and RRV-p14) to the aqueous environment. This energetically unfavourable event is not likely to occur, and most probably the FAST proteins attempt to bury their hydrophobic domains in the donor membrane. This idea is substantiated by evidence from collaborators that reveals, by atomic force microscopy and nuclear magnetic resonance analysis, that the RRV-p14 ectodomain forms a loop that only extends 0.7-1.5 nm from the membrane (M. Jericho, unpublished results) (112).

Thus, the small size and limited reach of the FAST protein ectodomains suggest they should be highly dependent on membrane proximity provided by surrogate adhesion molecules. The closer two membranes are brought together, the better chance the FAST protein has of interacting with a target membrane to cause fusion. This hypothesis is supported by the data described here. Influenza virus HA extends about 10-13 nm from the cell surface and binds sialic acid on the opposing membrane (280, 596). Interactions with sialic acid-modified lipids (e.g. gangliosides (7)) would bring cell membranes within 10-13 nm of each other which is considerably closer than the 20-40 nm distance generated by cadherin contacts (280, 312, 437, 611). There is some debate about the nature of cadherin-mediated adhesion. Each cadherin molecule extends about 20 nm from the cell surface and contains five extracellular domains that can interact with other cadherin molecules in *cis* on the same cell or in *trans* between cells (551, 611). Only the outermost extracellular domain is required for calcium-dependent adhesion and is the site of initial contact (82, 484). However, experimental evidence suggests that all five domains can be involved in binding causing the *trans*-interacting cadherins to interdigitate, effectively halving the intermembrane distance to about 20 nm and adding stability to the adhesion site (Figure 4.3) (312, 611). This is almost twice the estimated distance of HA-mediated cell-cell contact (10-13 nm). The increased proximity afforded

by HA may account for the observation that HA causes a greater level of fusion under low-calcium conditions compared to passive adhesion or EL over L cells. With a maximum reach of ~15 nm it is possible for a FAST protein ectodomain to engage cells held at HA-mediated distances, but would be much more difficult at cadherin-mediated distances.

The concept of distance as a critical factor contributing to FAST protein-mediated fusion may help explain the apparent inability of suspension cells to fuse. Even when Jurkat cells were stimulated by PMA to induce homotypic aggregation *via* the αLβ2 integrin LFA-1 and the immunoglobulin superfamily member ICAM-1 they did not demonstrate signs of fusion (12, 602). Cell membranes interacting in this way would be about 30-40 nm apart, since LFA-1 and ICAM each extend about 17-20 nm from the cell surface each and interact *via* binding sites on the distal portions of the proteins (12, 26, 249, 518, 602). This distance is about the same as the initial cadherin contacts of passive adhesion or EL cells, which fuse only slightly better than cells lacking cadherin engagement. A 40 nm span may not provide sufficient closeness to increase FAST protein-mediated fusion in Jurkat cells that fuse inefficiently or not at all to begin with. Alternatively, or additionally, fundamental differences between suspension and adherent cells might prevent Jurkat cells from participating in FAST protein-mediated fusion. For example, the cortical actin network in suspension cells might present a greater barrier to fusion than in adherent cells, or the inability to induce active adhesion in response to LFA-1-ICAM interactions might block fusion. Studies are currently underway to make stable Jurkat cell lines expressing RRV-p14 and/or influenza HA or E-cadherin, to determine if these adhesion molecules can render Jurkat cells susceptible to fusion.

In addition, the effect of binding distance on FAST protein-mediated fusion can be investigated by using a reductionist system, like liposomes, that is free of the multiple cellular binding proteins found in cell-cell fusion assays. Instead, membrane adhesion could be generated by using a combination of streptavidin and various biotin molecules containing different spacer arm lengths. Streptavidin could be covalently attached to one membrane and biotin to the other, or the streptavidin could be used to bridge two biotinylated membranes (77, 504). The variability in the spacer arm length would dock FAST protein-containing membranes at various distances and fusion would be monitored

to determine the relationship between binding distance and fusion efficiency. This approach could help determine the maximum distance from which the FAST proteins can initiate fusion. Remembering that the membrane is an essential and influential part of any fusion reaction, liposomes should be formulated to mimic the cellular environment as closely as possible. Alternatively, similar experiments could be conducted using cells or plasma membrane vesicles.

The ability of the FAST proteins to cause fusion in the absence of cadherins further supports the promiscuous nature of the FAST proteins and the idea that any process to support cell-cell adhesion can be exploited by the FAST proteins. Also consistent with the model that the FAST proteins exploit any adhesive machinery is the observation of residual fusion (~20% of normal calcium conditions) in FAST protein-transfected cells treated with low-calcium. Calcium depletion only disrupts cadherins and the adhesive structures that depend on the stability of cadherin contacts such as adherens junctions, tight junctions, desmosomes, and gap junctions (52, 474, 491). There is a group of calcium-independent cell adhesion molecules, called nectins, that act upstream of cadherins (531). The nectin family of proteins consists of four members (nectin-1, -2, -3 and -4) of the immunoglobulin superfamily each of which has several splice variants (254, 531, 532). Multiple nectins are expressed in most cell types and facilitate cell-cell adhesion *via* homotypic or heterotypic interactions between cells (Figure 4.3) (241, 532). With three immunoglobulin loops ~4 nm in length, each nectin molecule extends about 12 nm from the cell surface (254, 518). Since nectins pair *via* their outermost immunoglobulin loop, opposing cells are brought to within 20-24 nm of each other (254, 518). This distance is similar to interdigitated-cadherin junctions and could well be sufficient to enhance FAST protein-mediated fusion. Nectin-nectin interactions are weaker than cadherin interactions but they serve to recruit cadherins and initiate cadherin-dependent cell-cell adhesion and junction formation (239-241, 254). The presence of nectins could explain why there is residual fusion in low-calcium treated cells or why the cadherin-deficient L cells are still susceptible to fusion, although at much slower rates (20 hpt vs. 6 hpt RRV-p14 in QM5). It would be informative to determine of this residual fusion is due to nectin activity. This question can be addressed in future studies by using siRNA directed against nectin mRNA, nectin-deficient cell

lines or antibodies that block nectin binding to inhibit nectin function and monitor the effect on fusion. All of these observations are also consistent with the idea that the FAST proteins are unregulated and do not require a specific fusion activator such as low pH or receptor binding. The FAST proteins are the first example of a fusion protein that functions without a specific receptor-binding component or protein partner that regulates fusion.

4.4. Figures

A

Cell Line	RRV-p14	NBV-p10	ARV-p10	BRV-p15	Species	Morphology
QM5					Quail	fibroblast
Vero					Monkey	epithelial
HeLa			48	48	Human	epithelial
Hep-2		48	48	48	Human	epithelial
CHO-K1					Hamster	epithelial
L-929				48	Mouse	fibroblast
C2C12			72	72	Mouse	fibroblast
NIH-3T3		UND			Mouse	fibroblast
MDCK					Dog	epithelial
Jurkat					Human	leukocyte

Legend	
	Fusion Begins by X hpt
UND	Undetermined
X	Inconclusive at X hpt
	No Fusion at X hpt

B

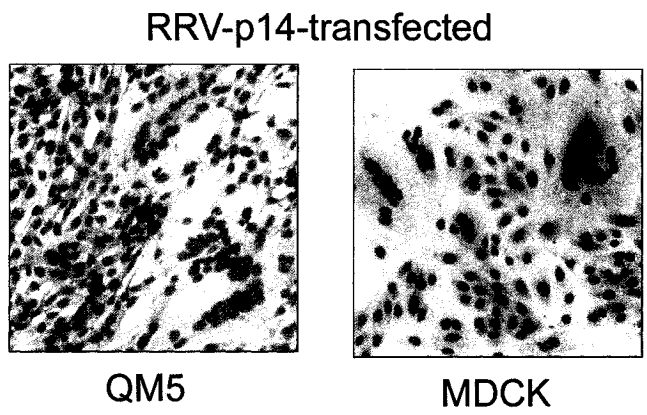

Figure 4.1 The FAST proteins are promiscuous fusogens. **(A)** Multiple cell lines from different species and tissue origin were transfected and assessed for their susceptibility to fusion by RRV-p14, BRV-p15, ARV-P10 or NBV-p10. **(B)** Giemsa stains of RRV-p14-induced syncytia in transfected QM5 (6 hpt) and MDCK (20 hpt) cells.

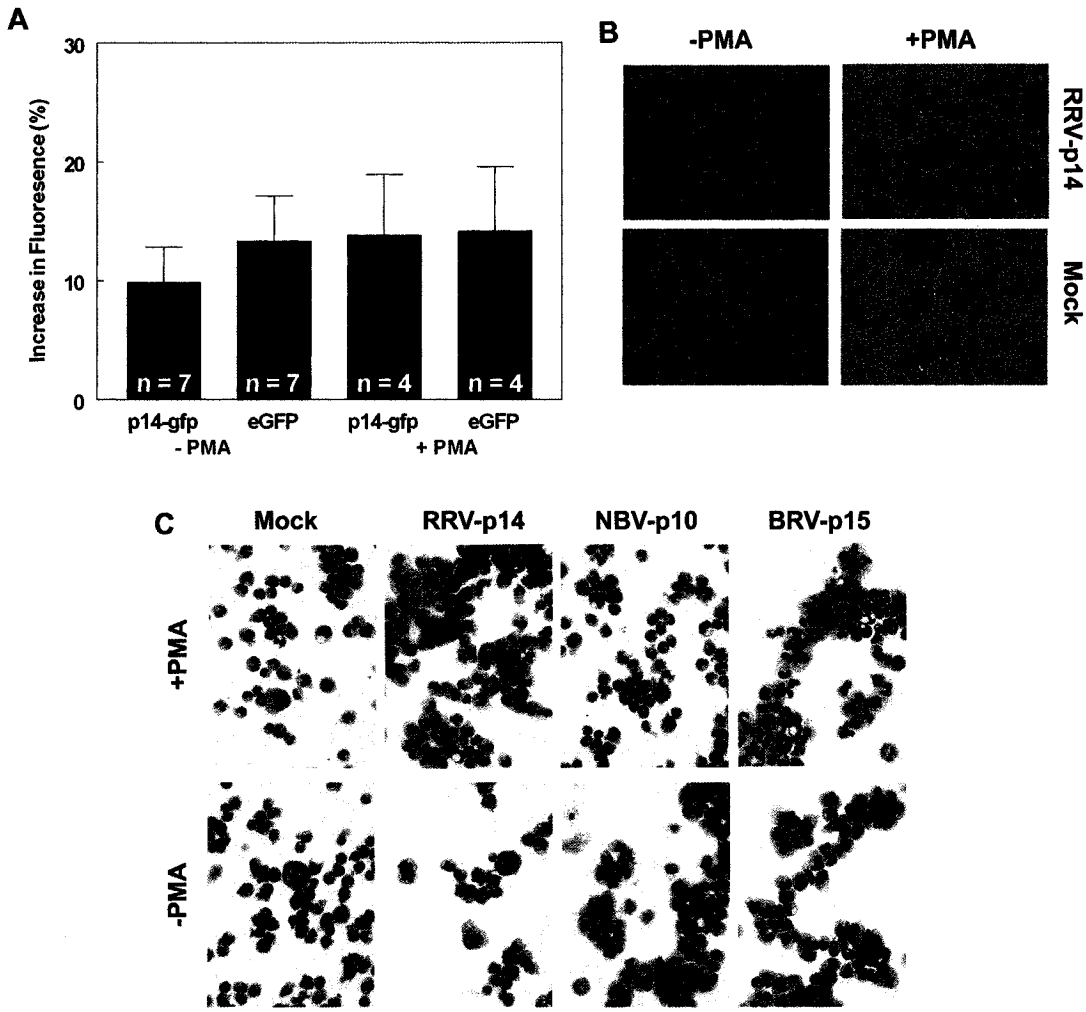

Figure 4.2 The FAST proteins do not fuse Jurkat cells. (**A**) FACS analysis was used to determine transfection efficiencies and protein expression in Jurkat cells by detecting increased GFP fluorescence. Overton subtractions were used to determine the percentage of p14-GFP or GFP transfected cells fluorescing above background 48 hpt with or without addition of 50 ng/ml PMA. (**B**) RRV-p14 or mock transfected Jurkat cells were treated with or without 50 ng/ml PMA and visualized at 24 h by light microscopy. Magnification = 200x. (**C**) Transfected Jurkat cells were treated with or without 50 ng/ml PMA, fixed at 48 hpt and Giemsa-stained to visualize nuclei and detect syncytium formation.

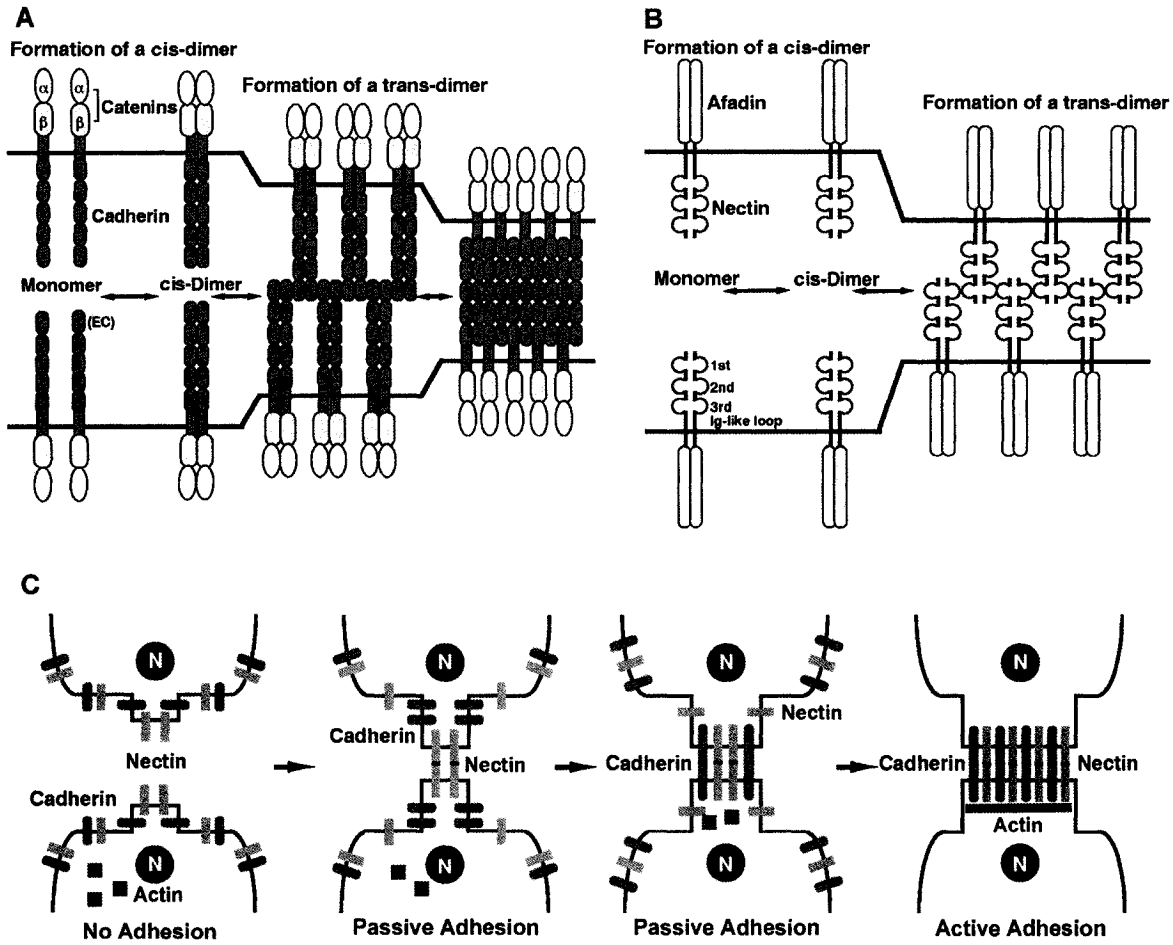

Figure 4.3. Cadherins and nectins mediate active and passive cell-cell adhesion. **(A)** Cadherins in opposing membranes assemble into *cis* dimers, which interact in a calcium - dependent manner to form *trans* dimers and mediate cell-cell contact. Cadherins can interdigitate to shorten the intermembrane distance and strengthen the adhesion site. **(B)** Similar to cadherins, nectins form *cis* and *trans* dimers to mediate adhesion. Nectins act upstream of cadherins in a calcium-independent manner. **(C)** Migrating cells not in contact with each other extend membrane protrusions with nectins at the tips (left cartoon). Nectin engagement recruits and stabilizes cadherin-mediated contacts prior to actin remodeling (passive adhesion, middle two cartoons). Recruitment of actin (purple) to the adhesion site stabilizes the contact and promotes junction formation (active adhesion, right cartoon). Modified from Takai and Nakanishi (2003) with permission from The Company of Biologists Ltd.

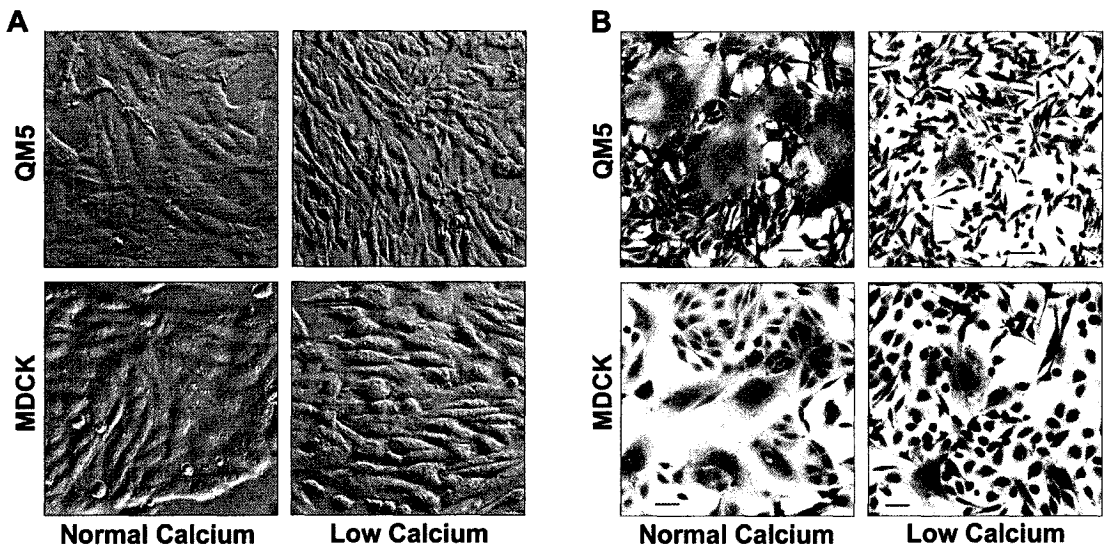

Figure 4.4 RRV-p14-mediated fusion is inhibited by disruption of cadherin-mediated cell-cell contacts. **(A)** Phase contrast microscopy of QM5 and MDCK cells under normal and low-calcium conditions. Magnification = 200x. **(B)** QM5 and MDCK cells were transfected with RRV-p14 and fusion progressed in the presence or absence of extracellular calcium. Cells were fixed and Giemsa-stained at 8 (QM5) and 20 (MDCK) hpt. Scale bar = 50μm.

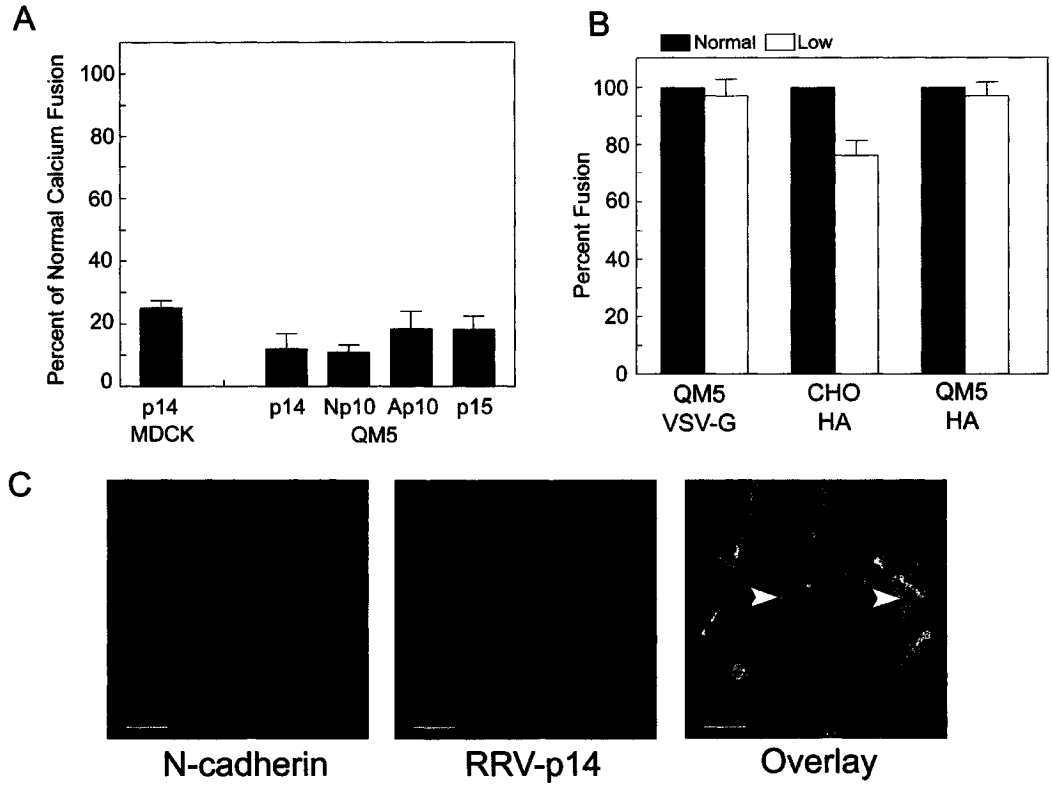

Figure 4.5 FAST protein-mediated fusion is sensitive to disruption of cadherin-dependent junctions. **(A)** FAST-transfected QM5 or RRV-p14-transfected MDCK cells were incubated in normal or low-calcium medium just prior to and during fusion. After fixation the average number of syncytial nuclei per field was determined by quantification of Giemsa-stained cells. Data are presented as the percent of fusion relative to that seen under normal calcium levels for each FAST protein in a given cell line. Values represent the mean ± the SE (n=3). **(B)** QM5 cells were transiently transfected with VSV-G or stably transfected with influenza virus HA (H1N1 strain) and fusion under normal or low-calcium conditions was quantified. Also, fusion was triggered in influenza virus HA-expressing (X-31 strain) CHO cells under normal and low-calcium conditions. Data are presented as percent fusion relative to the normal calcium conditions. Values represent the mean ± the SE (n=3-4). **(C)** Immunofluoresence and DIC images of N-cadherin (red) and RRV-p14 (green) in QM5 cells fixed at 4 hpt. Arrows point to regions of N-cadherin and RRV-p14 co-localization at cell-cell contacts as indicated by yellow pixels. Scale bar = 10μm.

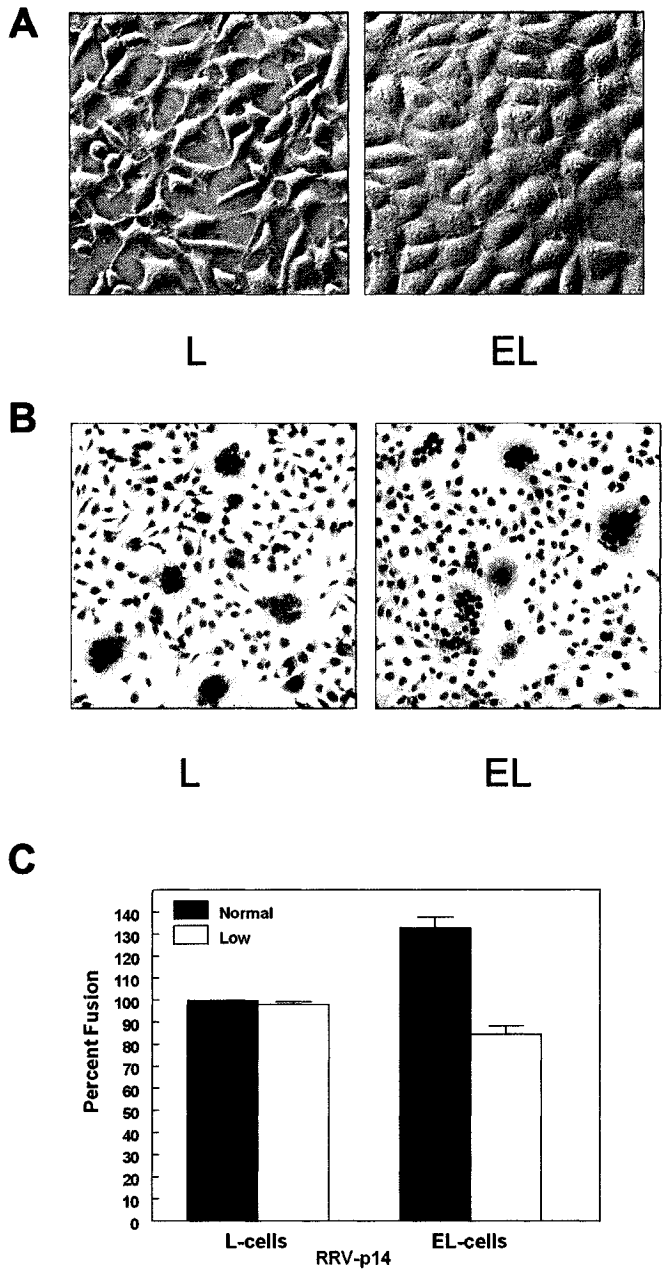

Figure 4.6 Cadherins moderately increase RRV-p14-mediated fusion. **(A)** Phase-contrast microscopy of L and EL cells (mag=400x). **(B)** Giemsa-stained L and EL cells fixed at 26 hpt with RRV-p14. (mag=200x). **(C)** L and EL cells were transfected with RRV-p14 in parallel, treated with normal or low-calcium medium, fixed at 26 hpt and syncytia formation was quantified. Data are presented as the percent fusion relative to L cells under normal calcium conditions. Values represent the mean ± the SE (n=3).

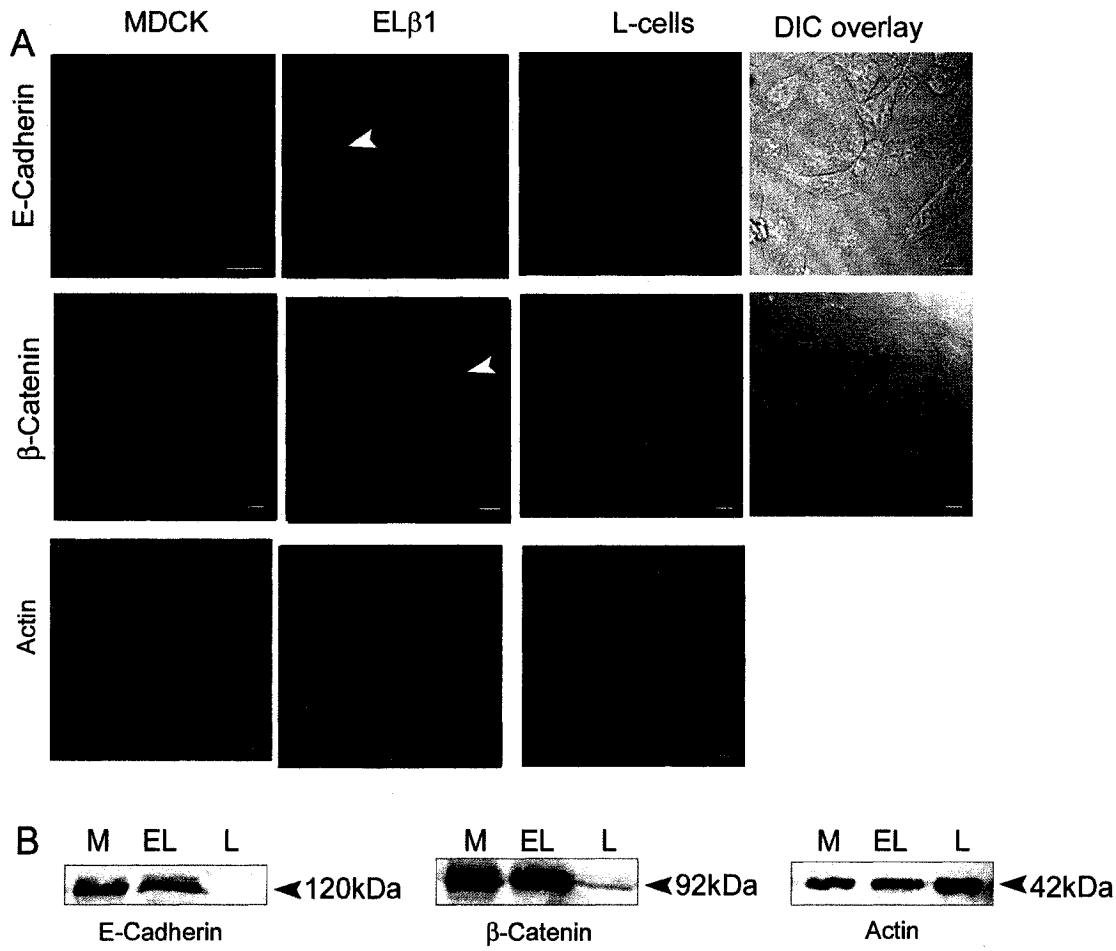

Figure 4.7 Different cell lines establish different adhesion phenotypes. **(A)** L, EL and MDCK cells were fixed and immunostained to determine E-cadherin, β-catenin and actin expression and localization. DIC overlays of L-cells indicate the number of cells in the field of view. Arrows indicate accumulation of the indicated protein at regions of cell-cell contact. Scale bar = 10 μm. **(B)** Western blot analysis of E-cadherin, β-catenin and actin expression in L, EL or MDCK (M) cell lysate.

Figure 4.8 Efficient p14-mediated fusion requires active adhesion. **(A)** Diagram of actin (red) and cadherin (green) localization under three different adhesion scenarios. Active adhesion occurs when cadherins pair in *trans* and remodel actin at the plasma membrane. Passive adhesion occurs when cadherins are engaged between cells but actin cannot remodel in response. No adhesion refers to the absence of both cadherin engagement and cadherin-dependent actin remodelling at the plasma membrane. **(B)** Fluorescent images of actin (red) and N-cadherin (green) in QM5 cells forming active, passive and no adhesion (top to bottom panels respectively) as described in **(A)**. These different adhesion scenarios were generated by the temporal addition and removal of extracellular calcium and/or cytochalasin D. Arrows indicate regions of cadherin-mediated cell-cell contact. The approximate locations of individual cell nuclei are marked with 'N'. (Scale bar = 10 μm). **(C)** QM5 cells were transfected with RRV-p14 and treated to generate the active-, passive- and no-adhesion phenotypes. Just prior to the onset of fusion (3 hpt), cells were subjected to calcium depletion (black and white bars) or maintained in normal calcium levels (grey bars, "(+)Ca to (+)Ca"). Immediately after depletion, cells were incubated in normal (white bars, "(-)Ca to (+)Ca") or low-calcium (black bars, "(-)Ca to (-)Ca") medium in the presence or absence of cytochalasin D as indicated and described in the text. The adhesion phenotype for each condition is inset in the bar (N = No Adhesion, P = Passive adhesion, A = Active adhesion). Cells were fixed at 6 hpt and fusion was quantified by syncytial indexing. Data are presented as the percent fusion relative to untreated cells and is the mean ± SE (n=5).

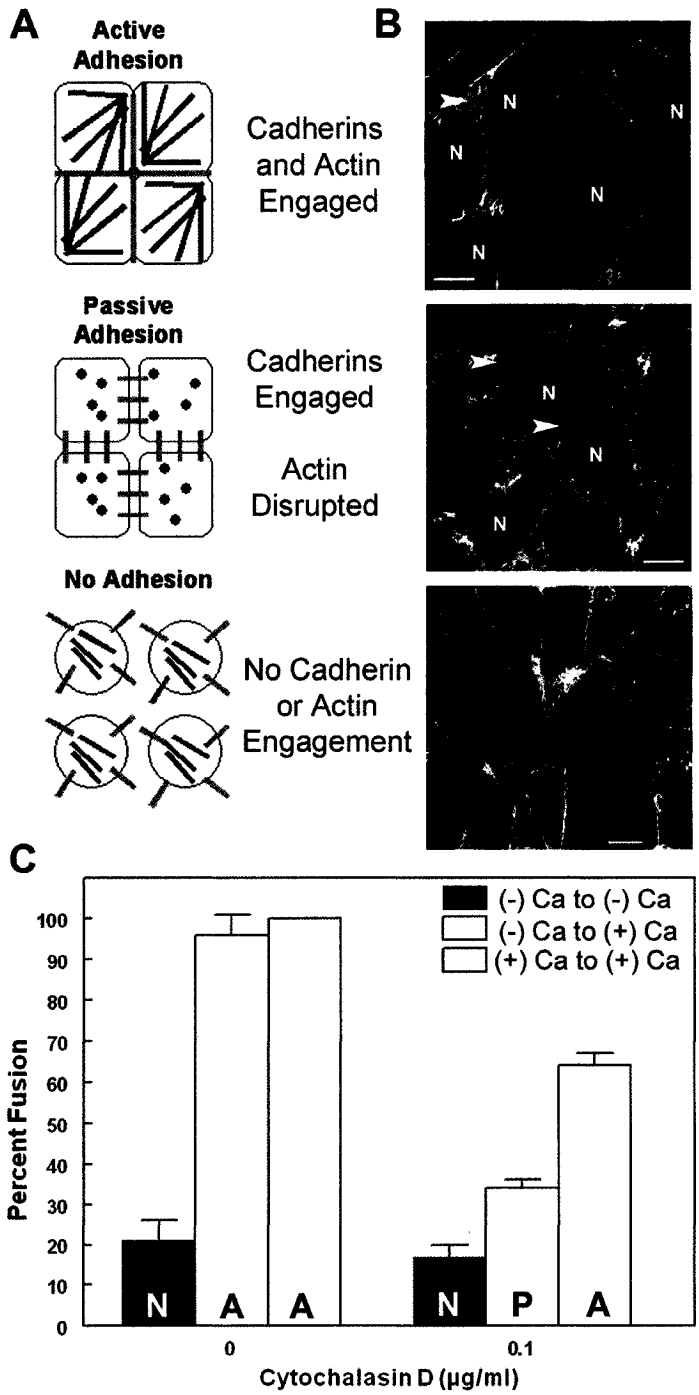

Figure 4.8 Efficient p14-mediated fusion requires active adhesion.

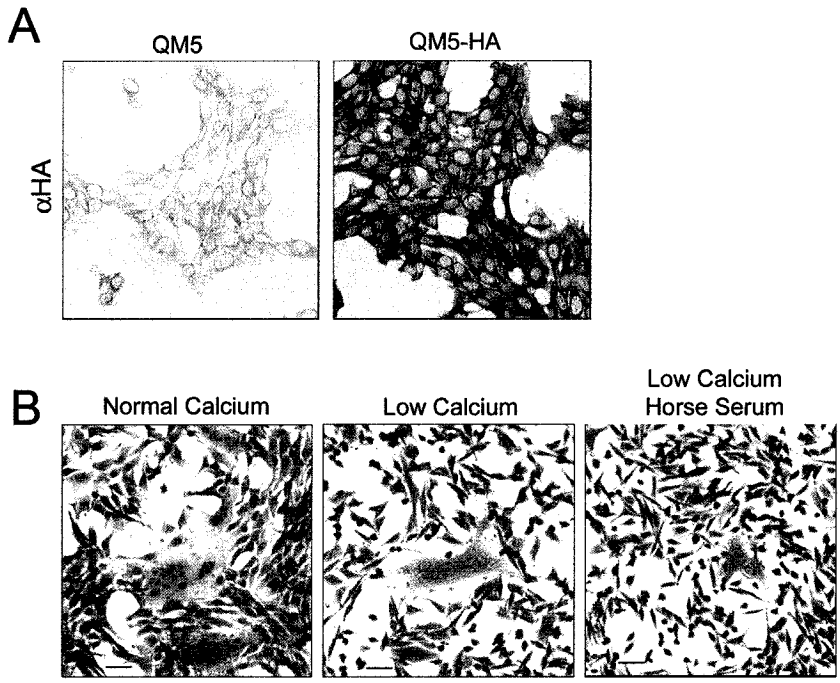

Figure 4.9 Influenza virus HA can function as a surrogate adhesion molecule for RRV-p14. **(A)** QM5 and QM5-HA cells were immunostained with αHA antibody to detect HA expression in the stably transfected QM5-HA cells. Magnification = 200x. **(B)** QM5-HA cells were transfected with RRV-p14 and treated as indicated with horse serum and/or low-calcium. Cells were fixed at 6 hpt and Giemsa stained. Scale bar = 50μm.

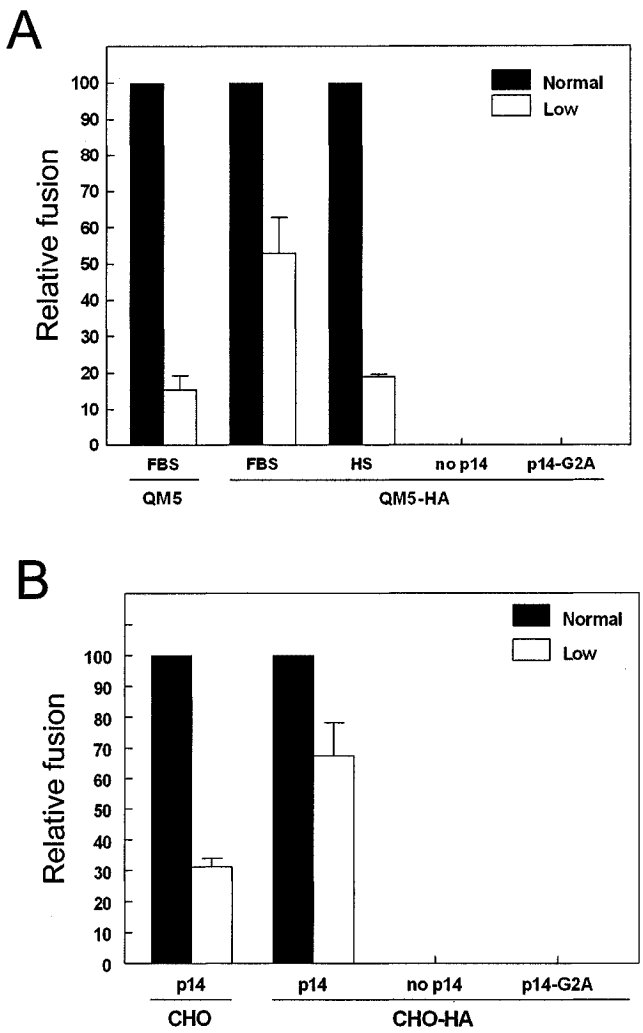

Figure 4.10 Influenza virus HA partially restores RRV-p14-mediated fusion in the absence of extracellular calcium. **(A)** QM5 and QM5-HA cells were transfected with RRV-p14 and fusion under normal (black bars) or low-calcium (white bars) conditions was quantified. QM5-HA cells were also transfected under similar conditions with the non-fusogenic mutant RRV-p14G2A or empty vector (no RRV-p14). Horse serum (HS) was added to RRV-p14-transfected QM5-HA cells to block HA receptor binding. Data are presented as the percent fusion relative to normal calcium conditions. Values represent the mean ± the SE (n=3-5). **(B)** CHO cells and influenza virus HA-expressing (X-31 strain) CHO cells (CHO-HA) were transfected with RRV-p14 and fusion under normal or low-calcium conditions was quantified. CHO-HA cells were also transfected under similar conditions with the non-fusogenic mutant RRV-p14G2A or empty vector (no RRV-p14). Values represent the mean ± the SE (n=3).

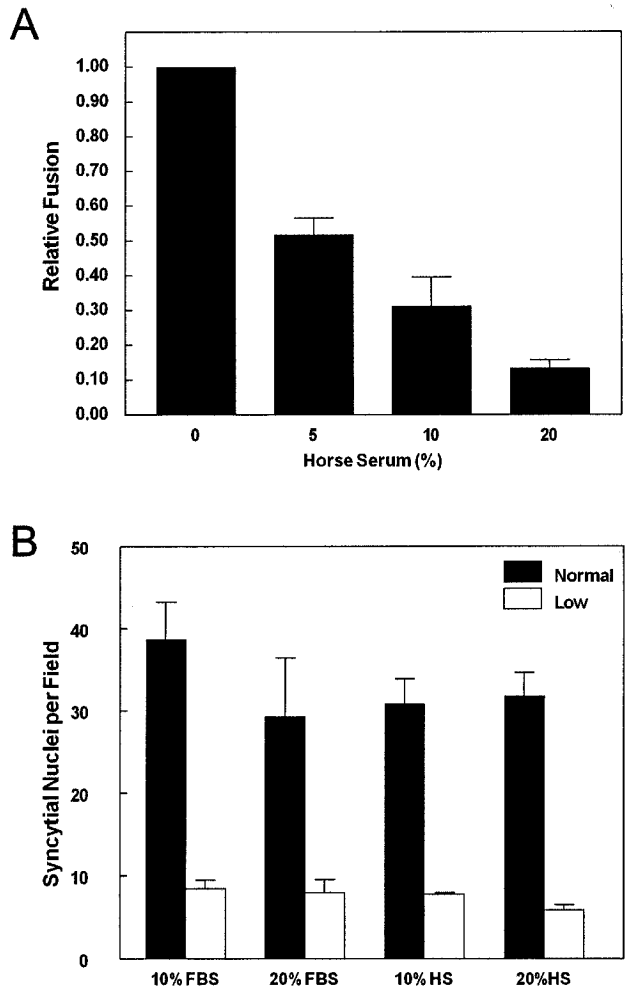

Figure 4.11 Horse serum inhibits HA fusion but does not affect RRV-p14-mediated fusion. **(A)** QM5-HA cells were incubated in the presence of 0, 5, 10 or 20% HS prior to triggering of HA fusion with low pH. Cells were fixed and the extent of fusion relative to untreated cells was quantified by Giemsa staining. Data are presented as the mean ± the SE (n=3). **(B)** RRV-p14-transfected QM5-HA cells were treated in parallel with 10% or 20% FBS or HS in the presence or absence of extracellular calcium. Cells were fixed at 6 hpt and the average number of syncytial nuclei in 5 random fields was determined by Giemsa staining. Data are from a representative experiment and is presented as the mean ± SD.

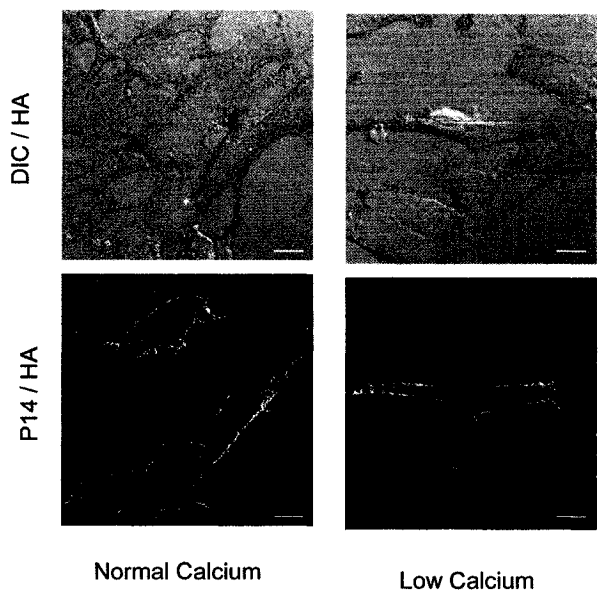

Figure 4.12 RRV-p14 and influenza virus HA co-localize at regions of cell-cell contact in normal and low-calcium conditions. Immunofluoresence and DIC microscopy of epitope-tagged RRV-p14 (red) and influenza HA (green) in QM5-HA cells under normal and low-calcium conditions. Yellow indicates regions of co-localization. Scale bar = 10μm.

CHAPTER 5

FAST Protein-Mediated Fusion Requires Cholesterol in the Plasma Membrane of Donor Cells

5.1. Introduction

The reovirus FAST proteins represent a unique class of membrane fusion proteins. The previous chapter describes the ability of extracellular adhesion molecules to promote FAST protein-mediated cell-cell fusion and suggestes that the efficiency of fusion is dependent on the environment in which the FAST protein finds itself. If this is so, then the other components of the fusion-enhancing environment, such as membrane composition and intracellular factors, must be identified. Now, our attention will turn to how the composition of the plasma membrane could affect FAST protein-mediated fusion.

The plasma membrane is a complex and essential component in any cell-cell fusion reaction. It contains a diverse range of lipid components, some of which might preferentially support FAST-protein-mediated fusion. Cholesterol is a key component of cellular plasma membranes and has important effects on membrane fluidity, protein trafficking and cellular signalling (561, 589). Of particular interest is the presence of plasma membrane subdomains termed lipid rafts (502). Lipid rafts are composed of cholesterol, sphingolipids and/or saturated phospholipids that pack together tightly to form a liquid ordered phase (422). The physical properties of a lipid or protein determine whether it will associate with membrane microdomains, giving the molecule a non-random distribution on the cell surface. Experimental evidence suggests the size of these membrane microdomains varies in range from ~20 nm to 200 nm (129, 416, 433). Confirming that a protein resides in these microdomains is difficult, as detergent extraction procedures and microscopic analysis can produce ambiguous, artefactual and conflicting results (328, 387, 502).

Methods for determining the biological relevance of a raft association of a molecule are also imprecise. The most common strategy is to use cyclodextrins to extract plasma membrane cholesterol and correlate that depletion with a change in function (97, 498). Plasma membrane cholesterol levels can also be depleted with pharmacological inhibitors of cholesterol synthesis and processing (273, 498). The results derived from

these methods can be difficult to interpret because depletion of membrane cholesterol, especially rapid depletion, can affect cell signalling and cytoskeleton remodelling (297, 498). Essentially, it is very difficult to determine whether a protein localizes to membrane microdomains, and even harder to assign functional significance to those findings.

In regard to membrane fusion, one might expect the rigidity of lipid rafts to resist the lipid rearrangements necessary for membrane merger. It is interesting, then, that cholesterol and membrane microdomains are important in the fusion reactions mediated by several fusion proteins. The fusion protein of Semliki forest virus requires cholesterol and sphingolipids in the target membrane to facilitate fusion during the entry process (281, 579). Influenza virions assemble and release from lipid rafts as a mechanism for increasing the concentration of the HA fusion protein on virion surfaces (534). Similarly, entry of certain retroviruses is inhibited if lipid rafts in target cells are depleted by cholesterol extraction resulting in decreased density of raft-localized receptors (334, 430, 572). Lipid rafts are also implicated in aggregating and coordinating cellular SNARE proteins for efficient exocytosis events (307).

It is conceivable that the FAST proteins may employ comparable strategies and share a similar requirement for plasma membrane cholesterol and lipid rafts to support enhanced cell-cell fusion. Many cell adhesion molecules, including cadherins and the gap junction-forming connexins have been identified as raft-associated proteins (75, 478, 483); thus the ability to also localize to lipid rafts could place the FAST protein in proximity to these molecules known to enhance fusion efficiency. Also, many viral fusion proteins function co-operatively as groups of multimers, with influenza virus HA requiring between 3 and 8 trimers (28, 120), rhabdovirus G requiring 13-19 trimers (448), and Semliki forest virus requiring 5-6 E-protein trimers to cause fusion (196). The multimerization status of the FAST proteins is currently being assessed, but the evidence suggests that RRV-p14 and BRV-p15 may form multimers (E. Clancy, unpublished data and (110, 125)) while similar experiments suggest that suggest ARV-p10 may not (496). Either way, raft localization could facilitate FAST protein aggregation, helping them to multimerize or accumulate to a high enough density to effectively mediate fusion. The data reported here indicate that the FAST proteins require membrane cholesterol for

efficient function and RRV-p14 specifically requires it in the donor membrane. Concurrent studies determined that RRV-p14 is associated with detergent resistant membranes (110) and, taken together, the data suggest that this association is necessary for function.

5.2. Results

5.2.1. Plasma membrane cholesterol is required for FAST protein-mediated fusion

Multiple approaches were used to deplete or eliminate cholesterol from the plasma membrane in an effort to disrupt membrane microdomains and determine the effect on FAST protein-mediated fusion. Plasma membrane cholesterol was extracted from ARV-p10-transfected QM5 cells using the cholesterol-binding drug MβCD (282) prior to the onset of fusion (Figure 5.1A). The presence of MβCD dramatically inhibited ARV-p10-mediated fusion. The efficacy of MβCD-mediated cholesterol removal was quantified under various serum conditions by detecting the amount of [^{3}H]-cholesterol extracted from pre-loaded QM5 cells using 2 mM or 20 mM MβCD (Figure 5.1B). As little as 2 mM MβCD was sufficient to remove >60% of the labelled cholesterol from the cell membrane and >90% was extracted with 20 mM MβCD (Figure 5.1B). The ability of MβCD to extract cholesterol was unaffected by the presence of 5% lipoprotein-deficient serum or 10% FBS indicating that these conditions could be used in further experiments without concern for the efficacy of the drug (Figure 5.1B). However, prolonged exposure (>2 h) to concentrations of MβCD greater than 5 mM appeared to be toxic to QM5 cells and they began to detach from the culture dish (data not shown). Therefore, FAST protein-transfected QM5 cells were depleted of membrane cholesterol using 2 mM MβCD prior to the onset of fusion and for the duration of the experiment.

RRV-p14 and NBV-p10 were the most sensitive to cholesterol depletion with fusion being inhibited in MβCD-treated cells by ~85% when compared to untreated cells (Figure 5.1C). Similarly, fusion induced by BRV-p15 and ARV-p10 was inhibited by >65% in both cases, indicating that the FAST proteins require membrane cholesterol to mediate efficient cell-cell fusion (Figure 5.1C). To confirm that this effect was FAST protein-specific, QM5 cells were transfected with the non-raft-associated vesicular stomatitis virus G protein (VSV-G) and treated with 2 mM MβCD. VSV-G protein-

mediated fusion was unaffected by cholesterol extraction consistent with the lack of dependence of VSV-G on membrane microdomains or cholesterol for function (Figure 5.1C). This observation also revealed that MβCD treatment did not render cells incapable of fusion and that the drug's effects seem to be specific to the FAST proteins. Further, fusion was restored when cholesterol was added back to MβCD-treated, RRV-p14-transfected cells using MβCD preloaded with cholesterol (Figure 5.1D) (572). This supports the assumption that the inhibitory effect of MβCD is due to cholesterol extraction and confirms that MβCD does not render the cell, or RRV-p14, permanently incapable of fusion (572).

That the inhibitory effect was due to cholesterol depletion was further demonstrated by using other cholestero-modulating drugs. Lovastatin, an HMG-CoA reductase inhibitor that prevents *de novo* cholesterol synthesis (5, 273, 582), and U18666A, a drug that alters cholesterol trafficking and inhibits cholesterol biosynthesis (171, 270, 326, 353), were used to deplete plasma membrane cholesterol levels. Both drugs inhibited RRV-p14-, NBV-p10- and BRV-p15-mediated fusion by 80-100% (Figure 5.2). Finally, cholesterol-auxotrophic CHO cells were used to confirm that membrane cholesterol is necessary for FAST protein-mediated fusion. The parental CHO-K1 cell line was readily susceptible to RRV-p14-mediated fusion and sensitive to MβCD treatment (Figure 5.3B). The same cells containing a deficiency in the chromosomal site-2 protease necessary for activation of SREBP-responsive genes (N-BP-2 cells) are unable to undergo *de novo* cholesterol synthesis without induction of the plasmid-encoded gene that compensates for the deficiency (409). Thus, culturing uninduced N-BP-2 cells without exogenous cholesterol renders them cholesterol deficient. N-BP-2 cells grown in cholesterol-free conditions for 24 or 48 h prior to transfection were only half as susceptible to RRV-p14-mediated fusion compared to cells containing cholesterol (Figure 5.3). Together, these data demonstrated the clear dependence of FAST protein-mediated fusion on cellular cholesterol.

5.2.2. RRV-p14-mediated fusion requires cholesterol in the donor membrane

To further investigate the requirement for membrane cholesterol in FAST protein-mediated fusion it was determined whether cholesterol is required in the FAST-

containing donor membrane, the target membrane or both. QM5 cells were labelled with different cell-tracker dyes (CMTMR [red] and CMAC [blue]) to identify distinct cell populations. Donor populations (red) were transfected with RRV-p14 and co-incubated with blue target cells to produce syncytia containing both red and blue nuclei indicating fusion of donor and target cells (Figure 5.4A). The average number of syncytial nuclei per random field of view and the relative number of donor to target nuclei were determined for each condition by microscopic analysis. When target cells were added to donor cells treated with 0, 10 or 20 mM MβCD the relative number and size of resultant syncytia decreased dramatically with increasing cholesterol extraction, suggesting that cholesterol is required in the donor membrane for fusion (Figure 5.4B). Also, the syncytia that formed under these conditions contained approximately equal numbers of donor and target nuclei, indicating that both cell types were equally susceptible to fusion (Figure 5.4C). Conversely, removal of cholesterol from target cells prior to the addition of donor cells had no effect on the extent of fusion and was comparable to the level of fusion when untreated target cells were added to untreated donor cells (Figure 5.4B). However, treatment of target cells with 20 mM MβCD resulted in a change in the ratio of donor to target nuclei within these syncytia to dramatically favour donor cells (Figure 5.4C). Initially this observation seemed to indicate that cholesterol is also required in the target membrane, although perhaps to a lesser extent. However, this observation is consistent with the interpretation that cholesterol is required in the donor membrane. Fusion of an untreated donor cell to a single cholesterol-depleted target cell would produce an RRV-p14-expressing syncytium with only half of the normal cholesterol content. Thus, fusion of donor cells to MβCD-treated target cells effectively reduces cholesterol in the donor membrane, and would result in a syncytium that cannot progress. Only syncytia that are formed by a majority of donor-to-donor fusions would be able to progress. Thus, the FAST proteins require cholesterol in the donor membrane for proper function.

5.2.3. The FAST proteins do not co-localize with classical lipid microdomain markers or each other

Cholesterol is a major component of membrane microdomains termed lipid rafts. It is possible that the cholesterol dependence of the FAST proteins is due to an association with membrane microdomains. J. Corcoran (collaborator, Dalhousie University) confirmed this association for RRV-p14 using detergent extraction and sucrose-gradient analysis (110). Her analysis revealed that most of RRV-p14 is not raft associated, but a significant proportion consistently associates with microdomains extracted with lubrol or triton X-100 (110). To further investigate FAST protein raft-association, immunofluorescence microscopy was used to determine if RRV-p14 co-localized with raft marker molecules or with other FAST proteins. QM5 cells were co-transfected with placental alkaline phosphatase (PLAP) and the non-fusogenic but raft-associated mutant RRV-p14G2A to prevent fusion and allow sufficient PLAP expression. Surface immunostaining was conducted on live, unpermeabilized cells at 4°C to maintain raft integrity and promote raft patching (220). Although both molecules displayed punctuate surface staining characteristic of raft proteins (220), there was very little co-localization of RRV-p14 and PLAP (yellow, Figure 5.5A). Authentic RRV-p14 and endogenous ganglioside GM1 showed more co-localization than RRV-p14G2A and PLAP, but the effect was not substantial (Figure 5.5B) Interestingly, the two raft markers GM1 and PLAP did not co-localize well with each other either (Figure 5.5C). Finally, RRV-p14G2A and HA epitope-tagged BRV-p15 (BRV-p152HAN) showed some co-localization with each other, especially at the cell edge (Figure 5.5D). Together, these data indicate that the FAST proteins only partially associate with each other and only randomly associate with typical membrane microdomain markers.

5.3. Discussion

Cholesterol is an important plasma membrane component that coordinates the formation of lipid raft microdomains. Multiple approaches used to deplete membrane cholesterol levels reveal that this sterol is also required for efficient FAST protein-mediated fusion. The cholesterol-dependent inhibition of fusion is also reversible, since replenishing membrane cholesterol restored cell-cell fusion activity. Further, the fusion

activity of the non-raft-associated fusion protein VSV-G was unaffected by cholesterol depletion, demonstrating that cyclodextrin-treated cells are still capable of undergoing fusion and that the inhibitory effect is specific to the FAST proteins. In addition, the heterotypic cell fusion assay determined that RRV-p14 requires cholesterol in the donor but not the target membrane. This observation is consistent with the hypothesis that localization to lipid rafts might be necessary for FAST protein function. Previous studies confirmed that RRV-p14 associates with detergent-resistant membranes, and that this association is lost when cells are similarly treated with cyclodextrin. The mechanism for FAST protein raft localization is unclear, but could be attributed to a shared structural element such as the transmembrane domain or the polybasic region. The transmembrane domain of HA and a polybasic region of CD4 have been implicated as raft-localization motifs for these molecules (20, 429). As shown by the data presented here, raft association is correlated with FAST protein function.

These conclusions are drawn with an appropriate amount of caution, since cholesterol depletion can affect cellular processes such as protein lateral mobility, intracellular signalling and cytoskeleton remodelling (297, 498). Thus, FAST protein raft-association might not be essential for fusion. Instead, intact rafts might render the cell more capable of supporting FAST protein-mediated fusion by ensuring the cellular adhesive, signalling and/or cytoskeleton machinery are functioning properly. Either way, membrane cholesterol must be maintained at physiological levels to support FAST protein-mediated fusion.

The data presented here are consistent with several models describing a role for cholesterol and lipid rafts in FAST protein-mediated fusion. The proposed models are not mutually exclusive, and indeed the actual requirement for membrane cholesterol in the FAST protein fusion reaction may be multifaceted. First, the FAST proteins may require membrane cholesterol because they are raft-associated proteins. This association may be necessary for function in a number of ways described below. Second, the effects of cholesterol, lipid rafts or raft-associated lipids on biological membranes may be mechanistically important for FAST protein-mediated fusion. Finally, pleiotropic effects of cholesterol depletion may influence the ability of the FAST proteins to mediate membrane merger.

5.3.1. Lipid raft association could promote FAST protein aggregation and association with cellular components necessary for fusion

Unlike other molecules which are exclusively located in lipid rafts, only a fraction of RRV-p14 is resistant to detergent extraction (110). This profile could be generated in two ways. First, random distribution of RRV-p14 on the cell surface would place some of the protein in rafts and some of it would be excluded from rafts. This would result in variable resistance to detergent extraction. Second, rafts are absent from the endoplasmic reticulum (ER) and early Golgi compartments (55, 589) but intracellular staining indicates that the majority of RRV-p14 is associated with intracellular membranes. Thus, surface-localized RRV-p14 might be entirely raft-associated but the large non-raft intracellular pool appears as the detergent-soluble fractions. It is unclear whether the raft profile of RRV-p14 represents a random distribution on the cell surface, or the population of protein in the raft-deficient intracellular membranes, or both. However, the punctate surface staining shown here is consistent with raft-localized proteins and may suggest that the non-raft population of RRV-p14 is predominantly intracellular. Combining the data presented here, RRV-p14 raft-association correlates with its fusion activity since cholesterol extraction results in loss of fusion and loss of raft association. The cholesterol-dependence of the other FAST proteins suggests that raft association may be a common characteristic of these proteins. There are several interesting models that incorporate raft association with the efficient function of these proteins.

Like other raft-localized proteins, the FAST proteins may associate with membrane microdomains as a way to aggregate and increase their local concentration. Other membrane fusion proteins, like HA, require both multimerization and aggregation to ensure a high enough protein density to enable fusion to proceed (69, 120). In the case of HA, at least three protein trimers are required to initiate fusion (28, 120). The virus ensures infectious progeny contain sufficient concentrations of HA by budding from lipid rafts (470, 534, 608). Thus, cholesterol is not involved in the HA fusion reaction directly, but infected cells do require intact rafts to produce infectious progeny (534). Similarly, the FAST proteins may require lipid rafts in the donor membrane to ensure high enough

local concentrations of protein to initiate fusion. The observation that RRV-p14 specifically requires cholesterol in the donor membrane is consistent with this hypothesis. Although initial evidence suggested that ARV-p10 does not form multimers (496), more recent data indicate multimerization is a common theme among the FAST proteins. Thus, the FAST proteins may be similar to HA in that they exploit lipid rafts to aggregate fusion-inducing multimers.

Cholesterol is also required for fusion in the donor membrane of human herpesvirus 6 (HHV-6) (243). MβCD extraction of cholesterol from the virion membrane causes a minor reduction in binding and a dramatic inhibition of fusion to target cells (243). Membrane cholesterol may be directly involved in the fusion reaction of these proteins, or it might facilitate the aggregation or interaction of the multiple components of the herpesvirus fusion complex. Similarly, extraction of cholesterol from the target cell membrane can inhibit HIV-mediated entry by preventing the clustering of the gp120 receptor and co-receptor, resulting in failure to activate the gp41 fusion subunit, suggesting that raft-mediated protein clustering in either the donor or target membranes can be important for fusion (430, 572).

Another plausible purpose of FAST protein raft-association is that it places the FAST proteins in proximity to raft-localized cellular adhesion proteins, such as cadherins. The previous chapter discusses the importance of cadherin-based junctions in the fusion reaction mediated by the FAST proteins. Analysis by confocal microscopy revealed that surface-localized RRV-p14 proteins does not significantly co-localize with the raft markers GM1 or PLAP. However, RRV-p14 does co-localize with N-cadherin in the same cells at regions of cell-cell contact and adhesion (Figure 4.4). This is consistent with an earlier hypothesis that RRV-p14 might preferentially associate with Lubrol resistant membrane fractions over Triton-X-100 resistant fractions (110). A variety of cell types generate highly curved, actin-filled membrane protrusions, generally termed microvilli, whose functions include, among other things, cell-cell adhesion and fusion (76, 597). Lipid rafts are commonly associated with cell adhesion molecules, and Lubrol rafts in particular appear to be localized to microvilli (458, 518). Thus, the localization of the FAST proteins to Lubrol rafts found at the tips of microvilli could place them in an environment that contains fusion-promoting curved membranes and adhesion molecules.

It is also interesting to note that epitope-tagged RRV-p14 (2HAN) co-localized well with influenza HA, a known raft-associated protein (Figure 4.11). These observations support emerging ideas in the field that membrane microdomains are dynamic and diverse with different rafts containing different protein profiles and different sensitivities to extractions with various detergents (151, 423). Consistent with the heterogeneity model, GM1 and PLAP also failed to co-localize, suggesting that multiple types of rafts may be present in QM5 cells. This concept of raft heterogeneity is further supported by observations that GM1 fails to co-localize with other GPI-linked proteins (e.g. Thy-1) in fibroblast cells (595)

A few caveats must be provided for the microscopic analysis however. Co-localization studies only provide information at about 200 nm resolution, far less than is required for confirming that two molecules are in the same rafts. Those conclusions require higher-resolution imaging techniques like FRET (275). Thus, co-localization of membrane proteins in this discussion is only meant to imply that those molecules are in the same region of a cell, not necessarily in the same microdomain. Interestingly, RRV-p14 and BRV-p15 did not strongly co-localize with one another except at the cell periphery. This might further reflect lipid raft diversity, but a proper interpretation requires further experimentation to confirm the localization of BRV-p15 in detergent-resistant membranes or its co-localization with N-cadherin or HA. Regardless, both proteins require membrane cholesterol, but not necessarily rafts, for function and it is entirely possible that each FAST protein has an individual requirement for this sterol.

A less likely role for cholesterol in FAST protein-mediated fusion might parallel the requirement of HIV entry on membrane rafts. HIV requires rafts in the target cell membrane to aggregate its entry receptors CD4 and CCR5 (334, 430). Disruption of adhesion-molecule localization in the donor and target membranes could be inhibitory to FAST protein-mediated fusion. However, by light microscopy, cell-cell adhesion in cholesterol-depleted cells was only marginally disrupted, if at all. Indeed, the concentration of MβCD used to extract cholesterol was chosen because of its ability to remove cholesterol and maintain cell adhesion. Further, the fact that fusion was decreased when donor but not target cells were depleted of cholesterol argues against this model (Figure 5.4). Donor cells seem perfectly capable of incorporating normal or

cholesterol-depleted target cells into syncytia. Only when high concentrations of MβCD (20 mM) were used did the donor-to-target-nuclei ratio of syncytia skew to favour donor-cell incorporation. This is not likely due to a deficiency in the cholesterol-depleted cell to be a fusion target, but rather that its incorporation into a growing syncytium can effectively reduce the donor-syncytium cholesterol content to below the threshold required for the FAST protein to initiate fusion. Thus, the most probable role for lipid rafts in the FAST protein fusion reaction is to serve as a platform for aggregation and association with adhesion machinery.

5.3.2. Membrane microdomain components may be fusion-promoting molecules

The fusion protein of Semliki forest virus (E1) requires cholesterol and sphingolipids in the target membrane to promote fusion (281, 579). This is not a raft-dependent effect, but instead indicates that direct SFV-E1 protein interactions with cholesterol are required for fusion activation (195, 524, 579). A direct interaction between the FAST proteins and cholesterol in the target membrane is possible but not likely, since the FAST proteins lack structural similarity to SFV-E1 and to each other. Further, RRV-p14 does not appear to require cholesterol in the target membrane to initiate fusion.

Interestingly, the FAST proteins all contain a potential cholesterol-binding motif in their endodomains. This cholesterol recognition/interaction consensus (CRAC) sequence was originally described in the carboxyl terminus of peripheral-type benzodiazapine receptor (320, 321) and recently identified in the FAST proteins (125). The CRAC sequence is $L/V(X)_{1-5}Y(X)_{1-5}R/K$ (320) which is satisfied in the FAST proteins by endodomain regions directly adjacent to the transmembrane domain (125). The functionality of the CRAC domain in the FAST proteins has not been assessed, but mutational analysis and cholesterol-binding studies should be conducted to determine if this motif is important for membrane localization or fusion activity. Interestingly, the CRAC domain of ARV-p10 and NBV-p10 encompass the palmitoylated cysteines adjacent to the transmembrane domain, which could potentially abolish cholesterol-binding activity. The presence of a cholesterol-binding motif could serve to target the

FAST proteins to membrane microdomains, or ensure cholesterol is present for the fusion reaction.

Raft-independent qualities of cholesterol could support FAST protein-mediated fusion. An important study conducted by Razinkov and Cohen (439) demonstrated that physiological cholesterol levels are required to promote fusion pore expansion when fusing HA to planar lipid bilayers. Conversely, sphingolipids promoted pore flickering and inhibited fusion pore progression. When both lipids were present, fusion kinetics were normal. The authors suggest that cholesterol can limit pore flickering by stabilizing the fusion pore structure by interacting with the lipids within the wall of the fusion channel, thus preventing reversion to hemifusion or collapse of the fusion pore (439).

Another interesting characteristic about cholesterol is its high rate of flip-flopping between the inner and outer membrane leaflets (214, 520). Perhaps this mobility is favourable to fusion as it could manoeuvre through the transitional structures that occur during fusion pore formation. In contrast to these ideas is the fact that cholesterol-rich lipid rafts are viewed as rigid structures lacking the flexibility needed to support fusion (9, 406, 434, 607). The long, saturated acyl chains of raft components would resist the membrane curvature needed to support the intermediate structures of a membrane during fusion. Perhaps these views are incorrect, or perhaps fusion occurs at the edges of fusion protein-containing rafts where the lipids are more miscible.

5.3.3. Cholesterol depletion has a variety of effects on cells that could alter FAST protein function

A common problem with assigning functional significance to lipid raft localization is that extraction of cellular cholesterol with MβCD can have a variety of effects on a cell other than raft disruption. Further, any method for disrupting lipid rafts will dramatically alter normal cellular processes that depend on microdomain integrity. However, the multiple approaches to cholesterol alteration employed in these studies and the observation that VSV-G retains function in the absence of cholesterol strongly indicate that the FAST proteins do require membrane cholesterol for function. Further, since VSV-G can still mediate fusion in cholesterol-depleted cells, whatever pleiotropic effects MβCD treatment has on cells, it does not render them incapable of fusion. Thus,

MβCD treatment is affecting the cells in manner that specifically inhibits FAST protein-mediated fusion.

Each pharmacological agent used in these studies has unique effects on treated cells, some of which may implicate other cellular processes in the FAST protein fusion reaction. MβCD is used to rapidly extract cholesterol from membranes, and this acute treatment alters the cytoskeleton and inhibits the lateral mobility of membrane proteins (218, 297, 498). Lovastatin decreases *de novo* cholesterol synthesis by inhibiting the cholesterol-biosynthetic enzyme HMG-CoA reductase. Cholesterol depletion by this method does not affect the lateral mobility of proteins but can still alter the cytoskeleton (218, 337, 498). HMG-CoA reductase is an important step in the protein prenylation (farnesylation and geranylgeranylation) pathway as well (561). Many acylated proteins, including phospholipases, G-proteins, and Ras and Rho GTPases are involved in coordinating cell adhesion, actin remodelling and cell signalling at the plasma membrane (reviewed in (85, 338, 508). Palmitoylation of the t-SNARE SNAP 25 is required for its membrane localization and involvement in exocytic fusion reactions (267, 288, 570). Lovastatin could inhibit the activity of these acylated membrane-interacting proteins and render the cell unable to support FAST protein-mediated fusion.

U18666A is a useful tool for depleting cholesterol from the plasma membrane, but its exact mechanism of action is unknown. One of its activities may include inhibition of cholesterol synthesis by inhibiting oxidosqualene cyclase (105). Thus, while U18666A decreases membrane cholesterol by altering recycling to the plasma membrane, it may also be inhibiting *de* novo synthesis (105, 171, 270, 326, 353). Interestingly, oxidosqualene cyclase inhibition occurs downstream of HMG-CoA reductase and prenylation; thus the side effects of lovastatin treatment could be avoided. Therefore, by preventing the negative effects of prenylation inhibition, U18666A may be a better tool than lovastatin for pharmacological inhibition of cholesterol synthesis. Given that the inhibition of FAST protein-mediated fusion is similar with the three drug treatments, it is most likely that the inhibitory effects are due solely to the cholesterol-depletion and not to a particular side effect. However, cytoskeleton alterations are a common theme with all cholesterol depletion methods and cannot be ruled out as a factor contributing to the efficiency of the FAST proteins. This is consistent with the data from

the previous chapter demonstrating actin-controlled active adhesion as necessary for efficient FAST protein function.

In summary, the data presented here demonstrate a clear requirement for cholesterol in the donor membrane of cells for effective FAST protein-mediated fusion. Collaborative experiments helped to correlate this effect to the localization of RRV-p14 and the other FAST proteins to detergent-resistant membrane microdomains. It is unclear if the cholesterol dependency is due to the effects of this sterol on membranes, the integrity of lipid rafts or alterations to the cytoskeleton as a result of cholesterol depletion. These possibilities are not mutually exclusive and the FAST proteins may have adapted to exploit the normal functions of these cellular molecules and processes to maximize their fusion potential. Continuing investigations are attempting to determine the raft dependency of the FAST proteins by replacing their transmembrane domains with that of the raft-excluded transferrin receptor and immunoprecipitation of rafts to identify the protein and lipid profile of FAST-containing rafts. Given the repeated appearance of cytoskeleton alterations as fusion-modulating cellular process, further investigation was warranted and is the subject of the next chapter.

5.4 Figures

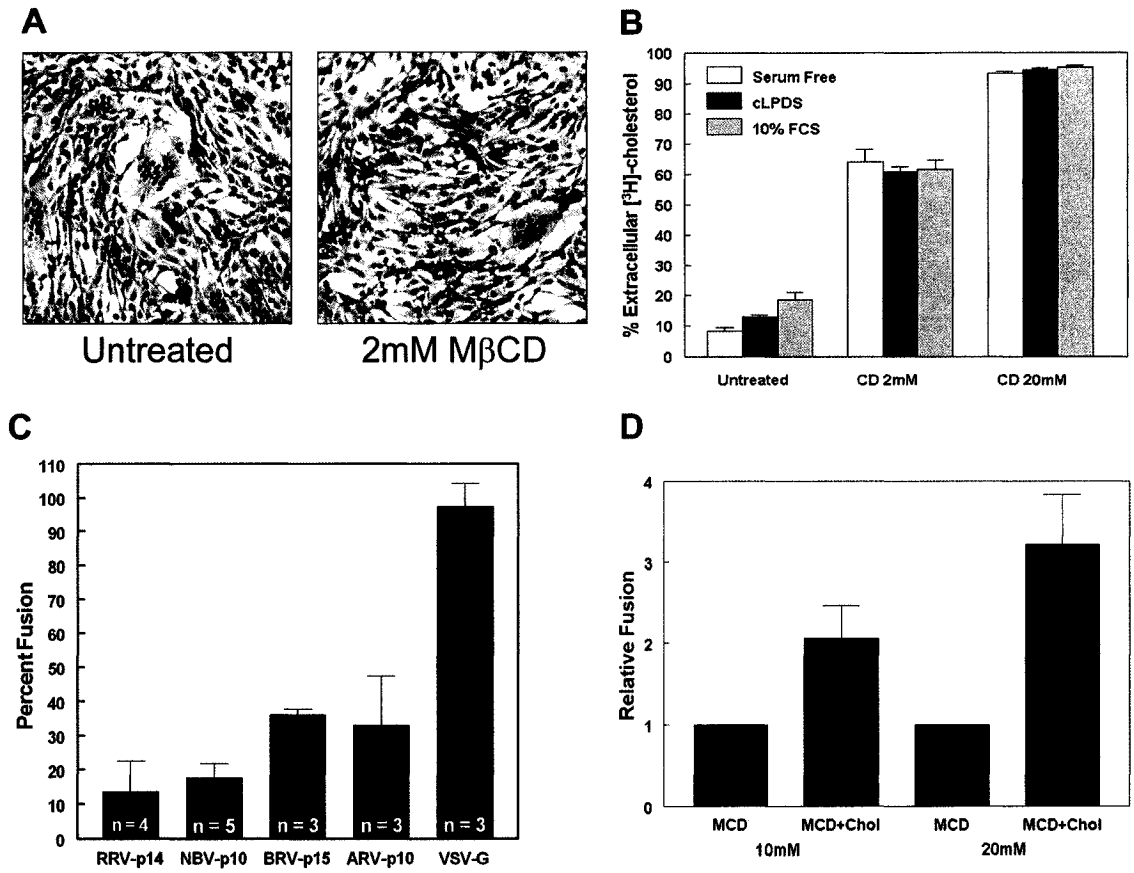

Figure 5.1 FAST protein-mediated fusion requires membrane cholesterol. **(A)** ARV-p10-tranfected QM5 fibroblasts were treated with 2 mM methyl-β-cyclodextrin (MβCD) prior to fusion to extract membrane cholesterol. Cells were methanol-fixed 6 h later (20 hpt) and Giemsa stained. Magnification = 200x **(B)** QM5 cells were loaded with [^{3}H] cholesterol prior to extraction with 2 mM or 20 mM MβCD in the indicated serum conditions and the percent of extracellular calcium determined by scintillation counting. Data are presented as the mean ± SE (n=3). **(C)** QM5 cells were transfected with RRV-p14, BRV-p15, NBV-p10, ARV-p10 or VSV-G and treated with 2 mM MβCD prior to and during fusion. Cells were fixed at 8 (RRV-p14), 12 (NBV-p10), 19 hpt (BRV-p15 and ARV-p10) or 24 hpt (VSV-G) and the percent fusion relative to untreated cells was determined by quantifying Giemsa-stained cells. Data are presented as the mean ± SE (n=3-5) **(D)** RRV-p14-transfected QM5 cells were treated with 10 mM or 20 mM MβCD for 30 min and fusion was allowed to progress in the presence or absence of 20 mM MβCD preloaded with cholesterol. The relative increase in fusion of cholesterol-reloaded cells (MCD + Chol) was determined by quantifying Giemsa-stained cells. Data are presented as the mean ± SE (n=3-5).

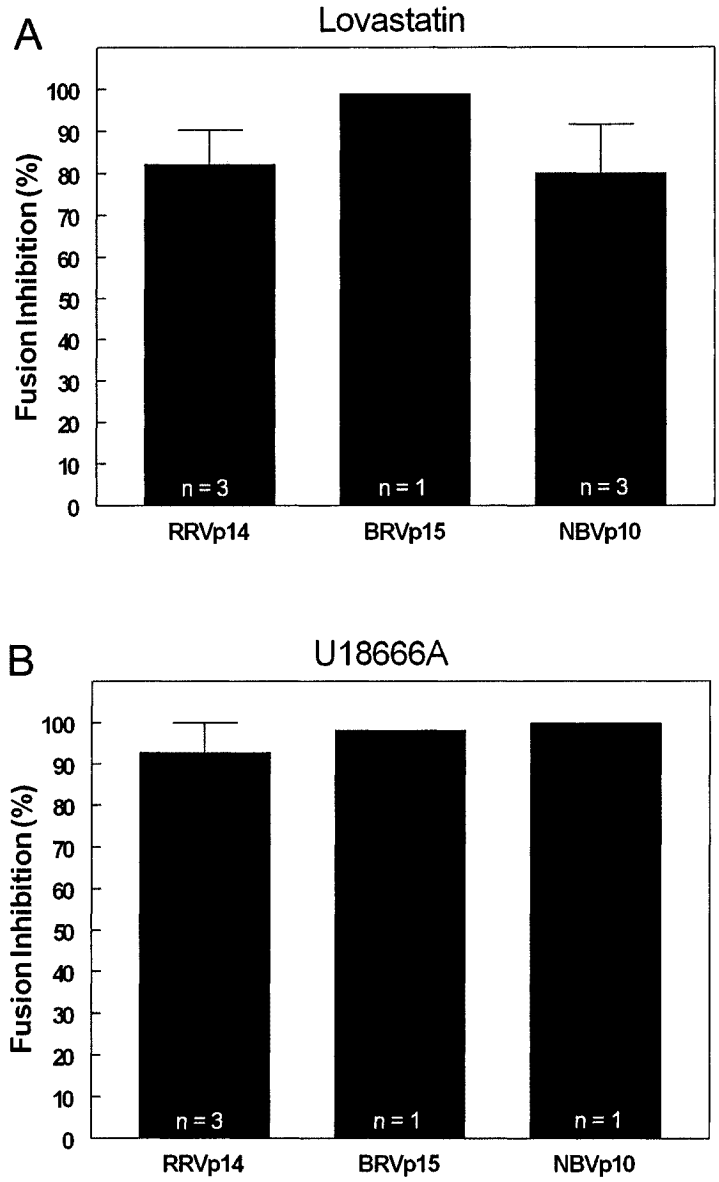

Figure 5.2 Cholesterol alteration inhibits FAST protein-mediated fusion. **(A)** QM5 cells were treated with 25 μM lovastatin 24 h prior to and during transfection with RRV-p14, BRV-p15 or NBV-p10. Cells were fixed at 8 (RRV-p14), 12 (NBV-p10) or 19 hpt (BRV-p15) and the percent inhibition of fusion was determined by quantifying Giemsa-stained cells. Data are presented as the mean ± SE. **(B)** QM5 cells were treated with 2 μg/ml U18666A 24 h prior to and during transfected with RRV-p14, BRV-p15 or NBV-p10. Cells were fixed at 8 (RRV-p14), 12 (NBV-p10) or 19 hpt (BRV-p15) and the percent inhibition of fusion was determined by quantifying Giemsa stained cells. Data are presented as the mean ± SE.

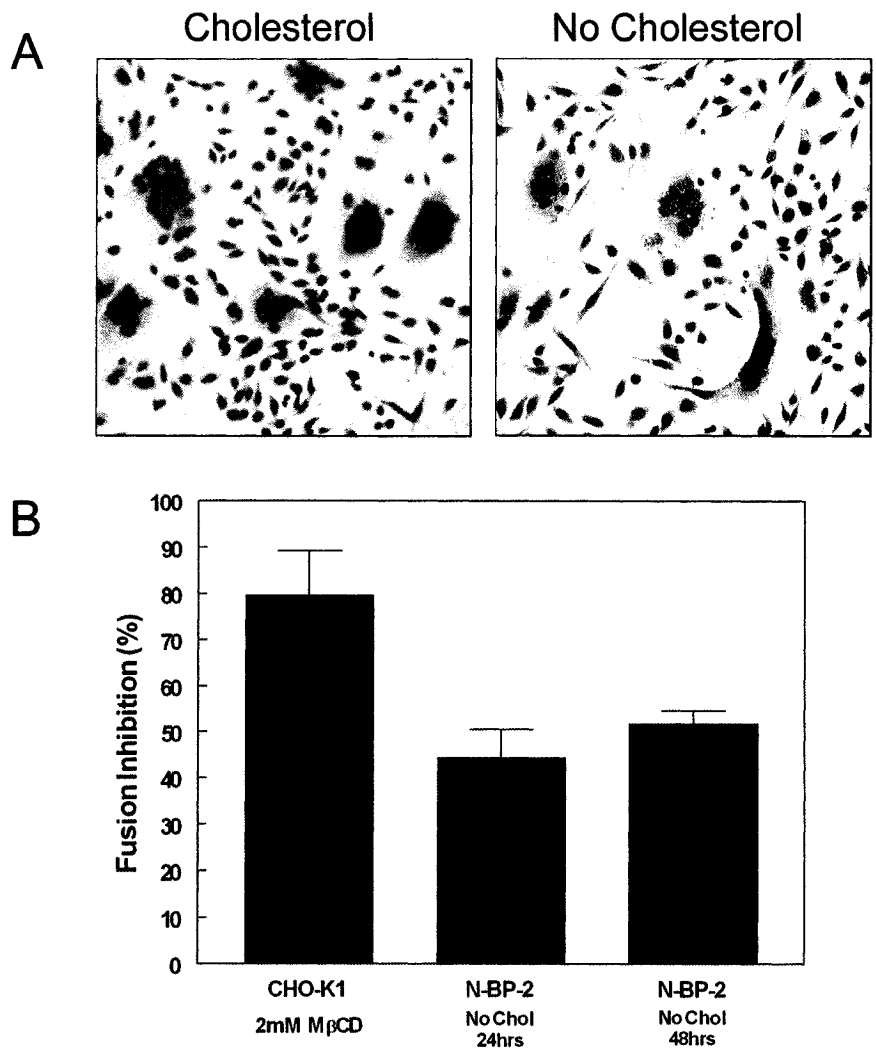

Figure 5.3 Cholesterol-deficient cell lines are less susceptible to RRV-p14-mediated fusion. **(A)** N-BP-2 cells were grown in the presence or absence of exogenous cholesterol and transfected with RRV-p14. Cells were methanol fixed 24 hpt and Giemsa-stained to visualize nuclei. Magnification = 200x. **(B)** CHO-K1 cells were transfected with RRV-p14 and treated with or without 2 mM MβCD prior to and during fusion. Cells were fixed at 12 hpt, and the extent of fusion determined by quantifying Giemsa-stained cells. N-BP-2 cells (CHO cells deficient in *de novo* cholesterol synthesis) were grown in the presence or absence of extracellular cholesterol for 24 h or 48 h prior to transfection with RRV-p14. At 12 hpt cells were fixed and the extent of fusion determined by quantifying Giemsa-stained cells. Data are presented as the mean ± SE (n=3).

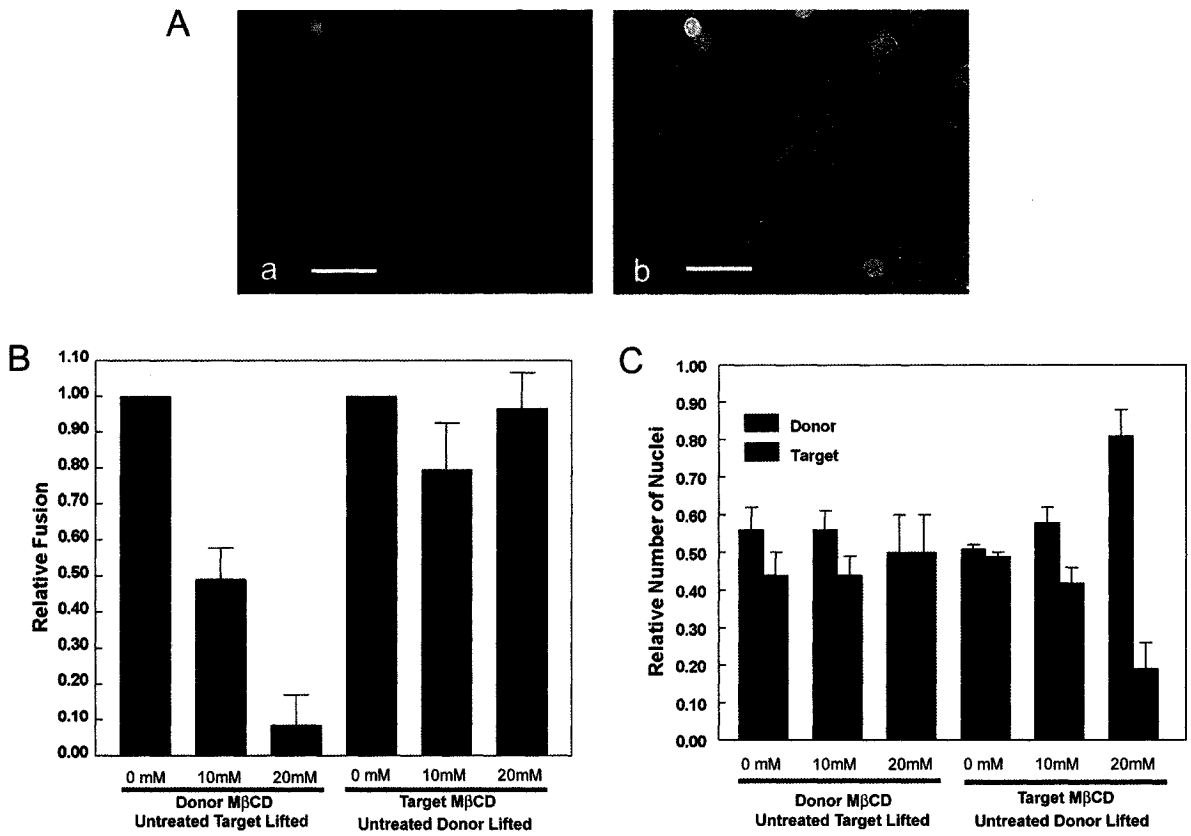

Figure 5.4 Cholesterol is required in the donor membrane. **(A)** RRV-p14-transfected QM5 donor cells (red) were added to QM5 target cells (blue) grown on coverslips, incubated for 2 h and methanol-fixed. Heterotypic fusion (donor to target) is detected by the presence of both blue and red nuclei in the same syncytium (a). DIC overlay indicates the borders of the syncytium (b). Scale bar = 10 μm. **(B)** CMTMR (red) or CMAC (blue) labelled QM5 cells grown on glass cover slips and serving as donor (RRV-p14 transfected) or target cells (untransfected) were left untreated or were cholesterol-depleted with 10 or 20 mM MβCD as indicated. Untreated donor or target cells were added to MβCD-treated cells for 4 h and methanol-fixed. Relative amounts of fusion were determined by determining the average number of fluorescent syncytial nuclei per field of view at 200x magnification. Data are presented as the mean ± SE (n=4). **(C)** The relative number of donor (red) and target (blue) nuclei in each condition in **(B)** is presented as the mean ± the SE (n=4).

Figure 5.5 The FAST proteins do not strongly co-localize with raft markers or with each other. **(A)** QM5 cells were transfected with PLAP and the fusion-dead RRV-p14G2A to prevent fusion and allow time for PLAP expression. At 24 hpt live cells were surface-stained at 4°C with anti-p14, anti-PLAP and fluorescently-conjugated secondary antibodies to detect protein localization. **(B)** QM5 cells were transfected with wild-type RRV-p14 and live cells were immunostained at 5 hpt using anti-p14 and AlexaFluor488-conjugated secondary antibody. Endogenous GM1 was detected at the same time with AlexaFluor555-conjugated cholera toxin B (CTB) and an anti-CTB secondary antibody. **(C)** QM5 cells were transfected with PLAP and immunostained live at 24 hpt for PLAP and GM1. **(D)** QM5 cells were co-transfected with RRV-p14-G2A and BRV-p15-2HAN and live cells were surface-stained at 24 hpt using rabbit anti-p14, mouse anti-HA and fluorescently-conjugated secondary antibodies. Scale bars = 10 μm.

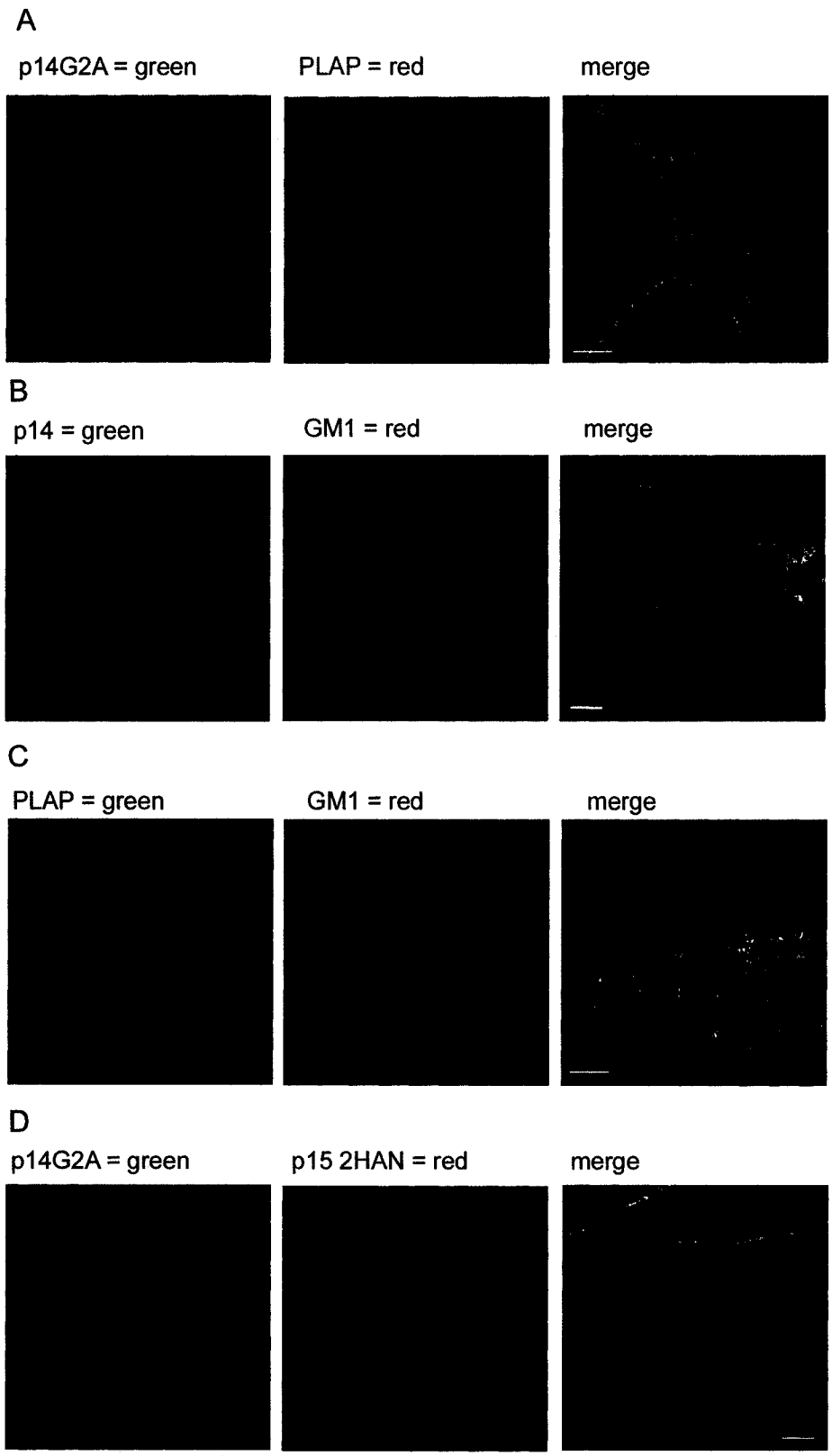

Figure 5.5 The FAST proteins do not strongly co-localize with raft markers or each other.

CHAPTER 6

FAST Protein-Mediated Fusion is Sensitive to Actin Alterations

6.1. Introduction

The previous chapters have identified components outside (cadherins) and within (cholesterol) the plasma membrane that affect FAST protein-mediated cell-cell fusion. Now our attention turns to factors inside the cell that might influence the susceptibility of the plasma membrane to FAST protein-mediated fusion. It is becoming increasingly clear from the data presented that alterations to actin remodelling can have dramatic effects on the function of the FAST proteins. Actin remodelling in response to cell adhesion (active adhesion) promotes fusion, while cholesterol extraction, which can disrupt actin, is inhibitory to FAST protein-mediated fusion. The ability of the cytoskeleton to influence the success of a fusion reaction is a topic largely ignored in the fusion field, but further investigation into the role of the cytoskeleton in FAST protein-mediated fusion is clearly warranted.

6.1.1. Components of the cytoskeleton

The cytoskeleton is a dynamic and complex network of fibre-forming proteins with diverse cellular roles including vesicle trafficking, cell migration, cell-matrix and cell-cell contact and adhesion, membrane dynamics and cell shape and polarity (135, 153, 228, 587). The three primary components of the cytoskeleton are actin-based microfilaments, tubulin-based microtubules and intermediate filaments. There are over 65 human intermediate filament proteins that are expressed in a tissue- and development-specific manner which include such proteins as keratin, the nuclear lamins and desmin (294). Intermediate filaments are composed of self-assembling, highly charged α-helical proteins that form coiled-coil structures and are primarily involved in providing structural support to the cell through interactions with desmosomes at regions of cell-cell adhesion (294, 605). Microtubules are assembled by polymerization of α-tubulin and β-tubulin heterodimers and are essential for organelle organization, vesicle trafficking, axonal transport, and mitotic spindle formation (15, 380, 399).

The actin microfilament network is the branch of the cytoskeleton network that has the greatest number of interactions with, and effects on, the plasma membrane. Actin is a globular, polarized protein with three isoforms (α, β and γ) in eukaryotic cells (reviewed in (142)). Monomeric actin (globular G-actin) polymerizes to generate filamentous actin (F-actin) microfilaments containing a barbed growing end and a pointed trailing end (142). G-actin binds to and dissociates from both ends of the F-actin filament, but with different affinities such that the rate of polymerization is highest at the barbed end and the dissociation rate is highest at the pointed end (142). This results in net polymerization in the direction of the barbed end in a process referred to as treadmilling (142, 412). The initiation of actin polymerization in response to various cellular stimuli is regulated by a variety of different actin-binding proteins (142).

6.1.2. Actin coordinates cell adhesion

The ability of actin to regulate membrane dynamics during cell migration and adhesion implicates this branch of the cytoskeleton as having the most potential to modulate the fusion ability of the FAST proteins. The process of an individual cell finding and binding a neighbour involves multiple and dramatic actin rearrangements and alterations to cell shape (124). Actin remodelling is typically coordinated by a series of small GTPases that respond to movement and to adhesion signals received at the plasma membrane (436). A description of the process of cell-cell junction formation between migrating cells that contact one another should suffice to introduce the host of molecular players involved in these processes.

Epithelial and fibroblast cells mediate cell-cell contact using nectins and cadherins, as described in Chapter 4 (Figure 4.3). Both nectins and cadherins can recruit actin to their cytoplasmic domains through different motifs that bind actin-adaptor proteins (530). Current models suggest that nectins are responsible for initiating cell-cell contact and for recruiting cadherins to contact sites to confer specificity and strength to the interaction (Figure 4.3) (241, 254, 531, 532). These initial contacts are mediated by a few molecules of nectins and cadherins, and for cellular junctions to form, the contact site must be extended (254). This involves remodelling the membrane and movement of the contacting cells to form a long, continuous contact (492).

The Rho family of small GTPases that regulate these membrane dynamics consists of over 20 members, of which three (RhoA, Cdc42 and Rac1) are the major players in cell migration and adhesion (14, 436). The GTPases are controlled by a series of guanine-nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine-nucleotide dissociation inhibitors (GDIs) (14). There are two major pathways involved in converting cell-cell contacts into cellular junctions. First, contact recruits actin to the cytoplasmic domain of the adhesion molecules in an effort to strengthen and expand the contact site (Figure 6.1) (3, 99). Second, actin polymerization promotes membrane protrusions and migration to the area to reshape the cells and deliver more adhesion molecules to the contact site (Figure 6.2) (53, 99, 492). These two pathways complement each other and result in the establishment of a cellular contact that incorporates cell- and junction- specific adhesion molecules, including desmins for desmosomes, claudins, occludins and junction adhesion molecules (JAM) for tight junctions and connexins for gap junctions (Figure 6.1) (241, 254, 283, 552).

Initial nectin interactions recruit l-afadin to their cytoplasmic domains, which in turn recruits actin (530). There also seems to be a physical connection between nectins and cadherins, although the exact mechanism for this interaction is unclear (Figure 6.1) (254). *Trans*-pairing cadherins also recruit actin to their cytoplasmic domains by recruiting β-catenin and several other adaptor proteins (2, 3, 427). Cadherins can potentially interact with nectins through α-catenin, which has afadin-binding capability (426, 529). Since this binding is relatively weak, it is believed the interaction may be more complicated, involving ponsin-vinculin and/or afadin DIL domain interacting protein (ADIP)-α-actinin bridges spanning the gap between afadin and α-catenin (13, 254, 340, 407). Actin is recruited to cadherins in a number of ways. First, β-catenin binds the cadherin and recruits α-catenin, which can bind actin directly (251, 257, 427). Also, α-catenin can recruit the actin-binding proteins vinculin, ZO-1, ZO-2 and α-actinin (Figure 6.1) (251, 427). The result of this lateral actin assembly is to promote recruitment of cadherins to nectin-based contacts, stabilize the adhesion site and facilitate its expansion and maturation into an adherens junction (AJ) (532, 567).

The molecules above generally form small but numerous points of contact. The formation of mature adherens junctions, which contain all the elements described above,

requires membrane remodelling to recruit more adhesion molecules to the contact site and "zipper up" the adjacent cells. The Rho family of GTPases accomplish this process through various effector proteins (14, 51, 180, 254). *Trans*-nectin interactions activate Cdc42 and Rac, which promote membrane protrusion and movement through formation of sheet-like lamellipodia (Rac) and finger-like filopodia (Cdc42) (Figure 6.2) (99, 182, 183, 533). *Trans*-cadherin interactions activate Rac, but not Cdc42, in a PI3K-dependent manner, which also promotes lamellipod formation (53, 254, 400, 604). Unpaired nectins in the vicinity of paired cadherins can block cadherin-dependent Rac activation, presumably to halt further junction formation until nectin pairing has occurred (Figure 6.2) (241, 532) This could serve as a regulatory process whereby simultaneous nectin and cadherin pairing ensure junctions are only formed between the proper cell types. The precise mechanism of GTPase activation in response to cell-cell contact is still being elucidated; however several GAPs, GEFs, and GDIs are recruited to sites of cell-cell adhesion which could interact with adhesion molecules directly or with associated adaptor proteins (113, 254).

The molecular targets of Rac include actin-binding proteins such as IQGAP1 and IRSp53/WAVE (179, 296, 370). IQGAP1 binds and recognizes the newly organized cytoskeleton assembling at nectin and cadherin contact sites where it can interact with activated Rac to initiate more actin polymerization (254, 271). In addition, Rac works through WAVE to activate Arp2/3 (135, 336, 542). Arp2/3 is a multi-protein complex that binds to F-actin and vinculin to initiate branching of actin filaments at 70° angles (40, 134, 135, 254). The resultant network of branched protrusions results in the formation of lamellipodia (17, 139). Similarly, Cdc42 interacts with IQGAP1 as well as N-WASP (323, 350). N-WASP is related to WAVE and interacts with the ARP2/3 complex to initiate actin remodelling and filopodia extension (1, 64, 348).

In summary, there are three main actin alterations that occur in response to nectin and cadherin engagement. First, nectins and cadherins initiate lateral actin assembly by recruiting adaptor proteins to their cytoplasmic domains. Second, activation of Cdc42 promotes membrane protrusions that increase the number of contact points. Finally, activation of Rac initiates the formation of lamellipodia that effectively connect the points of contact to form mature adherens junctions. At this point, other adhesion

molecules can be recruited to form other cell-cell junctions, such as tight junctions, desmosomes and gap junctions, which will further stabilize the adjoining cells (Figure 6.1).

6.1.3. Actin remodelling and fusion

The influence of the Rho GTPases and the actin cytoskeleton on modulating the function of fusion proteins in cell-cell fusion is not well explored. However, one might predict that activation of Cdc42 or Rac might enhance fusion as these molecules promote cell-cell contact. In the case of cortical actin and fusion, exocytosis requires that actin is transiently disassembled at the plasma membrane to facilitate docking of exocytic vesicles (153, 505, 549). Disassembly of the cortical actin network is also required for repair of torn membranes to allow vesicles to supply the lipids needed to patch the plasma membrane breach (358, 359, 376). With respect to viral fusion proteins, one of the few examples of actin affecting fusion is the ability of Rac activation and actin reorganization to support HIV entry and syncytium formation (428).

Actin remodelling could influence membrane fusion in a number of ways that are not mutually exclusive. First, cortical actin networks can restrict lipid mobility and pose a barrier to fusion pore expansion by increasing the energy required for lateral membrane movement (358, 410, 486). This may be why pore expansion has been reported to be the most energy costly step in some fusion reactions (104, 342, 364). Second, actin-coordinated membrane protrusions can change membrane curvature and lipid packing thereby altering the thermodynamic stability of a membrane (597). These alterations could either decrease or increase the energy required to initiate membrane merger, thus enhancing or hindering membrane fusion. Finally, actin remodelling at the plasma membrane to promote cell-cell contact and adhesion can increase the number, duration and stability of cell-cell contacts, thus increasing the opportunities for the FAST proteins to initiate fusion. Since cell membranes and actin remodelling are dynamic, certain regions of a cell may better support fusion at any given time.

Here, the role of actin in the fusion process mediated by the FAST proteins was investigated. The process of syncytium formation induced gross cytoskeletal rearrangements and disruption of F-actin assembly that was inhibitory to fusion. Further,

alteration of normal actin remodelling using cytoskeleton inhibitors and effector-domain mutants of Rho, Cdc42 and Rac revealed that FAST protein-mediated fusion is more sensitive to actin rearrangements that affect cell-cell contact. In addition, ARV-p10 displayed different sensitivities to actin alteration than did the other FAST proteins. Thus, the FAST proteins require an intact actin network for efficient fusion but have developed individualized responses to the molecules regulating actin organization.

6.2. Results

6.2.1. FAST protein-mediated syncytium formation causes dramatic actin remodelling

The implication of active adhesion in efficient RRV-p14-mediated cell-cell fusion (Chapter 4) prompted an investigation into how FAST protein-mediated fusion responds to changes in actin. Actin morphology in QM5 cells under various conditions was assessed by immunofluorescence microscopy. Untransfected QM5 cells adopt a typical fibroblast morphology with stress fibres connecting points of cell-substrate contact (Figure 6.3A and B). The leading edges of the cell in Figure 6.3A (red PI stain indicates nuclei of individual cells) contain several actin-filled protrusions that extend perpendicular to the concentric arcs of actin that lie parallel to the plasma membrane. This pattern is typical of a migrating cell. The cluster of cells in Figure 6.3B contains some actin localization at regions of cell-cell contact, consistent with the establishment of cellular junctions. Similarly, individual cells transfected with RRV-p14 (red) have actin that appears normal (Figure 6.3C) suggesting that that RRV-p14 does not directly affect actin organization. In contrast, syncytia formed by RRV-p14-mediated fusion contain dramatic cytoskeletal modifications (Figure 6.3D). Four main differences between syncytial cells and normal cells were consistently observed. First, syncytia showed a loss of stress-fibre density, forming fewer and thinner filaments that ran the length of the syncytium. Second, there was an increase in actin density at the borders of the syncytium, which was less pronounced at regions of cell-cell contact (Figure 6.3D). In addition, syncytia contained more cytoplasmic actin that assembled as large patches of fine, wispy filaments (Figure 6.3D). Finally, densely staining actin clumps appeared near the centre of the syncytium, generally over the syncytial nuclei (Figure 6.3D).

This actin organization might be particularly relevant to the fusion reaction, as indicated by the images in Figure 6.4. DIC and fluorescence images reveal what appears to be fusion between two syncytia or a syncytium and a target cell (Figure 6.4A and B). Of particular interest is the actin organization at the neck of the potential fusion site (Figure 6.4C). Figure 6.4C is a series of Z-axis sections of the area selected in figure 6.4B. As one moves from the top of the syncytium to the bottom, one sees that the actin completely surrounds the fusion pore except where the syncytium meets the substrate (Figure 6.4C). This essentially forms an actin tunnel that appears to be constricting the fusion site and preventing it from expanding. It is unclear if the actin ring is recruited to the fusion site or if it represents a pre-existing actin structure like cortical actin. Several actin alterations can be identified within syncytial cells, especially at what appear to be fusion sites; however, it seems as though the fusion process, not the fusion protein, is mediating these changes.

6.2.2. FAST protein-mediated fusion is sensitive to actin disruption

To further investigate the role of actin on FAST protein-mediated fusion, the ability of different cytoskeleton-altering drugs to modulate syncytium formation was analyzed. Cytochalasin D, a fungal metabolite that binds to F-actin and prevents addition of new actin monomers (295, 473), was used to disrupt actin. The addition of 1 μg/ml or more of cytochalasin D to QM5 cells completely disrupted actin, and caused the formation of actin plaques and deterioration of cell-cell contacts and junctions (Figure 6.5B). Low doses of cytochalasin D produced intermediate phenotypes where actin staining becomes more punctate, but normal structures such as cell-cell contacts (Figure 6.5B) and stress fibres (Figure 6.5A) were maintained. All of the FAST proteins displayed a dose-dependent sensitivity to cytochalasin D, although they did retain about 60-80% of their fusion activity with doses less than 0.5 μg/ml (Figure 6.5C). Higher concentrations of cytochalasin D resulted in more dramatic fusion inhibition with the notable exception of ARV-p10, which was more resistant to the drug's effects. Jasplakinolide, an irreversible actin-stabilizing drug, also inhibited RRV-p14-mediated fusion in a dose-dependent manner (Figure 6.5D).

Since junction formation is important for efficient FAST protein-mediated fusion (Chapter 4), wortmannin, a PI3K inhibitor, was used to interfere with normal adhesion pathways (432). PI3K is a central element in several signalling pathways, including those involving Rac- and Cdc42-mediated cell motility and actin remodelling in response to adhesion (45, 80, 391, 464, 546, 591). Inhibition of PI3K with wortmannin could reduce adherens junction formation by inhibiting cell motility and possibly limiting Rac-mediated active adhesion (391). Reduction of cell motility and active adhesion could both result in inhibition of FAST protein-mediated fusion by limiting the number, size and stability of cell-cell adhesions. Partial inhibition of fusion was observed with higher doses of wortmannin (200-800 nM) (Figure 6.6A), but it should be noted that these concentrations are 2-8 times the 100 nM dose routinely used to inhibit PI3K activity (319, 349, 417). In addition, actin was largely unaffected by wortmannin treatment; however, the highest dose (800 nM) produced increased actin staining at the cell periphery and the production of more defined finger-like projections not observed in untreated cells (Figure 6.6B). It is possible that QM5 cells might be more resistant to the drug, which could be confirmed by assessing PI3K phosphorylation activity at the various drug concentrations used in QM5 cells. The other alternative is that general drug toxicity, or pleiotropic drug effects are responsible for fusion inhibition at high wortmannin concentrations. These results suggest that PI3K inhibition has, at best, a marginal effect on FAST protein-mediated fusion, an observation that correlated with an inability of wortmannin to effectively disrupt actin.

To explore possible roles for other branches of the cytoskeletal system in FAST protein-mediated fusion, the tubulin-binding drug nocodazole was used to disrupt the microtubule network (95). The syncytia-forming ability of all the FAST proteins was unaffected by nocodazole treatment (Figure 6.6C). Although nocodazole is not an actin-disrupting drug, exposure to cells resulted in minor alterations to cell morphology, causing cells to become more shrunken and cuboid, and to develop actin-filled projections with a possible increase in the number of stress fibres (Figure 6.6D). Thus, the FAST proteins seem to be unaffected by disruption of the microtubule network.

The ability of other treatments that inhibited FAST protein-mediated fusion (i.e. calcium depletion and cholesterol extraction) to affect actin organization was also

assessed. QM5 cells were grown in the absence of extracellular calcium or treated with 2 or 10 mM MβCD, and the actin distribution was observed with phalloidin staining. Cells grown in low-calcium lost most of their cell-cell contacts and developed actin-dense membrane ruffles with occasional small actin-filled projections (Figure 6.7). Stress fibres in these cells were present, but less pronounced (Figure 6.7). These observations are consistent with what would be expected of cells lacking cell-cell contact. Such cells would limit stress-fibre formation in preparation for substrate exploration using actin-filled projections (183, 586). Further, the absence of cadherin-mediated contacts due to calcium depletion causes the cells to be rounded, thus requiring increased cortical actin to support the more spherical cell shape (586).

Cholesterol extraction with 5-10 mM MβCD has been reported to promote cortical-actin stability, production of actin-filled plasma membrane projections (microspikes), loss of stress-fibre density and general cytoplasmic actin instability (297). Here, treatment of cells with 2 mM MβCD did not dramatically affect actin organization (Figure 6.5). However, treatment with 10 mM MβCD correlated with the production of actin-filled plasma membrane projections (Figure 6.6), which could be the same structures as the microspikes reported previously (297). A loss of stress-fibre density was difficult to determine based on qualitative analysis of multiple cell images. Image analysis was also insufficient to make conclusions about the stability of cortical and cytoplasmic actin, but the development of microspikes is consistent with the results of MβCD treatment previously reported (297).

In summary, the FAST proteins appear to be sensitive to certain types of cytoskeleton alterations. Microtubules do not seem to be important for fusion, while gross changes to the cytoskeleton induced by cytochalasin D, jasplakinolide and possibly calcium disruption are inhibitory to fusion. Disruption of PI3K-dependent actin alterations and cell motility had only minor effects on fusion, and only at high concentrations of inhibitor. Finally, cholesterol extraction had minimal effects on actin morphology, but may have more dramatic effects on actin stability that could affect fusion. Thus, certain elements of the actin network appear to be involved in fusion while others are not.

6.2.3. GTPase-dependent cytoskeleton alterations have diverse effects on FAST protein-mediated fusion

The Rho family of small GTPases coordinates cell migration and cell-cell contact by regulating actin remodelling. The implication of active adhesion mediated by these GTPases to enhance FAST protein-mediated fusion (Chapter 4) suggests that alteration of the pathways controlled by these molecules might dramatically affect cell-cell fusion. A reasonable prediction is that GTPases that promote cell-cell adhesion (Rac and Cdc42) might enhance fusion, while pathways that promote cell adhesion to the substratum (Rho) might inhibit fusion. Wild-type and constitutively active GFP-tagged forms of several Rho GTPases (RhoA, Rac1 and Cdc42) in eukaryotic expression vectors were acquired from from Dr. Michael Way (354, 381) (Table 6.1). Although the GTPases used in these experiments are of human origin, they are 99-100% conserved between human and chickens, and should function normally in QM5 quail fibroblasts. The rates of GTPase expression in QM5 cells were determined by FACS analysis by detecting the GFP tag on all constructs and compared to GFP-tagged RRV-p14 expression (Figure 6.8). Protein expression increased over time, reaching maximum levels at 16-22 h post-transfection with about 70% of cells fluorescing above background levels of untransfected cells.

The functionality of the different constitutively active GTPases in QM5 cells was assessed by microscopic analysis of GTPase expression, cell morphology and actin organization. Both Cdc42 and Rac1 promote cell migration and cell-cell contact by initiating formation of filopodia and membrane ruffles, respectively (14, 51, 254). Expression of Cdc42CA in QM5 cells yielded unexpected cytoskeletal changes in the form of membrane ruffles produced by actin filaments running perpendicular from a cytoplasmic actin spine set parallel to the plasma membrane (Figure 6.9). This is the opposite observation than predicted. Similarly, cells expressing constitutively active Rac1 were expected to exhibit increased formation of membrane ruffles, but instead contained many actin-filled projections which originated from an actin belt running parallel to the plasma membrane, consistent with filopodia (Figure 6.9). These unpredicted results are likely due to the overlapping and redundant pathways in which

Cdc42 and Rac are involved (14, 162). The RhoCA and Cdc42CA clones were also re-sequenced to verify these unusual results.

Expression of RhoACA in QM5 cells produced the anticipated results. In most cells the basic fibroblast morphology was maintained, with an increase in the number, but not size, of stress fibres (Figure 6.9, third row). In about 10-20% of cells, however, the effect was even more dramatic and the number and size of stress fibres increased and the fibres formed around a single organizing centre (Figure 6.9, fourth row). These cells were smaller in size and contained many finger-like membrane projections formed as the cell contracted on itself (Figure 6.9). These actin-dense structures were readily visible by actin staining and scanning electron microscopy and are indicative of retraction fibres (Figure 6.9) (14).

Microscopic analysis confirmed and characterized the effects of the different GTPases on QM5 cells at 12 hpt. This time point is optimal for studying the effects of these changes on ARV-p10- and BRV-p15-mediated fusion since these two FAST proteins do not initiate fusion until after 12 hpt. RRV-p14 initiates fusion as early as 4 hpt, which is before the morphological effects of the GTPases are observed (data not shown). To ensure that RRV-p14-mediated fusion occurred after the GTPases were exerting their effects I used RRV-p14C88, a C-terminal truncation mutant of RRV-p14 missing the last 37 aa but retaining fusion activity (110). This RRV-p14 mutant fuses more slowly than does the wild-type protein, with fusion beginning at about 8-12 hpt, similar to NBV-p10, and minimizes the amount of fusion occurring before the effects of the GTPases appear.

It was predicted that Cdc42CA and RacCA should enhance FAST protein-mediated fusion by promoting cell-cell adhesion and junction formation, thus creating conditions more favourable for fusion. Conversely, RhoCA should antagonize endogenous Rac and Cdc42 activity, resulting in fusion inhibition. To test these hypotheses, QM5 cells were co-transfected with ARV-p10, BRV-p15, RRV-p14C88 or NBV-p10 and with pcB6-GFP (GTPase plasmid backbone that expresses GFP) or one of the constitutively active GTPases. Co-transfection with any given FAST protein and pcDNA3 (FAST protein-encoding plasmid backbone) or pcB6-GFP resulted in virtually identical levels of fusion (data not shown), indicating that GFP expression had no effect

on the ability of the FAST proteins to cause fusion. Data presented in Figure 6.8 are set relative to each FAST protein co-transfected with pcB6-GFP.

ARV-p10-mediated fusion was the most sensitive to GTPase expression (Figure 6.10). Consistent with the hypothesis, there was a trend towards increased fusion when ARV-p10 was co-expressed with RacCA and a dramatic ~9-fold increase in fusion when it was co-expressed with Cdc42 (Figure 6.10). The other FAST proteins had less pronounced responses to co-expression with Cdc42CA and RacCA, with changes in fusion efficiency never reaching statistical significance. However, BRV-p15 showed a tendency for increased fusion in the presence of RacCA, as did NBV-p10 in a preliminary result. RRV-p14C88 was generally unaffected by Cdc42CA or RacCA expression. Consistently, however, RhoCA was inhibitory to FAST protein-mediated fusion. RRV-p14C88 was the least affected and was inhibited by about 50%. The other FAST proteins were inhibited by over 80% and fusion was virtually abolished in cells expressing BRV-p15 and RhoCA. Thus, the FAST proteins are affected to different degrees by activated Rho GTPases, with the general trend for the protrusion-promoting Cdc42 and Rac to enhance fusion, and Rho to be fusion-inhibitory.

Based on its resistance to actin disruption by cytochalasin D and its sensitivity to cytoskeletal changes mediated by the Rho-family of GTPases, ARV-p10 appears to be an outlier in the FAST protein family when it comes to analysis of actin alterations. The only other experiment where ARV-p10 behaved dramatically differently from the other FAST proteins was when it was expressed in L-929 cells (Figure 4.1). In that cell line ARV-p10, usually the slowest fusogen, caused fusion with much faster kinetics than that caused by NBV-p10 or BRV-p15 and equivalent to that caused by RRV-p14 (Figure 4.1). Examination of the actin in L-929 cells revealed an unusual branched morphology (Figure 6.11A) that might be indicative of deregulated activation of the Arp2/3 branching machinery that is controlled by Rac and Cdc42 (40, 446, 525). The unusual actin arrangements in L-929 cells might account for the altered fusion kinetics of ARV-p10 in these cells.

Original investigations into whether the FAST proteins could remodel actin were conducted using RRV-p14 as a model system (Figure 6.3). Given the unusual behaviour of ARV-p10, it was necessary to revisit this question as ARV-p10-mediated actin

modulation could account for the dramatic differences observed with ARV-p10 over the other FAST proteins. To address this, the actin organization in QM5 cells co-expressing ARV-p10 and either RhoCA or Cdc42CA was examined. The few ARV-p10-mediated syncytia formed in RhoCA-expressing cells displayed actin organization typical of non-syncytial RhoCA-expressing cells, with large bundles of stress fibres forming within the syncytium (Figure 6.11B). Analysis of ARV-p10-mediated syncytia in Cdc42CA-expressing QM5 cells revealed actin distributions similar to those in syncytial cells (Figure 6.3D), with perinuclear disorganised actin (Figure 6.11B). Actin at the borders of the syncytium resembled the lamellar protrusions in Cdc42CA-expressing cells lacking ARV-p10 (Figure 6.9), although less pronounced and less organized (Figure 6.11C). Thus, ARV-p10 appears to be responding to actin alterations, not generating them. In general, it does not seem as though the FAST proteins directly alter actin organization, but they appear to have evolved individualized sensitivities to actin rearrangements.

6.3. Discussion

6.3.1. Cell-cell fusion causes dramatic actin remodelling

The results from previous chapters implicated actin remodelling as an important factor for FAST protein-mediated fusion. Preliminary investigations concerning this involvement were conducted using fluorescence microscopy, cytoskeleton-altering drugs and effector-domain mutants of the Rho family of GTPases that help regulate actin reorganization. Microscopic analysis revealed that the process of syncytium formation caused dramatic cytoskeleton remodelling as syncytial fibroblasts lost their normal morphology and developed several unusual actin-based structures (Figure 6.3D). In general, these structures appeared disorganized, forming aggregates of actin toward the centre of syncytia and non-symmetrical webs of fine actin toward the cell periphery. It is unclear if this organization is reflective of deregulation of actin pathways or if it is a controlled event. Adherent cells that are freshly deposited on a substratum often display similar actin organization in an attempt to stabilize the atypical spherical morphology (586). The observed actin remodelling appears to be in response to syncytium formation and not to the presence of the FAST protein itself, since non-syncytial RRV-p14-transfected cells appeared normal (Figure 6.3C). There is no evidence to indicate that the

FAST proteins directly remodel actin, but with half or more of the FAST protein in the cytoplasm, the potential for interaction with cellular factors should be further investigated.

Interestingly, the fusion pores, or necks, between two syncytial cells stain strongly for actin, forming what appeared to be an actin-based ring around the fusion pore that could be restricting or regulating the rate of pore expansion (Figure 6.4). It should be noted that the fusion pores observed by microscopy are incredibly large compared to the minute fusion pore present at the site of fusion initiation. However, the presence of actin at the fusion site could have the same restrictive effects on a small pore as on a large one. The mechanism for signalling actin reorganization around a fusion pore is unknown. It is possible that a change in the membrane tension and/or curvature that results from fusion, or disruption of cortical actin during the fusion event can activate the cell to apply actin around the fusion pore to increase stability or repair the gap in the cortical actin network (357-359, 376). The presence of membrane-bound actin might contribute to the high amount of energy required to promote pore expansion as recently described for HIV gp41-mediated fusion a cell-cell fusion assays (104, 342, 364). It was noted that energy is required at the end of the fusion reaction to force lipids to adopt the energetically unfavourable bilayer intermediates required to support pore expansion (104). In addition to the natural resistance of fusion pores to expand, energy might be required to disrupt the cortical cytoskeleton that can restrict lipid movement (153).

6.3.2. A functional actin network is required for fusion

The actin reorganization described above refers to those changes that occur after fusion has occurred. However, the actin organization of a cell can dictate whether FAST protein-mediated fusion will proceed. Inhibition of actin polymerization with cytochalasin D or jasplakinolide prevented RRV-p14-mediated fusion. Similarly, cytochalasin D also inhibited the fusion activity of the other FAST proteins. Inhibition of fusion by cytochalasin D coincided with a progressive loss of actin at the plasma membrane and disruption of cell-cell junctions. This observation is consistent with previous data that revealed a need for active adhesion to support efficient FAST protein-

mediated fusion (Chapter 4). Proper cellular junction formation requires actin remodelling in response to adhesion-molecule engagement. The ability of cytochalasin D to bind monomeric G-actin and the ends of F-actin filaments (468, 473, 556) provides two mechanisms for disrupting cell-cell adhesion. First, binding G-actin would prevent formation of new actin filaments required for junction formation. Second, capping existing filaments would alter the rate of G-actin incorporation required to maintain those filaments, shifting the balance to favour actin disassembly over filament maintenance. Blocking new junction formation and disassembling old junctions could both be inhibitory to FAST protein-mediated fusion. It should be noted that while fusion-inhibitory doses of cytochalasin D (~1 μg/ml) cause loss of actin from the plasma membrane, cell-cell contacts remain readily observable, indicating a more dramatic loss of active rather than passive adhesion.

The observed inhibition of FAST protein-mediated fusion appears to be specific to the actin branch of the cytoskeleton, as the microtubule inhibitor nocodazole did not affect fusion over a range of concentrations widely used to disrupt the microtubule network (34, 37, 286). At concentrations of 2.5-10 μM, cell shape was altered and several actin-filled projections were visible. It is difficult to determine with static images if these represent retraction fibres or active projections, but the former is more likely, since nocodazole has been reported to promote cell contractility and stress-fibre formation through activation of Rho (37, 122, 408). If the nocodazole-induced actin rearrangements are actually the consequence of Rho activation, the lack of fusion inhibition in nocodazole-treated cells must be reconciled with the dramatic fusion inhibition observed when RhoCA is co-expressed with the FAST proteins (Figure 6.8). This important point will be revisited below.

Wortmannin, a PI3K inhibitor, was used to inhibit cell motility and certain GTPase-dependent actin remodelling events (134, 254, 289, 391). Inhibition of cell motility would limit the number of cell-cell contacts, potentially reducing the number of FAST protein fusion opportunities. In addition, wortmannin treatment could potentially block actin remodelling in response to nectin- and cadherin-activated Cdc42 and/or Rac1, thus preventing cells from forming active adhesion structures, and resulting in fusion inhibition (241). Consistent with the proposed outcome, all of the FAST proteins were

sensitive to wortmannin at the higher concentrations used; however, these preliminary observations must not be overstated. Nectins may be able to activate Cdc42 and/or Rac1 in a PI3K-independent manner, allowing active adhesion in the presence of wortmannin (254, 492, 531). In addition, non-paired nectins can inhibit PI3K-dependent Rac activation in response to cadherin engagement (Figure 6.2), making it more likely that the inhibitory effects of wortmannin treatment are due to decreased cell-cell contact caused by limiting cell mobility. Furthermore, PI3K is a central molecule in several different signalling pathways; thus global effects of PI3K inhibition could lead to general disruptions in cell signalling, making the results presented here difficult to interpret on their own (36, 522). Finally, the concentrations of wortmannin required to inhibit fusion were 2-8 fold higher than the 100 nM concentration commonly used to inhibit PI3K activity (134), making the possibility of undesired toxicity or pleiotropic effects more likely. Future investigations will have to confirm the ability of wortmannin to inhibit PI3K and/or generate a passive-adhesion phenotype.

The effect of cholesterol extraction and extracellular-calcium depletion on actin was also assessed to better understand the effects of these treatments on the cytoskeleton, and to determine if the fusion inhibition caused by these treatments (Chapters 4 and 5) might be partially attributed to actin modulation. With respect to calcium depletion, although actin alterations were observed, loss of fusion efficiency was likely due to loss of cell-cell contact rather than to actin disruption. This conclusion is supported by the observations with L and EL cells (Chapter 4), were fusion inhibition in low-calcium was determined to be dependent on the presence of E-cadherin and not on some pleiotropic effect of calcium depletion.

The development of plasma membrane projections observed with 10 mM MβCD treatment were consistent with previous reports (297), but these projections were absent from cells treated with 2 mM MβCD. Since the lower concentration of MβCD is sufficient to inhibit ~80% of FAST protein-mediated fusion (Chapter 5) without noticeable actin remodelling, it is unlikely that the actin changes observed with 10 mM concentrations are responsible for fusion inhibition. However, the ability of cholesterol extraction to stabilize existing actin structures would not be observable by microscopy,

but could inhibit fusion by preventing new membrane contacts and by impeding lipid mobility through strengthening of the cortical actin network.

6.3.3. GTPases can alter FAST protein-mediated fusion

One of the most reasonable models for how actin could enhance FAST protein-mediated fusion is by promoting and stabilizing the cell-cell contacts that enhance FAST protein function (Chapter 4). Several Rho-family GTPases control cell motility and adhesion. Cdc42 and Rac1 respond to cell-adhesion-molecule engagement and recruit actin to promote membrane contact and to stabilize junctions (181, 254). Thus, activation of these two GTPases could enhance FAST-protein-mediated fusion by increasing the efficiency of cell adhesion (181). In contrast, activation of the Rho pathway could inhibit fusion by antagonizing the activity of Rac1 and Cdc42, promoting anchoring of cells to the substrate and restricting cell motility and adhesion (181). Implicating these molecules in the FAST protein fusion reaction would help define a role for actin remodelling in FAST protein-mediated fusion and would lend further support to the model that the FAST proteins exploit active-adhesion pathways for efficient fusion.

Expression of constitutively active Rho GTPases in QM5 cells produced dramatic effects on actin dynamics, which correlated with altered FAST protein fusion efficiency. Cdc42CA and RacCA induced actin-mediated plasma membrane extensions but, in general, this correlated with only minor increases in FAST protein-mediated fusion in RacCA-transfected cells. The most dramatic effect of GTPase expression was a ~9-fold increase in ARV-p10-mediated fusion in Cdc42CA-transfected cells. The implications of this result will be discussed shortly, but first the effects of RhoCA expression on FAST-protein-mediated fusion must be addressed.

6.3.4. Rho activation inhibits FAST protein-mediated fusion

RhoCA caused substantial increases in stress-fibre formation, occasionally to the point of causing cell retraction. Consistently, FAST protein expression in RhoCA cells was inhibited by 50-90% depending on the fusogen, suggesting that the effects of RhoCA are inhibitory to fusion. There are a couple of ways in which the activity of RhoCA could be inhibitory to fusion. First, retraction of cells would physically pull

adjacent cells apart, preventing cell-cell contact and inhibiting fusion by inhibiting the binding step of the fusion reaction. Prior to, or in the absence of, the extreme scenario where cells are forced to dissociate, Rho activation could simply antagonize the effects of Cdc42 and Rac to prevent the formation of new cell-cell contacts, thus limiting the opportunities for fusion initiation.

Several lines of reasoning suggest that RhoCA-dependent fusion inhibition is due to cell retraction. First, although only 10-20% of RhoCA-transfected cells display the retraction phenotype at 12 h post-transfection (Figure 6.9), this percentage may have dramatically increased by ~19 h post-transfection when syncytia formation was quantified. This would allow retraction-mediated fusion inhibition to affect more than 20% of cells and account for the ability to cause 80-90% inhibition of fusion. Typically, the majority of RhoCA-transfected cell monolayers expressing the FAST proteins contained retracted cells. In addition, the ARV-p10-mediated syncytia formed in RhoCA-expressing cells display thick stress fibres (Figure 6.11).

Also consistent with the retraction model is the observation that the FAST proteins with the faster fusion kinetics in QM5 cells are less sensitive to RhoCA activity. This may reflect the gradual progression of RhoCA-expressing cells toward a non-fusogenic phenotype. RRV-p14C88 begins to cause fusion at about 8 h post transfection, when less than 10-20% of transfected cells adopt the retraction phenotype. Thus, RRV-p14C88 might be able to cause fusion in cells expressing RhoCA until the stress-fibre formation becomes extreme enough to cause cells to retract, at which point fusion is inhibited. The slower-fusing BRV-p15 would only begin to cause cell-cell fusion at about 13-15 hpt when a larger proportion of cells would be retracted, resulting in less overall fusion.

A final observation in support of the retraction model is the inability of nocodazole to inhibit FAST protein-mediated fusion. Nocodazole-treated cells display some signs of Rho activation including small retraction fibres, change in cell shape and a possible increase in stress fibres. Similar nocodazole-induced effects were reported elsewhere using lower drug concentrations (2 μM vs 10 μM); however the cellular changes were more pronounced (37). Even though Rho might be activated in nocodazole-treated cells, cell-cell contact was largely unaffected (data not shown) and

the effects on actin remodelling were not as severe as in RhoCA-expressing cells. The lack of significant cell retraction may explain the lack of fusion inhibition.

Combined, these data suggest that Rho-dependent fusion inhibition is the result of extensive stress-fibre formation leading to cell retraction and loss of fusion-promoting cell-cell contacts. The biological relevance of this observation is questionable, since normal signalling pathways are unlikely to produce such extreme Rho activation that cells would actually retract. It does, however, support the conclusion from Chapter 4 that normal cellular junction formation is required for enhancement FAST protein-mediated fusion. These results further suggest that the ability of Rho to antagonize the adhesion-promoting activity of Cdc42 and Rac may not be a major factor in FAST protein-mediated fusion since Rho activation does not inhibit fusion until cell retraction occurs. Further, activation of Cdc42 and Rac generally has only minimal effects on FAST protein-mediated fusion, with Rac activation supporting a moderate fusion increase, suggesting that these adhesion-promoting molecules might play a minor role in the FAST protein fusion reaction.

Future investigations will have to further characterize the molecular consequences of GTPase activation over expression. Since these GTPase signalling pathways are redundant and overlapping, there is the potential to produce unexpected actin remodelling or GTPase activation in transfected cells, which could limit the ability to properly interpret the results.

6.3.5. GTPase-independent actin remodelling may be sufficient to support FAST protein-mediated fusion

The ability of FAST protein-mediated fusion to be progressively enhanced by passive, then active, adhesion was demonstrated in Chapter 4. The preliminary results presented here suggest that Rho GTPases may not be key regulators in the pathways responsible for establishing fusion-promoting active adhesions. However, GTPases help regulate only two of the three main actin-remodelling events that occur subsequent to adhesion-molecule engagement. Rac and Cdc42 are responsible for orchestrating the membrane movements that promote cell-cell adhesion and junction formation, but are not

essential for the recruitment of actin to the cytoplasmic domains of the cadherins and nectins, which helps to stabilize newly formed contacts (254).

GTPase-independent lateral actin assembly forms parallel to the plasma membrane and connects engaged adhesion molecules, thus stabilizing and expanding the contact site (3, 16, 290, 567). These structures might be more relevant to the FAST protein-mediated fusion reaction by supporting fusion in a couple of ways. First, initial cell-cell contacts are often made at sites of membrane protrusion where the increased membrane curvature and the tension provided by the actin-mediated extension could serve as fusion-promoting factors (272). In addition, the increased stability provided by actin at these initial contact sites might enhance fusion by providing a stable platform for aggregating FAST proteins in relatively small area and providing them with sufficient time to exert their fusion activity. The dependence on GTPase-independent actin remodelling might help to rationalize the substantial fusion inhibition observed with cytochalasin D, which would disrupt all actin assembly pathways, and the relatively minor responses to GTPase alteration, which affect only a subset of actin remodelling pathways.

Steps should be taken to map the effects of constitutively active GTPase expression in FAST protein-transfected cells by detecting the activation status of all three major Rho GTPases (Cdc42, Rac and Rho) for each constitutively active mutant. This will help to confirm that actual events correspond to the predicted effects of GTPase expression instead of relying solely on microscopic evidence. In addition, expression of Cdc42CA and RacCA was predicted to increase cell-cell adhesion as a mechanism of enhancing fusion. This predicted increase in adherence should be confirmed. If it can be established that Cdc42CA and RacCA cause increased adhesion as predicted without causing unexpected signalling events, there would be a strong case that the FAST proteins respond differently to actin modification and steps should be taken to determine why.

6.3.6. ARV-p10 has unique responses to actin alterations

ARV-p10 responded to actin alterations in more dramatic ways than did the other FAST proteins. In particular, co-expression with Cdc42CA resulted in a ~9-fold increase

in fusion, suggesting Cdc42-mediated cytoskeletal alterations could significantly enhance ARV-p10-mediated fusion. Consistent with this hypothesis, fusion mediated by ARV-p10 was also enhanced relative to the other FAST proteins in L-929 cells (Figure 4.1). These cells appear to have an actin-remodelling defect that results in branching of actin and many actin-filled protrusions reminiscent of Cdc42 or Rac activation (Figure 6.7 and 6.9A). Thus, unlike the other FAST proteins, ARV-p10 seems particularly responsive to GTPase-mediated actin remodelling. In addition, ARV-p10 was the least sensitive to cytochalasin D treatment, with even the highest dose tested (2 μg/ml) being insufficient to completely abolish ARV-p10-mediated fusion. These observations may appear contradictory since in one case ARV-p10 is sensitive to actin alterations while in the other it is resistant. A possible explanation is that ARV-p10 is a self-sufficient but opportunistic fusogen. It may have less of a dependence on actin than do the other FAST proteins to initiate fusion, hence its resilience to cytochalasin D treatment. However, it may be the best FAST protein at exploiting opportunities provided by GTPase-dependent actin-mediated cell protrusions. Despite the proposed enhancement of ARV-p10-mediated fusion by GTPase-dependent actin reorganization, it is still a less robust fusogen than RRV-p14 in L-929 cells and in QM5 cells expressing Cdc42CA, with RRV-p14 causing more and larger syncytia in both cell types.

6.3.7. FAST protein-mediated fusion is influenced by actin status

The actin organization in ARV-p10-induced syncytia expressing Cdc42CA or RhoCA is similar to that of cells expressing these proteins in the absence of ARV-p10, suggesting that, like RRV-p14, ARV-p10 does not directly alter actin organization. Instead, FAST protein-mediated fusion is influenced by the state of actin. However, the unusual responses of ARV-p10 to actin modulation might be attributed to specific sequences or motifs in the ARV-p10 protein that promote interactions with cellular factors involved in actin reorganization that facilitate ARV-p10-mediated fusion.

Similarly, the other FAST proteins might have evolved unique sequences or structural properties that allow them to respond to the cellular environment and exploit it in different ways to facilitate cell-cell fusion. The diverse sizes, sequences and functional motifs of the FAST protein endodomains means different FAST proteins could respond

differently to the plasma membrane changes mediated by actin remodelling. GTPase-dependent and GTPase-independent actin remodelling can enhance or inhibit cell-cell adhesion, membrane curvature and lipid mobility. Any of these alterations would change the energy required to initiate membrane fusion, with stabilizing forces (e.g. cortical actin) raising the energy barrier and destabilizing forces (e.g. membrane curvature) lowering the energy required for fusion initiation. Different FAST proteins may be able to deal with each modification in unique ways. For example, RRV-p14 has the largest polybasic region of all the FAST proteins. The association of the RRV-p14 polybasic region with negatively charged PIPs could exclude PIP-binding proteins from the plasma membrane (486). Several of these proteins are important for mediating actin-membrane interactions, including those that assemble cortical actin (202, 369, 486, 586). Thus, the RRV-p14 endodomain may be better able to antagonize cortical-actin assembly, or other actin-membrane interactions, than the other FAST proteins, which could prevent or counteract actin-induced increases in membrane stability. Similarly, the polyproline region in the RRV-p14 endodomain could potentially interact with cellular SH3 domain proteins, many of which are involved in regulating actin remodelling and membrane dynamics (89, 352, 373). Although speculative, these examples should help to illustrate the point that the structural diversity of the FAST proteins could allow them to respond in individual ways to actin and membrane dynamics.

Determining if the FAST proteins have any cellular binding partners that might allow them to interact with, or be affected by, the cytoskeleton would advance our understanding of how these unusual proteins exploit cellular machinery to enhance fusion. Passing cell lysates over affinity tagged FAST protein endodomains bound to a column could help to identify potential FAST protein-interacting proteins. Mass spectroscopy could be used to identify candidate FAST protein-binding proteins that may be associated with cytoskeleton remodelling, or possibly cell adhesion. Preliminary results obtained by using this strategy with RRV-p14 have yielded some candidate RRV-p14-interacting proteins (D. Top, unpublished results).

6.3.8. Rho GTPases prime cells for fusion

A very recent study parallels the one presented here using the fusion proteins from paramyxoviruses (476). In this work, Schowalter *et al.* compared cell-cell fusion mediated by the Hendra virus F and G proteins and SV5 F and HN proteins in the presence of GTPase effector-domain mutants. In many ways, paramyxovirus F-protein-mediated cell-cell fusion is analogous to FAST protein-mediated cell-cell fusion. In both systems, fusion does not require a low-pH trigger and depends only on expression of the fusion proteins on the cell surface. Thus, fusion gets progressively better the longer the transfection proceeds. The main difference between the two systems is that paramyxovirus protein-mediated fusion requires the expression of two viral genes, the F (fusion protein) and the G or HN receptor-binding proteins that must directly interact for fusion to proceed (304). The presence of a receptor-binding protein ensures that paramyxovirus protein-mediated syncytium formation can occur with less dependency on cell adhesion molecules than for the FAST proteins. However, increased cell-cell contact and adhesion would increase the chances for G or HN to find its receptor (ehprin-B2 and sialyllactose, respectively) on the target cell to initiate fusion (46, 606). Also, alterations to membrane curvature and cortical actin could affect the energy required for F-protein-mediated fusion in the same manner as it would affect the FAST proteins.

The results reported for paramyxovirus F protein-mediated fusion are strikingly similar to those presented here. Constitutively active GTPase mutants that promote cell-cell contact and adhesion (Cdc42 and Rac) enhanced fusion, while Rho inhibits fusion (476). Typically, those authors report fusion enhancement of less than 2-fold (similar to the RacCA data presented here). The maximum amount of fusion enhancement reported was only 3-fold with Cdc42CA, compared to the ~9-fold enhancement observed with ARV-p10 (476). Those authors also report diverse reactions to the GTPases which were dependent on the fusion protein and cell type (476). These data have several implications for the results presented here with the FAST proteins.

First, the ability of different GTPase mutants to affect paramyxovirus F-protein-mediated fusion in a similar fashion as FAST protein-mediated fusion supports the conclusion that the FAST proteins do not directly interact with cellular machinery affecting actin remodelling. Instead, the most probable explanation is that the GTPase

mutations alter the susceptibility of a cell to protein-mediated fusion by altering cell-cell adhesion, membrane curvature and the supporting cytoskeleton network. That these cellular changes are exploited by fusion proteins with very different structural organization (i.e Class I versus FAST) indicates that both types of fusion proteins, while mechanistically distinct, are similarly affected by certain membrane alterations.

Also, the data presented here help to reinforce the importance of considering the membrane and its associated components as part of the fusion reaction. Actin can change the energy required to initiate fusion in a number of ways. The cortical actin network found lining the plasma membrane can restrict lipid mobility and apply tension to the membrane, inhibiting fusion pore formation and expansion (81, 293, 358, 410, 486). Activation of Cdc42 and Rac can prime the cell for fusion by promoting actin-filled plasma membrane extensions that enhance cell-cell contact and introduce membrane curvature and instability, which can be favourable to fusion (614). Various fusion proteins are subject to these changes and must employ strategies to exploit or evade these alterations in order to initiate fusion. The FAST proteins may be particularly susceptible to actin remodelling and actin-induced membrane alterations because of their dependence on cell-cell adhesion to provide optimal fusion environments. This dependence is understandable, however, since in nature the reovirus-infected cells that express the FAST proteins provide environments with abundant cell-cell contacts and adhesions.

6.4 Figures

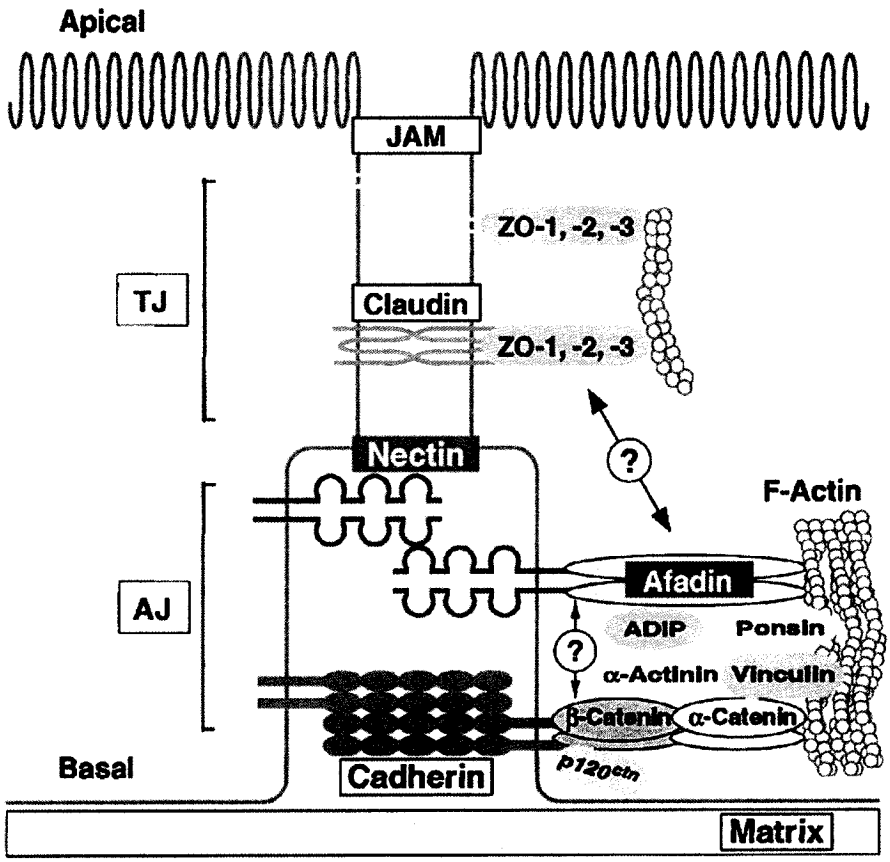

Figure 6.1 Cadherins and nectins recruit actin through their cytoplasmic domains. Adherens junctions (AJ) are formed by cadherin- and nectin-based interactions. The homotypic *trans*-pairing of cadherins and nectins recruits adaptor proteins to their cytoplasmic domains that can bind to one another, interact with cellular signalling molecules (e.g. small GTPases) and recruit actin. Actin polymerization at the site of adhesion helps to stabilize the contact site and promote active adhesion and junction formation. Once established, AJs can promote the assembly of other junctions, depending on the cell type, which include tight junctions (TJ) and gap junctions (not shown). This figure is modified from Shimizu and Takai (2003) and reprinted with permission from Oxford Journals.

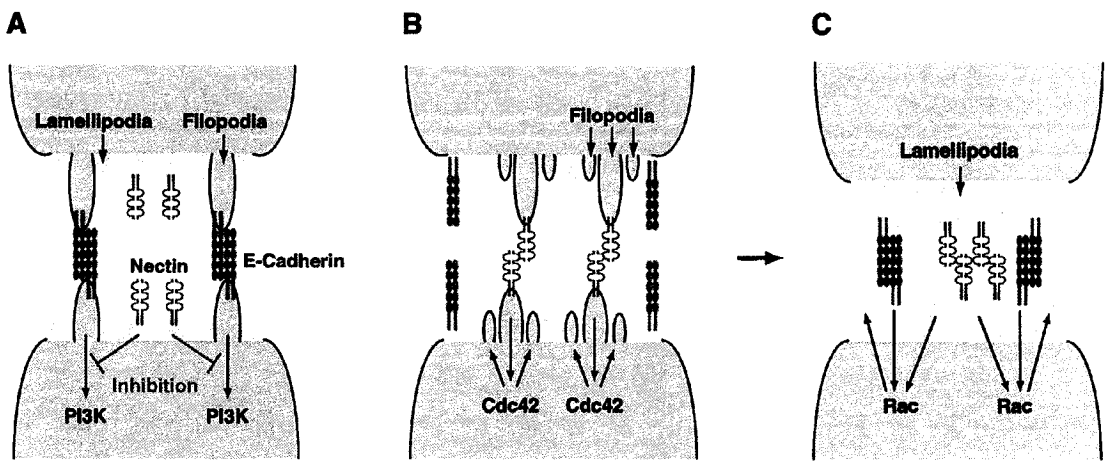

Figure 6.2 Rho family GTPases coordinate actin remodelling in response to cell-cell adhesion. **(A)** Migrating cells make initial contact through cadherins and nectins at the tips of filopodia and lamellipodia. The *trans*-pairing of cadherins can activate Rac in a PI3K-dependent manner, but this process can be inhibited by unpaired nectins in the vicinity. **(B)** Nectin engagement activates Cdc42 and filopodia extension in a PI3K-independent manner, which promotes further cell-cell adhesion through nectin-nectin and cadherin-cadherin *trans* interactions. **(C)** Paired nectins and cadherins activate Rac in both PI3K-dependent and PI3K-independent pathways, resulting in lamellipodia formation, which causes the cells to "zipper up" and form active adhesion structures such as adherens junctions, gap junctions and possibly tight junctions. This figure is modified from Shimizu and Takai (2003) and reprinted with permission from Oxford Journals.

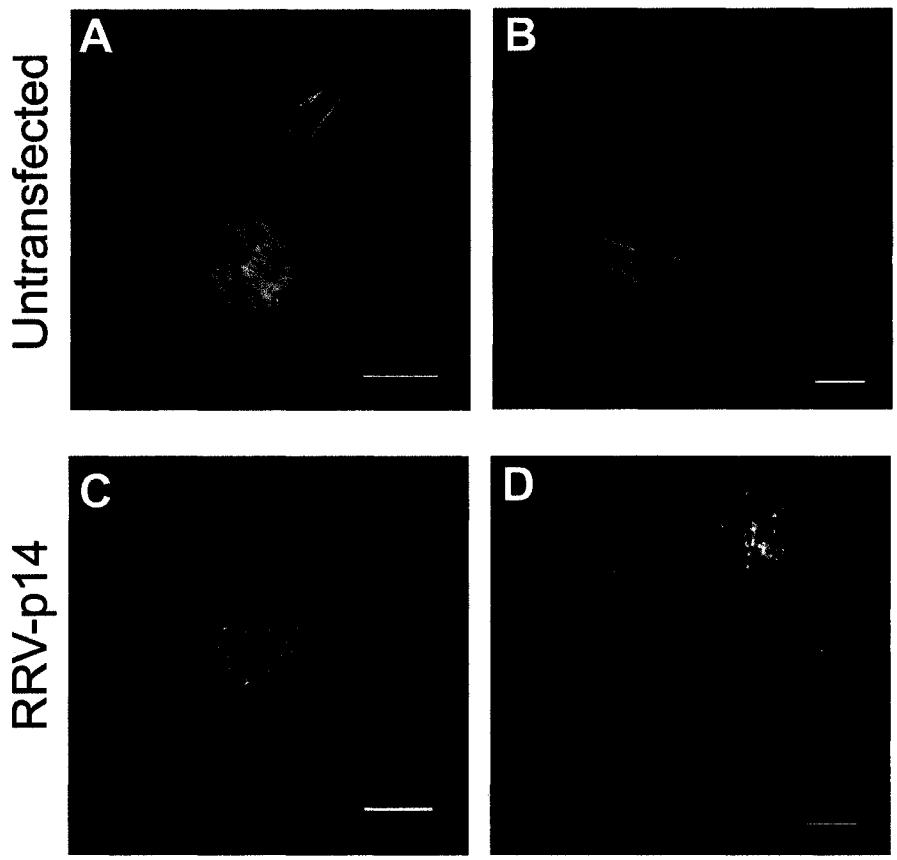

Figure 6.3 Syncytia formation, not the presence of RRV-p14, alters actin organization. **(A and B)** Representative images of untransfected QM5 fibroblasts stained with AlexaFluor488-conjugated phalloidin to visualize actin (green) and propidium iodide to visualize DNA (red) in individual cells **(A)** and in contacting cells **(B)**. **(C and D)** RRV-p14 expression (red) and actin organization (green) were assessed in p14-transfected QM5 cells 8 hpt for individual cells **(C)** and syncytia **(D)**. Scale bar = 10 μm.

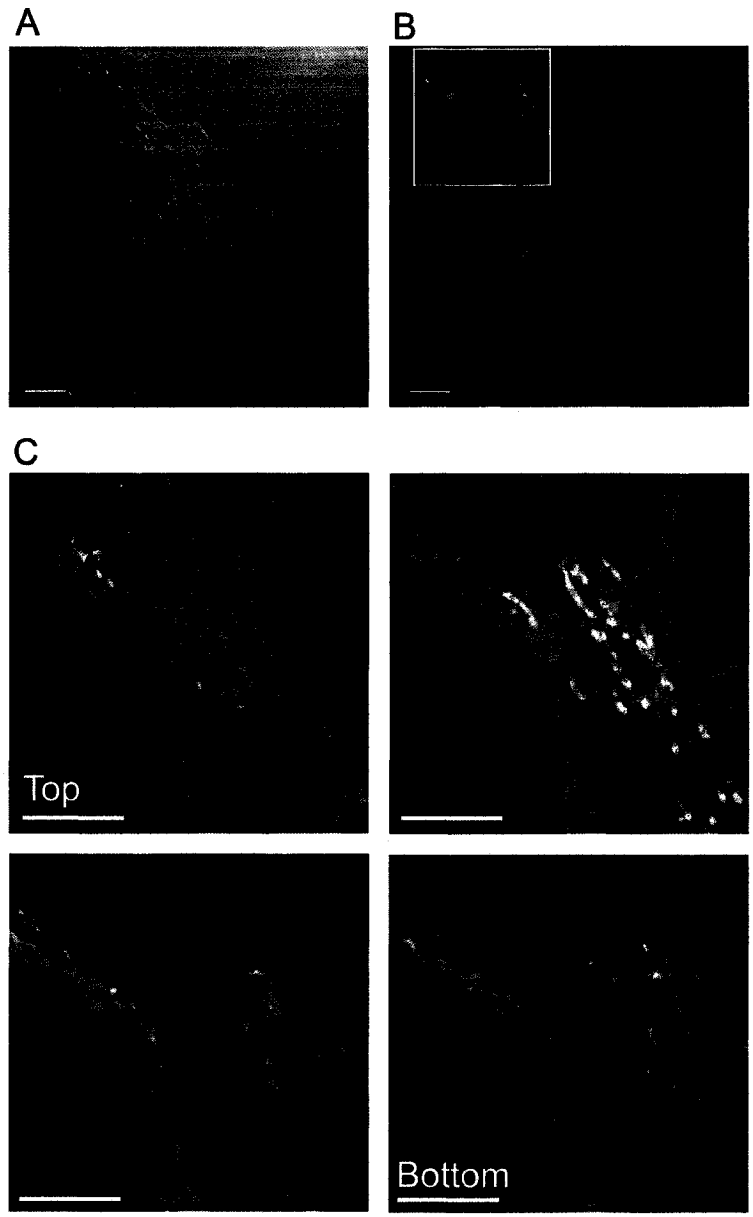

Figure 6.4 Actin is present at fusion pores. **(A)** DIC image of a QM5 syncytium induced by RRV-p14 8 h post-transfection. **(B)** Fluorescence image of **(A)** co-stained to detect actin (green) and RRV-p14 (red). **(C)** Sequential Z-axis cross-section images (left to right, top to bottom) of the portion of the syncytium highlighted by the white box in **(B)** showing unusual actin organization at a potential fusion site. Scale bar = 10 μm.

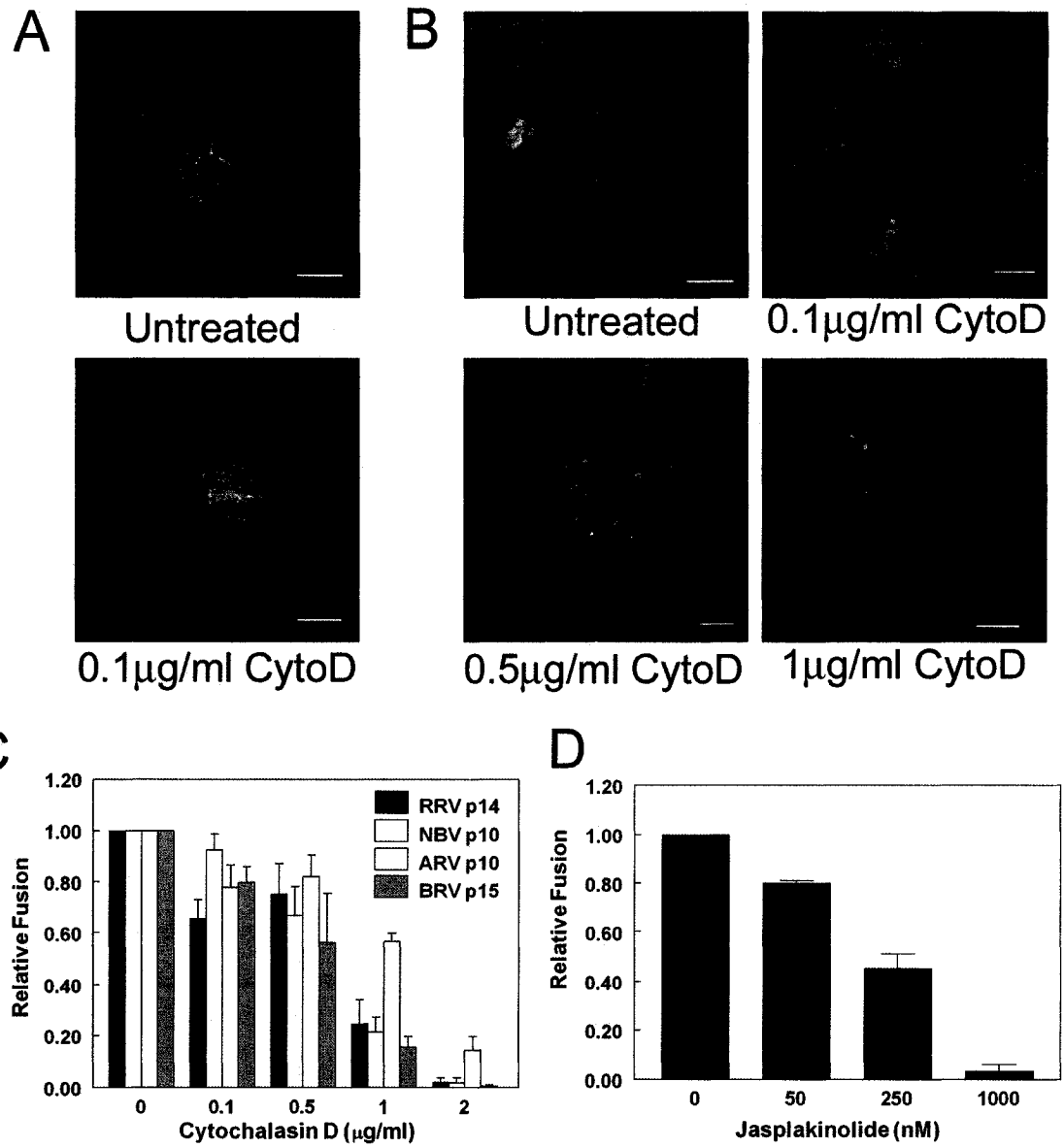

Figure 6.5 FAST protein-mediated fusion is sensitive to actin disruption. **(A and B)** QM5 cells were treated with the indicated concentrations of cytochalasin-D for 30 minutes prior to fixation and staining with propidium iodide (red) to visualize nuclei and Alexa488-conjugated phalloidin (green) to visualize actin in isolated cells (**A**) or in contacting cells (**B**). Scale bar = 10 µm. **(C)** QM5 cells were transfected with the different FAST proteins and treated with the indicated concentrations of cytochalasin D prior to and during fusion. Once syncytia in control wells had progressed, cells were fixed with methanol, Giemsa-stained and fusion was quantified. Data are presented as the percent fusion relative to untreated cells and is the mean ± the SE (n=3). **(D)** QM5 cells were transfected with RRV-p14, treated with the indicated concentrations of jasplakinolide prior to and during fusion. Cells were fixed at 8 hpt and fusion was quantified. Data are presented as the mean ± the SE (n=3).

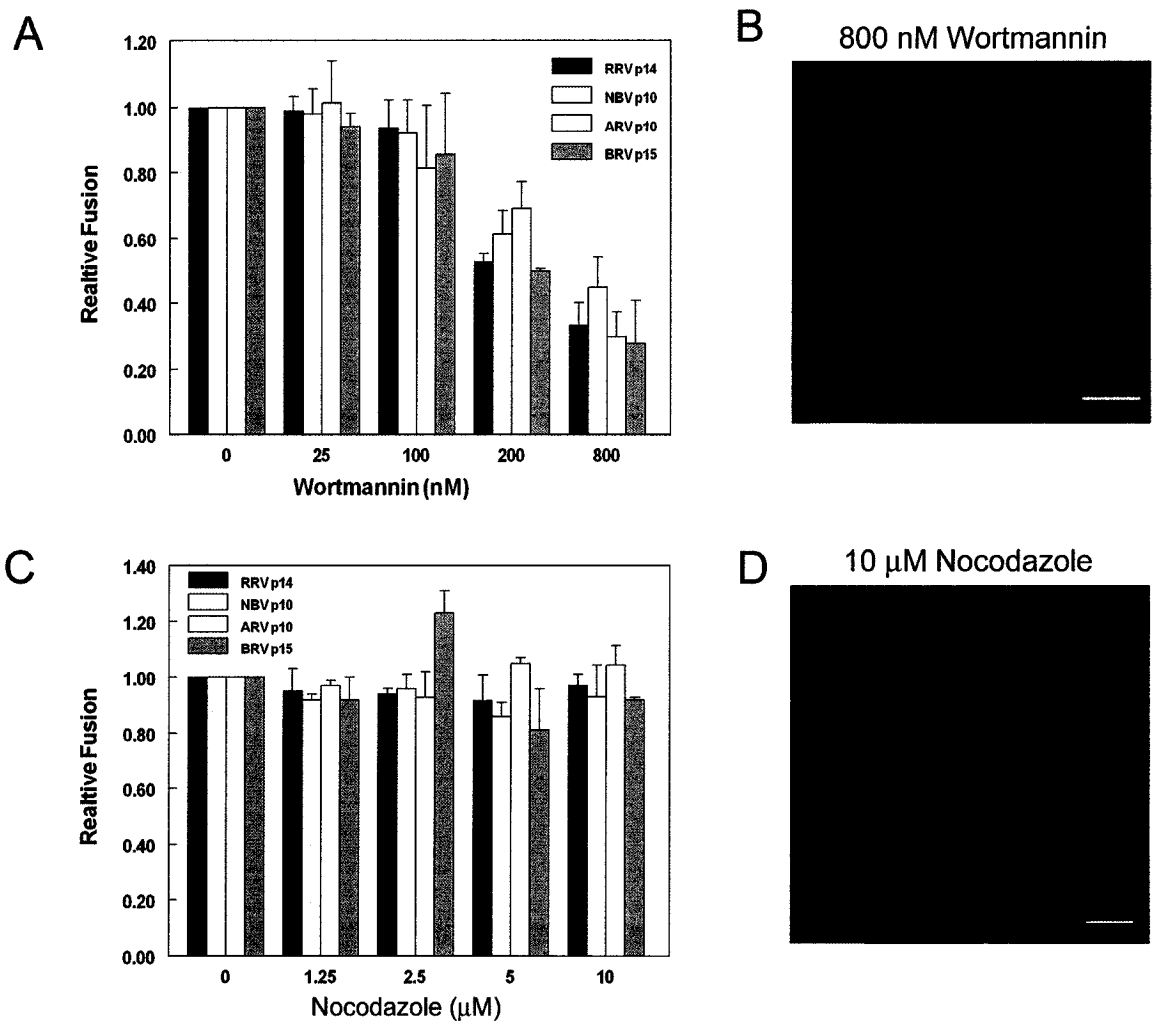

Figure 6.6 Wortmannin, but not nocadazole, inhibits FAST-mediated fusion. FAST protein-transfected QM5 cells were treated with the indicated concentrations of wortmannin **(A and B)** or nocodazole **(C and D)** prior to and during fusion. Once syncytia had progressed, cells were fixed with methanol, Giemsa-stained and fusion was quantified. Data are presented as the percent fusion relative to untreated cells and is the mean ± the SE (n=3). Wortmannin-treated **(B)** or nocodazole-treated **(D)** QM5 cells were fixed and stained with propidium iodide (red) to visualize nuclei and Alexa488-conjugated phalloidin (green) to visualize actin. Scale bar = 10 μm.

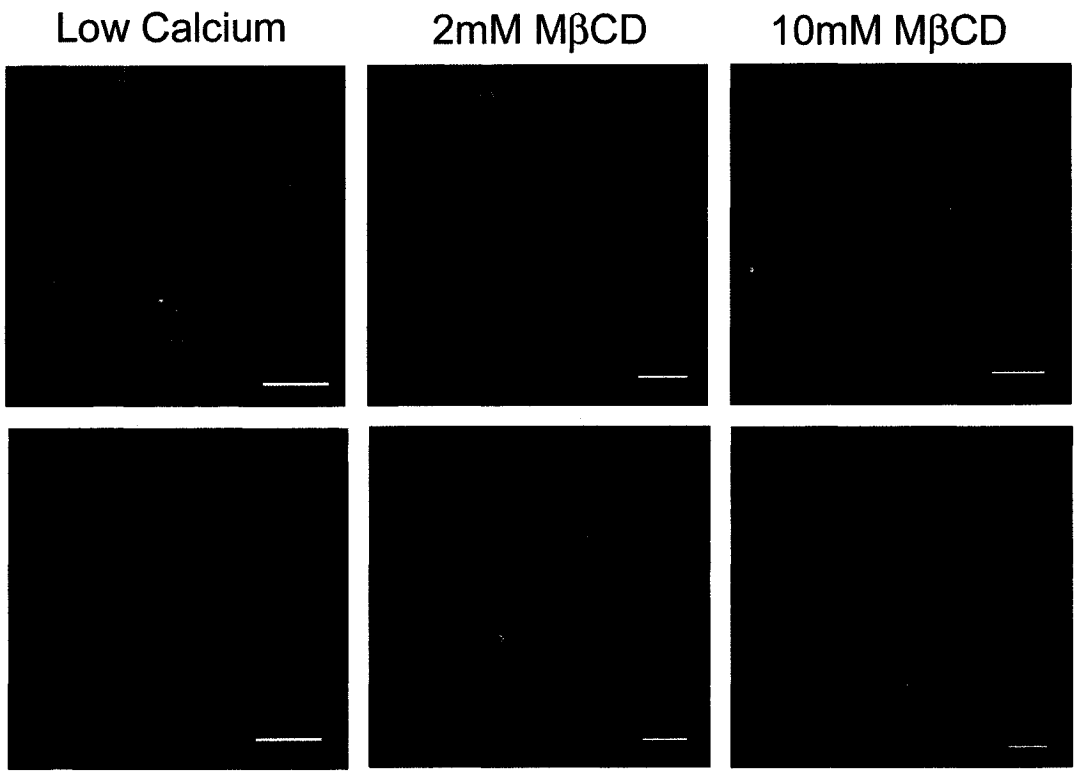

Figure 6.7 Calcium depletion, but not cholesterol depletion, alters actin organization. Representative images of QM5 cells treated without extracellular calcium or with the indicated concentrations of methyl-β-cyclodextrin (MβCD). Cells were fixed and stained with propidium iodide (red) to visualize nuclei and Alexa488-conjugated phalloidin (green) to visualize actin. Scale bar = 10 μm.

Table 6.1 The Rho-family GTPases and mutants used in this study.

Rho GTPases				
Clone Name	GTPase	Mutation	Function	Structure Affected
Rho	Rho-A	none	Wild Type	Stress Fibres
RhoCA	Rho-A	V14	Constitutively Active	Stress Fibres
Rac	Rac-1	none	Wild Type	Lamellipodia
RacCA	Rac-1	L61	Constitutively Active	Lamellipodia
Cdc42	Cdc42	none	Wild Type	Filopodia
Cdc42CA	Cdc42	L61	Constitutively Active	Filopodia

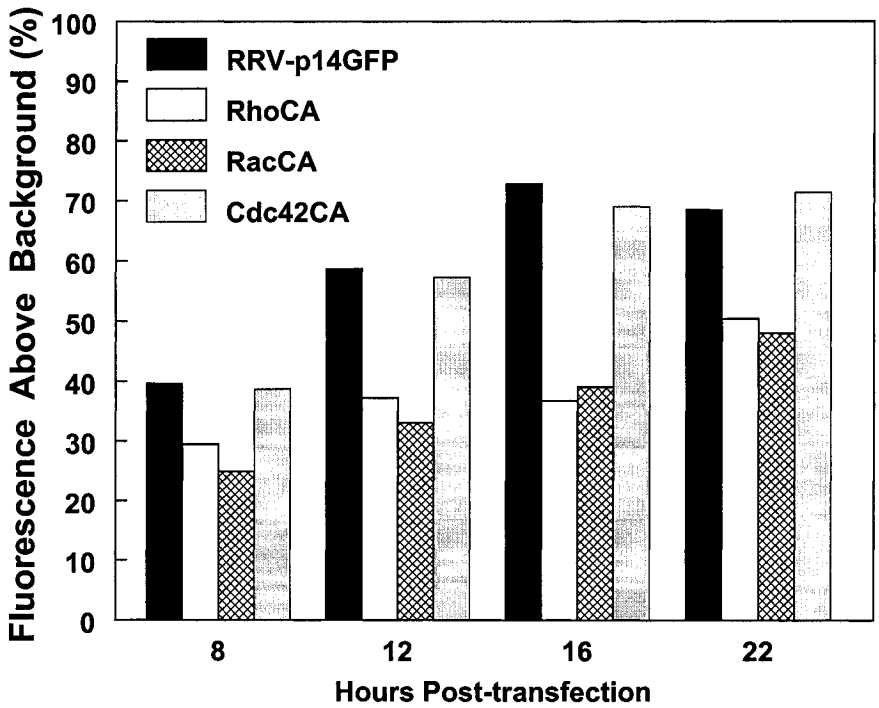

Figure 6.8 GTPases and RRV-p14 are expressed in QM5 cells. QM5 cells were transfected with GFP-tagged RRV-p14 (RRV-p14GFP) or GFP-tagged RhoCA, RacCA and Cdc42CA. At the indicated times post-transfection, protein expression was analyzed using flow cytometry to detect GFP fluorescence. The percent of fluorescent cells above background compared to untransfected cells from a representative experiment is shown.

Figure 6.9 Constitutively active Rho family GTPases alter actin in QM5 cells. QM5 cells were transfected with GFP-tagged RacCA, Cdc42CA or RhoCA (green), fixed at 12 hpt and stained with Alexa555-conjugated phalloidin (red) to visualize actin. Scale bar = 10 μm. The morphology of some cells (RhoCA) was also analyzed by scanning electron microscopy (SEM).

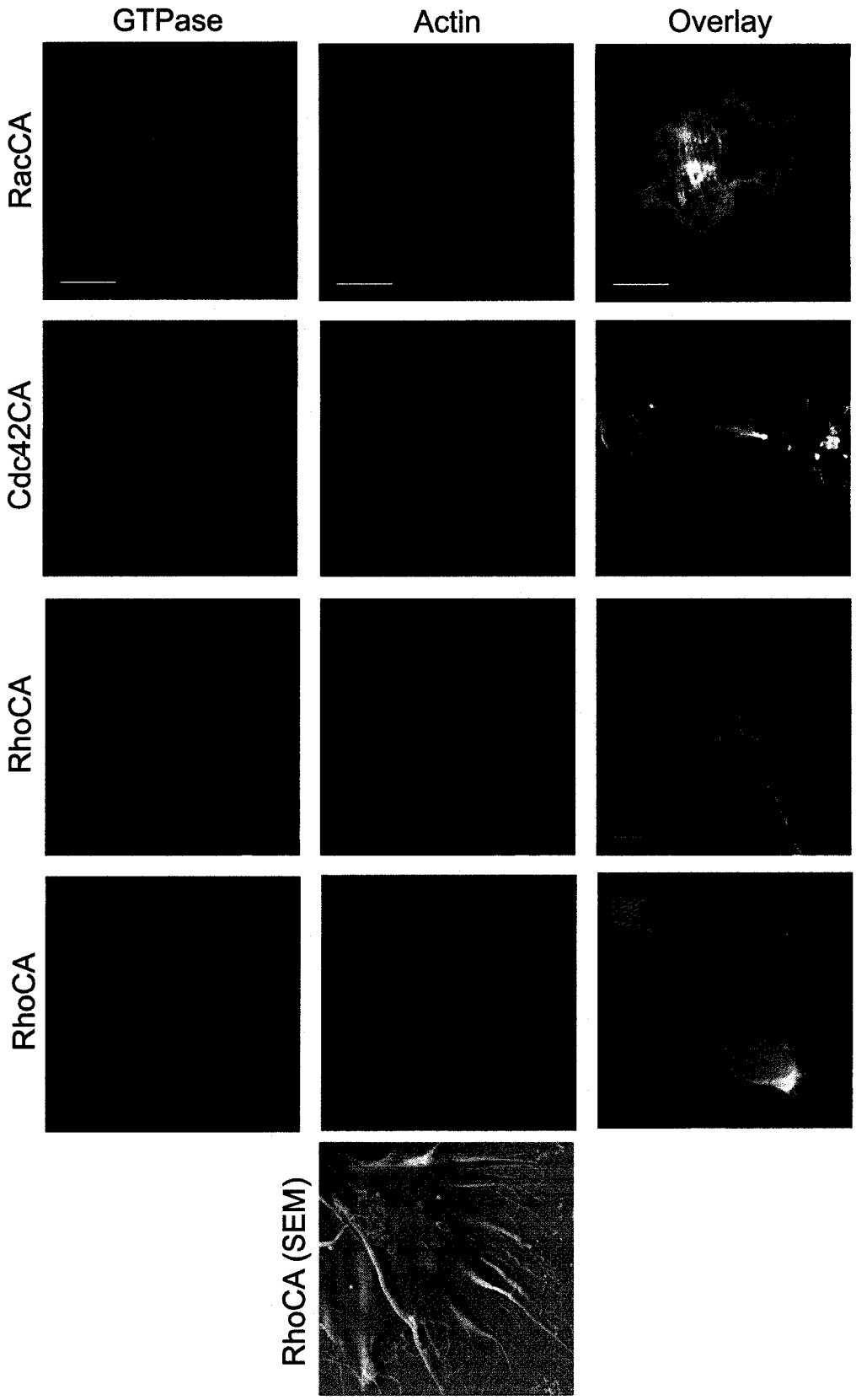

Figure 6.9 Constitutively active Rho GTPases alter actin in QM5 cells.

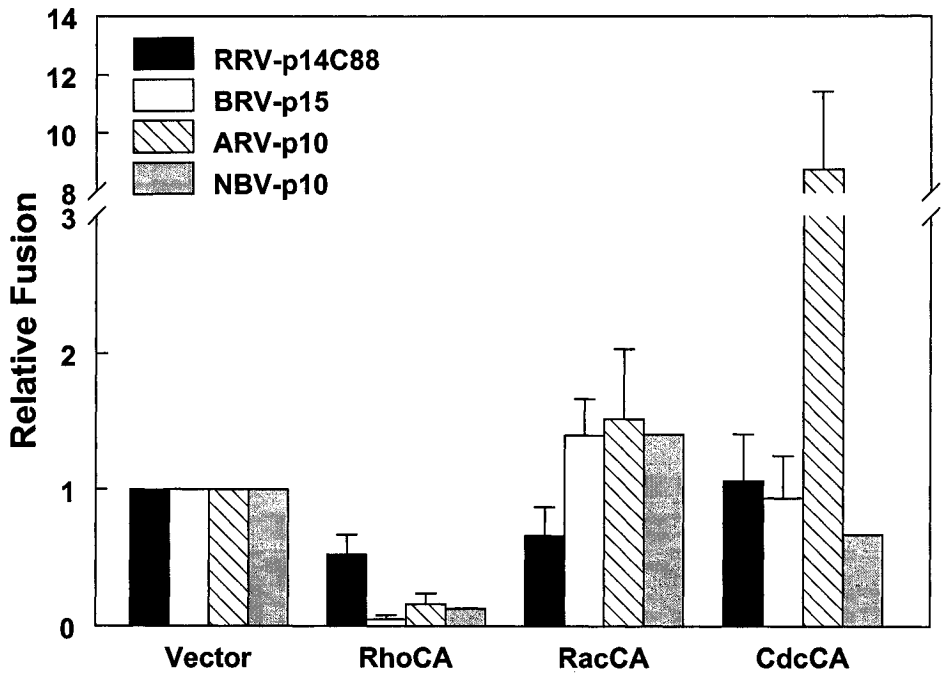

Figure 6.10 Constitutively active Rho family GTPases have varying effects on FAST protein-mediated fusion. QM5 cells were co-transfected with RRV-p14C88, BRV-p15, NBV-p10 or ARV-p10 and either RhoCA, RacCA, Cdc42CA or vector plasmid without a GTPase. The average amount of fusion in the presence of a GTPase, relative to empty vector, was determined by quantification of Giemsa-stained cells. Data are presented as the mean ± SE (n=5) except for NBV-p10 (n=1).

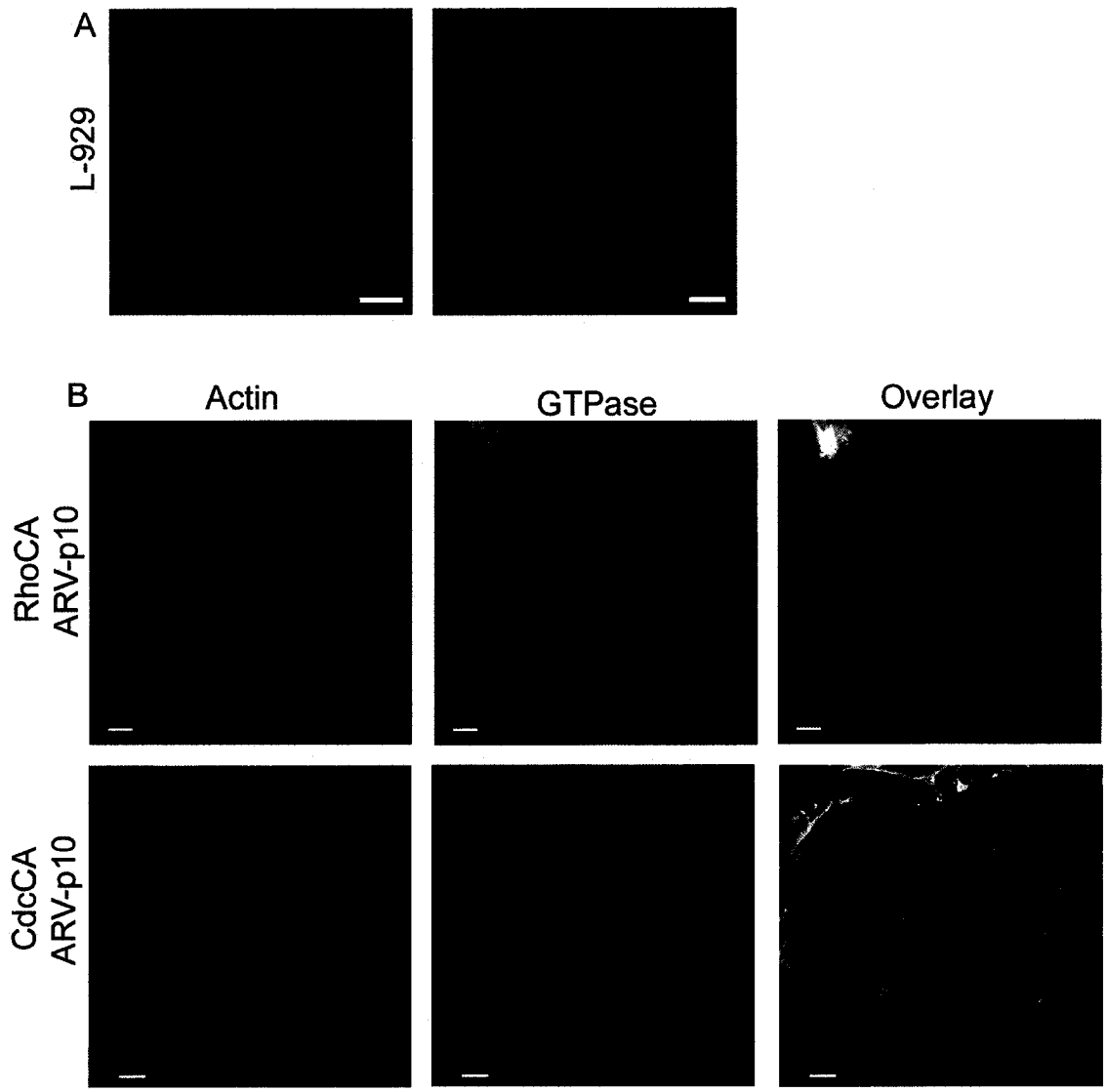

Figure 6.11 Specific actin alterations appear to influence ARV-p10-mediated fusion more dramatically than the other FAST proteins. **(A)** Actin organization detected by Alexa488-conjugated phalloidin in L-929 cells which are highly susceptible to ARV-p10-mediated fusion. Note the numerous actin-filled plasma membrane protrusions. **(B)** RhoCA or Cdc42CA were co-expressed with ARV-p10 in QM5 cells and actin was detected with Alexa555-conjugated phalloidin (red). The distribution of GFP-tagged Cdc42CA or RhoCA (green) was also examined. Scale bar = 10 μm.

CHAPTER 7

Discussion

7.1. Summary of salient findings

The FAST proteins are an unusual group of membrane fusion proteins that play an atypical role in the reovirus replication cycle. Cell-cell fusion is a common cytopathic effect for enveloped viruses that express pH-independent fusion proteins on the surface of infected cells (63, 74, 255, 269, 449). These proteins are required for virus entry and must be incorporated into the virion structure prior to virus release. In contrast, the FAST proteins are non-structural proteins encoded by non-enveloped viruses that do not require membrane fusion for entry. Instead, the FAST proteins are efficient mediators of non-leaky cell-cell fusion. They are not membrane-disruptive viroporin-like proteins as has been suggested (43, 494). Extensive FAST protein-mediated cell-cell fusion results in the formation of large syncytia that eventually die by apoptosis. These observations lead to a dual role for fusion in the reovirus replication cycle where fusion allows rapid cell-cell transmission early in infection and eventual syncytial cell death, which facilitates systemic dissemination of the infection (Figure 3.10).

Their small sizes and lack of similarities to other known fusion proteins suggest that the FAST proteins may be minimalist fusion machines that function in a fundamentally different manner than the paradigmatic enveloped-virus fusion proteins or SNARE proteins. While cellular proteins are not required for fusion activity, the FAST proteins do rely on cellular factors to dramatically enhance fusion efficiency. Most significantly, the FAST proteins require the establishment of cadherin-dependent cellular adhesion structures to support fusion by providing membrane proximity (Chapter 4). Fusion is enhanced by the formation of active adhesion, which allows the establishment of cellular junctions that could bring membranes into closer proximity than do cadherin interactions alone. This requirement for closeness can be partially satisfied by non-cellular adhesion machinery (e.g. HA), suggesting a generic requirement for membrane adhesion rather than a specific dependence on a particular adhesion molecule or type of cell junction. Thus, the FAST proteins are the first fusion proteins that do not rely on specific receptor-binding activity to trigger the lipid mixing steps of the fusion reaction.

The membrane itself is also important for FAST protein-mediated fusion. Specifically, cholesterol is required in the donor, but not the target, membrane for efficient fusion to occur (Chapter 3). Some evidence suggests that the FAST proteins localize to cholesterol-rich membrane microdomains, and that this association is necessary for fusion activity (110). Microscopic analysis indicates that the FAST proteins do not co-localize with classical raft markers such as GM1 and PLAP, or with each other, suggesting that there may be lipid raft heterogeneity within cells. However, RRV-p14 does co-localize with raft-localized cadherins and HA at regions of cell-cell contact, supporting the role of adhesion molecules in FAST protein-mediated fusion. It should be noted that due to the limits of imaging resolution, co-localization by microscopy is not necessarily indicative of raft co-localization.

Finally, the intracellular environment also influences the fusion activity of the FAST proteins. Disruption of the actin cytoskeleton severely inhibits the ability of the FAST proteins to function (Chapter 6). This effect might be due specifically to disruption of Rho GTPase-regulated actin remodelling in response to cell-cell adhesion. However, the FAST proteins respond quite differently to activation or inhibition of these pathways. In general, constitutively active effector-domain mutants of Cdc42 and Rac1, which promote cell-cell contact and adhesion, enhanced FAST protein-mediated fusion. However, the degree of fusion enhancement was dependent on the FAST protein and the activated GTPase, indicating that the FAST proteins respond differently to the effects of these adhesion-promoting molecules. Conversely, activated Rho pathways, which antagonize Cdc42 and Rac, inhibited FAST protein-mediated fusion.

In summary, the FAST proteins are dedicated cell-cell fusion proteins that have evolved a dependence on host cell factors, both within and on either side of the plasma membrane, to support efficient fusion. While the requirement for cell-cell adhesion is shared, it is apparent that individual FAST proteins may have evolved distinct methods for responding to this adhesion machinery. However, despite their differences, the FAST proteins appear to employ a similar and novel mechanism for protein-mediated membrane fusion (discussed below in Section 7.4.)

7.2. Setting aside the "enveloped virus fusion protein bias"

By now it should be evident that the current models for membrane fusion based on the principle that large conformational rearrangements supply energy as a driving force for fusion are not adequate to describe FAST protein-mediated fusion. These models have been shaped over the last few decades largely by analysis of enveloped-virus fusion proteins such as the influenza HA protein and HIV gp41, creating a bias that permeates the fusion field (86, 104, 280, 526, 596). This bias has been reinforced by the abundance of HA-like Class I fusion proteins in nature and the shared feature of coiled-coil domains between Class I fusion proteins and SNARES. Potential challengers to the HA paradigm are Class II fusion proteins, which are structurally distinct from Class I fusion proteins (8, 196, 224). However their role as viral fusion proteins has led to conservation of some mechanistic elements. A recent review of Class II fusion proteins by Kielian and Rey (280) describes the mechanistic similarities between Class I and Class II fusion proteins (Figure 1.4). It also briefly addresses the often-ignored challenges to the HA paradigm raised by unclassified fusion proteins such as VSV-G, herpesvirus glycoproteins and the FAST proteins stating that "...it will be important to understand how [the FAST proteins] fit into our growing knowledge of the mechanism of protein-mediated membrane fusion" (280).

This is an important statement for two reasons. First, it indicates a growing trend in the fusion community that is beginning to recognize the deficiencies of the HA fusion model to adequately explain several fusion reactions (86, 138, 258, 301, 360, 442, 603). Second, the statement appropriately establishes a context for the remainder of this discussion. The research presented here provides a timely response to Kielian and Rey and offers a plausible explanation for where the FAST proteins fit into our understanding of protein-mediated fusion. In order to fully appreciate FAST protein structure and function, it is necessary to set aside the HA bias and adopt the "FAST perspective" that each biological fusion reaction is unique and is executed by proteins well adapted to the nuances of each event.

7.3. The FAST proteins are a unique class of fusion protein

The FAST proteins form a novel class of fusion proteins because of their unusual structural organization as well as their unique role as mediators of cell-cell fusion that supports viral replication. Before delving into a description of FAST protein-mediated fusion, it is necessary to clarify and reinforce a few recurrent themes that appeared throughout this document. Careful explanation of key terms is essential, as subtle variations in usage hold larger implications for our understanding of fusion.

7.3.1. The FAST proteins are "cellular" fusion proteins

The first recurrent theme is that the FAST proteins are "cellular" fusion proteins. That is, their primary, if not only, purpose is to cause cell-cell fusion. However, the promiscuous fusion activity of the FAST proteins should not be confused with the highly regulated and coordinated process of physiological syncytium formation required for tissue development (86, 234, 244, 537, 576). Rather, the FAST protein-mediated cell-cell fusion reaction is unique in nature and limited to the fusogenic reoviruses. Understanding how the FAST proteins work as mediators of unregulated and untriggered cell-cell fusion provides an opportunity to broaden our understanding of protein-mediated membrane fusion.

Another point to keep in mind throughout the ensuing discussion is that the FAST proteins have evolved in the context of virus infections, and have developed a dependence on host cell factors for increasing the efficiency of fusion. The ability of RRV-p14 to cause liposome-liposome fusion indicates that these host factors are not required for fusion (547), but it has been demonstrated here that these factors greatly affect the efficiency of fusion and must be included when developing models for FAST protein-mediated fusion.

7.3.2. FAST proteins are not triggered or regulated by specific interactions with binding proteins

Another theme that emerges is the concept of regulating and triggering fusion. It was noted in the Introduction (Chapter 1, Section 1.9) that for viral fusion proteins, binding and fusion are closely coupled. The same holds true for cellular SNARE proteins

(27, 192, 242, 585). The FAST proteins, however, are different. Given the sequence diversity of the FAST proteins, the variety of cell types susceptible to FAST protein-mediated fusion and the diversity of adhesion molecules that can enhance FAST protein-mediated fusion, the FAST proteins are unlikely to be dependent on interactions with a cognate receptor-binding protein to trigger exposure of potential fusion-inducing domains. Therefore, the FAST proteins are the first fusion proteins described to have completely uncoupled binding from fusion, which results in the removal of one of the major regulatory elements governing the activity of other fusion proteins. This is in direct violation of the HA bias that suggests that fusion proteins must be spatially and temporally controlled through interactions with receptor-binding components.

This is not to say that the FAST proteins are completely untriggered or unregulated, but it means that they are triggered and regulated in a fundamentally different manner. For other viral fusion proteins, the fusion trigger exposes a fusion peptide that is sequestered in a multimeric pre-fusion structure. In the case of ARV-p10, it appears as though the hydrophobic patch is solvent-exposed during synthesis and trafficking to the cell surface (493). These results suggest that the FAST proteins are synthesized in a fusion-active state. However, close membrane apposition mediated by cellular adhesion molecules and cell-cell junctions could trigger dynamic interactions of hydrophobic elements, such as the hydrophobic patches and/or myristic acid moieties, with both donor and target membranes. The implications of such structural plasticity on models of FAST protein-mediated membrane fusion are discussed below.

For the FAST proteins, regulation does not occur at the level of receptor binding but at the level of protein expression. Factors such as the number of viral gene copies available, poor translation from the sub-optimal start codons and protein degradation can affect the rate of FAST protein arrival at the cell surface, where the proteins cause fusion without the regulation of specific receptor-binding proteins (493, 497). In essence, it is the rate of fusion that is regulated, not the activity of an individual FAST protein. This is not the case with other fusion proteins where binding regulates the activation of a specific fusion protein.

Most membrane fusion proteins need to control where and when the fusion reaction occurs, while the FAST protein fusion reaction is best described as causing

fusion "wherever and whenever" possible. In this sense, the FAST proteins are untriggered and unregulated, and this is the meaning assigned to these terms when used to describe the FAST proteins to ensure the difference between these unusual proteins and the other fusion proteins is emphasized.

7.3.3. The FAST proteins redefine a fusion machine

A third theme that arises from these studies is the need to define exactly what constitutes a fusion protein and a fusion machine. The definitions provided in Chapter 1, Section 1.8, are sufficient to describe every known fusion system except the FAST proteins. For example, influenza HA is both a fusion protein and a fusion machine. In contrast, by the definition in Chapter 1, individual SNARE proteins are neither fusion proteins nor fusion machines. However, t-SNAREs and v-SNAREs can be combined to form the core fusion machinery of the vesicle-trafficking fusion machine (242, 526, 585). Somewhere in between these two extremes are the paramyxovirus F proteins that serve as the fusion proteins in a bipartite fusion machine with the cognate receptor-binding component (H/HN/G) (209, 304). Thus, there is considerable diversity in how a fusion machine distributes the protein elements required for completing membrane merger.

While the FAST proteins can certainly be considered fusion proteins because they are "the individual component of a fusion machine that is necessary and sufficient to cause membrane merger once the fusion trigger requirement has been satisfied," the definition for a fusion machine provided in Chapter 1 requires revision to include the FAST proteins. They are not the "minimal components of a protein complex that is necessary and sufficient to mediate all of the five fusion steps" because they lack receptor-binding ability. Further, they do not directly interact with a cognate receptor-binding protein to execute fusion, but rely on membrane proximity provided by generic surrogate adhesion molecules. Thus, the FAST protein "fusion machine" or "fusion complex" is better understood in terms of the "fusion reaction" where individual fusion steps are executed without direct communication between the fusion and binding components. In this way, the FAST proteins are orphan fusion modules that exploit and convert adhesion sites into fusion sites. The "fusion machine" then is the "minimum molecular machinery necessary and sufficient to execute the five fusion steps." In the

case of the FAST protein fusion reaction, the adhesion and membrane-merger components of the fusion machine do not directly interact to execute fusion. This new definition might be important for other cell-cell fusion systems where fusion and binding might also be uncoupled (86).

7.3.4. Summary

The FAST proteins cannot be properly understood in the context of an HA bias. The unique role of the FAST proteins in the viral replication cycle has created a novel type of fusion reaction and a novel type of protein to mediate it. Armed with a clearer understanding of how the FAST proteins fit into and are set apart from the HA paradigm, we can begin to explore the mechanism of FAST protein-mediated fusion

7.4. A model for FAST protein-mediated fusion

7.4.1. Overview

The most striking feature of the FAST proteins as membrane fusion proteins is their small size. They are structurally distinct from other fusion proteins and are unlikely to support the energy-releasing conformational rearrangements characteristic of the traditional Class I, Class II or SNARE fusion machinery (69, 196, 585). An accumulating body of evidence supports the model that the FAST proteins mediate fusion through a signature arrangement of membrane-altering motifs that reduce the energy barrier preventing membrane fusion (111, 112, 127, 493, 495, 496, 547). Thus, instead of forcing membranes to fuse with large inputs of mechanical energy (e.g. HA), the FAST proteins may increase disorder both within, and on either side of, the membrane, coaxing fusion between closely apposed membranes. The evidence provided here supports these models and expands our understanding of how host cell factors contribute to FAST protein-mediated fusion. The following discussion will focus primarily on how the FAST proteins can satisfy the initial membrane-binding and close-proximity steps of the fusion reaction.

7.4.2. Step 1: Binding

The paradigm

With the exception of the FAST proteins, all of the known fusion systems are regulated by binding and contain a receptor-binding domain or cognate receptor-binding protein. In general, binding anchors opposing membranes about 10-15 nm apart and confers specificity to the fusion reaction (280, 304, 596). In the case of enveloped viruses that fuse at neutral pH (e.g. retroviruses, paramyxoviruses, herpesviruses), binding also serves as a trigger for fusion (185, 304, 516).

Opportunistic fusion

In practical terms, relying on cellular adhesion machinery to provide favourable fusion environments makes the FAST proteins opportunistic fusogens, devoid of the regulatory constraints imposed on other fusion systems by their receptor-binding components. Microscopic analysis reveals that the FAST proteins are found abundantly on the cell surface and do not appear to localize or target to a particular place (111, 127, 494). However, the cholesterol dependence of fusion (Chapter 5) suggests the FAST proteins may localize to membrane microdomains, many of which contain cell adhesion molecules, including cadherins and connexins (75, 478, 483). The ability of the FAST proteins to co-localize with HA and cadherins indicates that these sites of membrane contact may also serve as sites for FAST protein-mediated fusion. Thus, the membrane-binding step of the fusion reaction can be provided by any number of adhesion processes that can establish environments with very different properties such as intermembrane distance, membrane curvature and membrane composition. These structures would include cadherin-mediated adherens junctions (common to all adherent-cell types) and the downstream junctions, such as gap junctions, desmosomes or tight junctions, which are dependent on cadherin engagement (241, 254, 283, 552). Thus, the plasma membranes of adherent cells within an infected animal would contain a myriad of potential fusion sites with membranes from opposing cells ranging from about 3-20 nm apart (111, 127, 494, 513), consistent with the distances mediated by the binding proteins of other viruses. The FAST proteins appear to be able to cause fusion at these sites with variable effectiveness; however, the most efficient fusion occurs under the most

physiological conditions, those of active cell-cell adhesion, demonstrating that the FAST proteins have evolved to be excellent cell-cell fusion proteins. Cellular adhesion molecules and active adhesion would maximize the opportunities for FAST protein-mediated fusion by satisfying the binding step wherever adhesion molecules are engaged. In the three dimensions of infected animal tissues, where cells can be bordered on all sides by other cells, the entire plasma membrane could be a potential site for initiation of FAST protein-mediated fusion.

7.4.3. Step 2: Close membrane apposition

The paradigm of structural remodelling

It is clear that the FAST proteins do not mediate the binding step of the fusion reaction. However, it is unclear if they are involved in bringing membranes to within the limit of hydration repulsion (1-2 nm) to facilitate hemifusion (103, 424, 614). For other fusion proteins, this close membrane apposition is achieved by the cast-and-retrieve model for membrane fusion (69, 258). Activation of the fusion protein results in exposure of a hydrophobic fusion peptide that inserts and anchors in the target membrane (69). Subsequent conformational rearrangements generate a hairpin structure (e.g. six-helix bundle for HA) that causes the fusion protein to jackknife, pulling the opposing membranes into close proximity (69, 280).

Several plausible options exist for how the FAST proteins may mediate the close membrane-apposition step of the fusion reaction. The prevailing theory presented here for the FAST proteins, uncoupling binding from fusion, presents two distinct challenges for successful close membrane apposition. First, the FAST proteins must contend with the intermembrane distances generated by cell adhesion (10-20 nm) and bring membranes within about 2-3 nm of each other where hydration repulsion becomes a significant force. Second, the FAST proteins need to overcome the hydration forces to promote membrane contact and stalk formation (104). This two-step approach to close apposition is unique to the FAST proteins and is a direct consequence of an uncoupled fusion reaction. The FAST proteins are essentially initiating fusion from a step other than membrane binding.

FAST protein close apposition phase 1: from binding to hydration repulsion

Active pulling

First, the FAST proteins could extend and pull membranes together in a modified cast-and-retrieve model. This scenario is unlikely, as the FAST proteins lack the obvious structural elements to facilitate such activity and their ectodomains, when completely extended and unstructured, could span only ~7-15 nm. It is unlikely that this extended conformation is a plausible structure, and evidence suggests that RRV-p14 only extents about 0.7-1.5 nm from the membrane surface (M. Jericho, unpublished data, (112)).

Active pushing

A second option is that the FAST proteins can "push" membranes together, rather than pull them. This could be accomplished several ways, with the FAST proteins either stimulating actin remodelling or exploiting normal cellular process that extend actin-filled membrane protrusions into neighbouring cells (Figure 7.1) (574, 597). These processes could provide excellent fusion locations because of the membrane strain supplied by the curvature of the protrusion and the membrane stress from the actin pushing it (597, 614). In addition, Rho-family GTPases generally regulate these actin alterations, to which the different FAST proteins display varying sensitivity (Chapter 6). Thus, the different sensitivities of the FAST proteins to actin alteration might indicate that some of these actin-mediated adhesion events are more favorable for one FAST protein than another.

Passive pushing

In a second model for "pushing" membranes together, the FAST proteins might be able to promote close apposition by causing the disassociation of the membrane from the cortical actin network. Phosphotidylinosititol-4,5-bisphosphate (PIP_2) is a central molecule in coordinating actin dynamics at the membrane (325, 369, 413, 469, 486, 487). Binding of the FAST protein polybasic regions to PIP_2 could disrupt normal actin remodelling by competing with actin-adaptor proteins for binding to PIP_2 and displace them, and actin, from the membrane. Dissociation of the cytoskeleton from the plasma membrane could facilitate the formation of membrane blebs and protrusions that could

function to passively interact with neighbouring membranes (486). Microscopic analysis does not reveal major changes in cortical actin or the production of membrane blebs in FAST protein-transfected cells, but the FAST protein-mediated effects might occur transiently or over small areas and be missed by this type of analysis.

Opportunity knocks

Alternatively, the FAST proteins might employ a very non-sophisticated strategy to satisfy the initial phase of close apposition by relying on random membrane fluctuations to provide close contact between cells. Membranes are dynamic structures and even cells that are held together by adhesion molecules will move and reorganize. Cell-cell adhesion is more analogous to stacking water balloons than stacking building blocks. The active and passive membrane movements inherent to adherent cells could occasionally result in membranes "bumping into" each other. If the FAST proteins were present during such an event, fusion could result.

Active adhesion and gap junctions

While the closeness generated by cadherin-mediated cell-cell contacts (passive adhesion) was sufficient to cause an increase in RRV-p14-mediated fusion, proper junction formation involving actin remodelling (active adhesion) was essential for highly efficient fusion. Passive adhesion mediated by *trans*-cadherin interactions leads to actin remodelling and subsequent formation of adherens junctions (51, 99, 427). This allows other cellular junctions to form, including gap junctions, which are generated by both epithelial and fibroblast cells (Figures 7.1 and 7.2) (302, 491, 513). Gap junctions place membranes within 2-3 nm of each other, which is close to the hydration-repulsion distance (~2-3 nm) and the predicted range of RRV-p14 ectodomains (0.7-1.5nm). Thus FAST protein localization to gap junctions could provide and environment very favourable for initiation of FAST protein-mediated fusion.

All of the treatment conditions used in the previous chapters that result in dramatic loss of FAST protein function, including actin disruption, calcium depletion and cholesterol extraction, could disrupt gap-junction formation. Thus, gap-junction formation as a result of active adhesion might represent a key cellular factor responsible

for the efficient cell-cell fusion observed under active adhesion conditions. Characterizing a role for gap junctions in FAST protein-mediated fusion could be accomplished using RNA silencing techniques and dominant-negative mutants directed at the connexins that comprise the gap junctions (170, 176, 324, 339). Disassembling gap junctions without disrupting active adhesion (e.g. adherens junctions) would help address whether the increase in FAST protein-mediated observed under active-adhesion conditions is due to the these downstream adhesive structures. In addition, immunofluorescence microscopy would be useful for identifying the establishment of gap junctions and determining their localization relative to the FAST proteins.

A passive fusion strategy is effective

FAST protein reliance on the cell, or chance, to bring membranes closer than adhesion molecules allow would make them opportunistic fusion proteins, which is consistent with an unregulated fusion mechanism. One might expect that this is an inefficient process, which compared to other fusion proteins might be true, but the effectiveness of the FAST protein fusion strategy is readily noticeable in the time-lapse movie presented in Figure 7.4, where almost all the cells in the field of view have fused within 2 h. There is strength in numbers for the FAST proteins, and their fusion strategy is similar to the replication strategy of the viruses that encode them. Where the virus releases many copies of itself into its surroundings with the hope of finding an environment favourable for infection, the FAST proteins place lots of proteins in a membrane in the hope of finding an environment favourable for fusion.

FAST protein close apposition phase 2: from the hydration layer to membrane contact

The FAST proteins seem particularly well adapted for promoting membrane contact and lipid mixing if membranes can be brought within the nanometre distances where hydration-repulsion forces become strong. At this short range the FAST proteins might be able to employ a small-scale cast-and-retrieve mechanism, promote local dehydration of membranes or induce membrane perturbations.

The FAST protein ectodomains are likely to mediate membrane contact once opposing membranes are brought to within nanometers of each other. Although each

FAST protein ectodomain has a unique sequence and structural arrangement, there are some common elements that could promote membrane contact. Each ectodomain is predicted to form a loop containing a hydrophobic domain and/or modification with myristoylation (112, 127, 493, 495). The ectodomain sequences not involved in loop formation may be unstructured and allow movement of the other ectodomain elements (112).

Membranes in very close proximity might be induced to contact one another by the presence of hydrophobic motifs on the FAST protein ectodomains. In particular the ability of myristic acid to reversibly insert into membranes could disrupt lipid packing in a way that could be favourable for fusion (356, 444). For example, the reversible insertion of myristic acid into the donor membrane could introduce disorder by exposing its own hydrophobic structure or displacing membrane lipids such that their hydrophobic tails are partially exposed. At these short intermembrane distances, exposed hydrophobic surfaces will attract each other and drive membrane contact and stalk formation (104, 614). Another plausible method for promoting membrane merger is based on the prediction that exposure of the myristate moiety or the hydrophobic domains would displace water, possibly facilitating the close apposition of hydrophilic lipid head groups over small areas to promote stalk formation. These processes would add to the disorder of the lipid bilayer and lower the energy barrier preventing fusion (226, 614). Thus the FAST proteins may produce an environment that promotes membrane apposition rather than generating it by force.

Summary

Close membrane apposition is a requirement for membrane fusion, but it is not a required function of a fusion protein. It is still unclear exactly how the FAST proteins bring membranes within nanometer distances, but the process is not likely to involve HA-like energy-releasing structural remodelling on the part of the FAST protein. The small sizes of the FAST proteins and their lack of structural elements capable of undergoing gross conformational rearrangements reveals that such structures, common to the best-described fusion systems, may not be necessary for mediating fusion. Whether the FAST proteins themselves use a novel mechanism for mediating close membrane

apposition or if they delegate the role to cellular machinery, they are the first examples of fusion proteins that do not accomplish this task through the types of triggered conformational rearrangements associated with all other well-described fusion machines.

7.4.4. Step 3- Lipid mixing: From hemifusion to pore expansion

Introduction

The remaining three steps of the fusion reaction, hemifusion, pore formation and pore expansion, will be discussed together. The structural diversity of the FAST proteins suggests that they may have individual methods for executing each of the lipid mixing steps, but this discussion will attempt to present a unifying model for FAST protein-mediated fusion that is in stark contrast to those predicted for other fusion systems. A precise understanding of how the FAST proteins execute each step of the fusion reaction awaits further investigation. However, the identification of cellular factors that influence the activity of the FAST proteins offers support for current models and provides directions to aim future experimentation.

The paradigm

One of the fundamental concerns regarding the fusion mechanism of SNARE proteins and fusion proteins from enveloped viruses is identifying where the energy released from conformational reorganization is applied during the fusion reaction. Clearly some of it must be used to pull membranes into close contact, but how much remains to be applied to the hemifusion, pore formation and pore expansion steps is debated (47, 104, 210, 258, 360, 555). Some models suggest that close apposition is sufficient to at least initiate and possibly complete the fusion reaction, while others report that the pore expansion step is the most energy-demanding step of the fusion reaction (104, 362, 364, 366, 441). The precise role of the fusion peptide is also debated, with some suggesting it only serves to anchor the fusion protein in the target membrane and others describing a more active role where it creates lipid disorder to promote fusion (31, 258, 301, 538). Generally, however, it is accepted that the same rearrangements that promote membrane apposition are responsible for causing fusion (258). In addition, the presence of transmembrane anchors is critical for completion of the fusion reaction, but

their exact role remains unclear (360, 440). It is proposed that the transmembrane domains may restrict lipid movement during fusion or they may apply force to the membrane to promote pore formation and/or expansion (91, 104, 316, 440).

The FAST proteins

Early models of FAST protein-mediated fusion proposed that fusion might be initiated by the interactions of the hydrophobic ectodomains with the donor and/or target membranes (111, 112, 127, 495). These interactions were predicted to cause membrane perturbation and disruption, leading to a decrease in the activation energy required to initiate lipid mixing, thus making fusion more favourable. This type of fusion model would be far more sensitive to the effects of lipid composition and membrane curvature on altering the activation energy required for fusion (614). Models for FAST protein-mediated fusion are based in part on evidence that synthetic peptides based on the ectodomains of ARV-p10 and RRV-p14 can induce lipid mixing (112, 495). While these types of interactions may occur, the data presented in Chapter 3 indicate that the FAST proteins are not inherently membrane-disruptive but that FAST protein-mediated cell-cell fusion is a non-leaky process. The ability of the FAST proteins to promote hemifusion is likely to be largely ectodomain-based; however, contributions from the transmembrane and endodomains should not be excluded. In contrast, pore formation and expansion might be predominantly facilitated by the transmembrane and endodomains through their interactions with membrane lipids and cytoplasmic factors. How these factors lend support to the current models for FAST protein-mediated fusion is discussed below.

Cholesterol

Cholesterol is important for the formation and expansion of fusion pores generated by HA (439). It is possible that the cholesterol requirement of the FAST proteins (Chapter 5) has significance for the trafficking of the proteins, as well as mechanistic importance for the fusion reaction itself. The ability of cholesterol to flip-flop between leaflets of a membrane might allow it to stabilize hemifusion intermediates by filling void spaces, or it may lend structural support to the fusion pore (439). The FAST proteins may interact with cholesterol through a putative cholesterol-binding

domain (CRAC) to facilitate membrane localization and/or recruit or hold cholesterol at the fusion-site (125, 321). The functionality of this potential cholesterol-binding motif should be assessed for all of the FAST proteins to help support this model for fusion site remodelling through recruitment of specific lipid components.

Phosphatidylserine

Another mechanism for the FAST proteins to promote or stabilize the hemifusion intermediate might be to recruit phosphatidylserine (PS) to areas of membrane fusion. Externalization of PS to the outer membrane leaflet would promote negative curvature and support the hemifusion structure. PS externalization has been implicated in the fusion reactions during muscle and placenta development (123, 557) and VSV-G entry (161). Preliminary results suggest that PS externalization might also occur during FAST protein-mediated fusion since exogenous addition of a PS-binding protein, annexin V, to the culture medium inhibits fusion (Figure 7.3). Similar experiments demonstrated inhibition of myotube formation between C2C12 mouse myoblasts (557). A putative binding motif for anionic PS is the polybasic region common to all the FAST proteins, since other basic proteins can organize these negatively charged phospholipids into microdomains (136). The mechanism for PS externalization is uncertain, but it could be mediated by inhibiting the cellular lipid transporters (e.g. flippases and scramblases) that maintain PS in the inner leaflet, or the FAST protein could directly orchestrate externalization (174, 562). Interestingly, although PS is generally excluded from lipid rafts, it can be organized into microdomains with raft-localized PIPs by basic proteins (136). Further, lipid rafts have been linked to PS externalization during apoptosis and cell motility (174, 256). This link between rafts, PS and basic proteins might provide yet another rationale for FAST protein raft-localization.

Actin

Another contribution this work makes to the FAST protein fusion reaction is implicating actin in the process. ARV-p10-mediated fusion is very responsive to actin alterations that promote actin-filled plasma membrane extensions, and all of the FAST proteins share a requirement for normal actin dynamics in host cells (Chapter 6). With

regards to hemifusion and pore formation, initiating fusion from the tips of actin-filled protrusions would favour fusion because of the curvature strain applied to the membrane by the actin filament. With respect to fusion pore expansion, cortical actin could pose a substantive barrier. The association of actin with the membrane can prevent lateral lipid mobility in the cytoplasmic leaflet of the membrane and could prevent the formation of a fusion pore or the expansion of it (358, 410, 486). Local disruption of cortical actin through PIP_2 binding, *via* FAST protein polybasic regions, and displacement of cytoskeleton adaptor proteins could help to eliminate this barrier over small areas (486).

Whether or not the FAST proteins need to locally disrupt cortical actin to initiate pore formation, this actin still presents a barrier after the pore has formed. The movie of RRV-p14-mediated fusion in QM5 cells associated with Figure 7.4 shows the process of incorporation of two target cells (a and b) into a syncytium. This process occurs over the course of 2 h for cell "a". Note how the cell is seemingly sucked into the syncytium, rounding up and undergoing membrane blebbing. It appears as though the fusion pore is stable enough not to collapse, expands from the site of fusion, but is restricted for the duration of the fusion event. Cortical actin could be providing this restrictive force as indicated by the ring of actin surrounding the possible fusion pore in Figure 6.4. Thus, while cortical actin may not be a sufficient force to restrict the establishment of a fusion pore it can potentially prevent its rapid expansion. This is not always the case however, as cell "c" is incorporated into the syncytium more quickly and without rounding and blebbing.

Summary

In summary, the FAST protein fusion reaction begins with binding, which is actually a two-step process where cellular adhesion molecules first promote membrane contact at distances of about 10-40 nm. The FAST proteins must then wait for cellular machinery (e.g. gap-junction formation) or chance to bring membranes within the limit of the hydration layer (1-3 nm). From this distance, the dehydrating activity of the FAST protein ectodomains could promote close membrane apposition (<2 nm) and contact leading to stalk formation (92). At this point the FAST proteins could promote lipid mixing and fusion by direct interactions with the membrane and orchestrating lipid

rearrangements, as well as recruit or antagonize cellular proteins to make fusion more favourable. Given that the FAST proteins place more of their sequence inside the cell than outside, which is highly atypical of a fusion protein, it is not surprising that the FAST proteins might use the concerted efforts of their endo-, ecto- and transmembrane domains to complete the fusion reaction without the need for large conformational rearrangements.

7.5. The FAST proteins' form fits their function in the viral replication cycle

By relying on cellular adhesion machinery to facilitate cell-cell contact, the FAST proteins remove the key regulatory element of receptor-binding specificity common to other fusion systems. As a result, FAST protein-mediated cell-cell fusion is not subject to the same types of spatial and temporal control required for other fusion events. The fusogenic reoviruses, therefore, might benefit from a less regulated fusion protein that fuses plasma membranes of adjacent cells indiscriminately. This allows the virus to spread quickly and access cells that may not express the receptor for virus entry. By relying on generic, rather than specific, adhesion molecules and structures to facilitate fusion, the FAST proteins allow the replicating reovirus to infect cells and tissues that are not exposed in the initial routes of infection. Our understanding of the role of surrogate adhesion proteins in the process of FAST protein-mediated cell-cell fusion adds to the proposed model of the role of syncytium formation in the reovirus replication cycle and in viral pathogenesis, as presented in Chapter 3. In addition, the wide range of species and tissues susceptible to FAST protein-mediated fusion would potentially allow the virus to change its host organism, by mutation affecting the receptor-binding protein, without compromising the ability to cause syncytium formation or limiting the advantages conferred by it. This process may have occurred with NBV, which closely resembles avian reovirus despite its isolation from a mammalian host (146, 494). Thus, the structural elements of the FAST proteins are well adapted to their function as mediators of untriggered and unregulated cell-cell fusion to promote virus replication.

7.6. Consequence of non-essential fusion on FAST protein evolution

The diversity of the FAST proteins suggests they may employ similar, but not necessarily identical, fusion mechanisms. The FAST proteins may be tailored to cause efficient fusion in their natural hosts by exploiting different membrane environments and cell-cell adhesion structures for fusion. It is curious, however, that the fusion proteins from the same virus genus should be so diverse between species. By comparison, the fusion proteins from other viruses are generally very well conserved in domain structure and organization between species. If one considers the similarities between the different Class I or Class II fusion proteins (Table 1.1), there is even structural and organizational conservation between the fusion proteins from different virus families. The following section speculates how such diversity might have evolved among the FAST proteins.

The non-essential nature of the FAST proteins may help explain their sequence and structural diversity and evolution. If syncytium formation is beneficial, but non-essential, for virus replication, FAST protein-encoding viruses can undergo mutations that eliminate fusion activity without eliminating virus replication. Under ideal health and living conditions for the host, fusion might confer only a slight replication advantage, allowing fusogenic and non-fusogenic virus strains can co-exist as quasispecies within a host but slightly favouring the fusogenic forms. However, whenever the balance is tipped in favour of the virus, the replication advantages conferred by syncytium formation could allow the fusogenic strains to replicate better and become the dominant species. Subsequent virus generations may acquire new mutations to the FAST proteins that restore, enhance or abolish fusion activity to repeat the selection cycle. If this model were true, one would expect to be able to isolate viruses with non-functional FAST proteins from healthy hosts. This has not been reported, but this information has also not been sought-after through random sampling of a species population.

There is however, evidence to support this theory. If the FAST proteins had less selective pressure than other viral proteins, because of their non-essential nature, then the sequence divergence among FAST proteins from different fusogenic reoviruses should be greater than the diversity among other viral proteins. Sequence analysis supports this hypothesis. The major core structural proteins of the NBV capsid share about 60% amino

acid identity with those of the closely-related ARV while NBV-p10 is only 33% identical to ARV-p10 (146, 494). Further, the more divergent ARV, RRV and BRV share about 20-30% amino acid identity for their structural proteins, with some higher conservation of domain organization (494). The opposite is true for the FAST proteins encoded by ARV, RRV and BRV, which have no amino acid identity and limited conservation of domain structure and organization (111, 128, 494). Although the different FAST proteins contain some common structural elements such as a transmembrane domain, hydrophobic patch and a polybasic region, the sequences, positions and lengths of these elements are non-conserved. Thus, it appears as though the non-essential nature of the FAST proteins decreases any selective pressure that would force them to maintain a certain structure or organization. Free of this constraint, the FAST proteins have evolved many different structures suited to exploiting the cellular environment to facilitate membrane merger. In addition, they will continue to evolve new strategies for fusion and interactions with the host and constantly generate diversity within the FAST protein family, providing lots of potential for the evolution of novel fusion mechanisms.

7.7. Implications for the fusion paradigm

Every fusion reaction is different, with protein form fitting function

To address the comment by Kelian and Rey (280) that began this discussion, the FAST proteins "fit into our growing knowledge of the mechanisms of protein-mediated membrane fusion" by demonstrating that the HA paradigm does not apply to all fusion systems. The FAST proteins help us to understand that protein-mediated membrane fusion can be accomplished by a number of ways, and that the form of each fusion machine can be as diverse as the type of fusion reaction it needs to perform. Proteins involved in virus entry are specialized fusion proteins, not generalized ones. Their function is to regulate virus entry into cells and they do that masterfully. Similarly, SNARE proteins are adapted to vesicle trafficking in cells where the increased genetic capacity of the cell can allow multiple proteins with various functions to coordinate a more complex fusion reaction. However, since vesicle fusion and virus entry share similar functional properties (cargo delivery), the fusion proteins that execute these fusion reactions share some structural properties (e.g. helix-bundle formation).

Fusion steps are separable

The ability to uncouple one fusion step from another has substantial significance for understanding membrane fusion. If binding and fusion can be uncoupled, can hemifusion and pore formation, or pore formation and pore expansion, also be uncoupled? In addition, modelling the FAST proteins to a five-step fusion reaction created a problem with the close apposition step that was partially resolved by subdividing it into two phases. This implies that other fusion systems could also merge or divide fusion steps based on how they assign different fusion functions (e.g. binding, hemifusion) to different components of the fusion machinery.

This model of uncoupled FAST protein fusion represents an extreme example of, and offers support for, the concept currently taking shape in the fusion field that the steps of the fusion reaction can be separated and distributed among various components of the fusion machinery. Traditional fusion models hold that formation of helix bundles or hairpins provides the free energy that drives the fusion reaction. In these scenarios, progression from close apposition to pore expansion was believed to occur in ordered succession as the protein refolds (69, 280). This perception has given way in recent years in favour of models for HA where bundle formation may only execute certain steps of the fusion reaction with the transmembrane domains and fusion peptides facilitating the remainder (93, 177, 343, 366, 403). However, there is much discrepancy over exactly which steps of the fusion reaction helix-bundle formation is required for. In addition, the fusion peptide is being re-evaluated and described as a membrane disruptive element that promotes fusion, not unlike the model proposed for the FAST protein ectodomains (31, 258, 301, 538). However, current understanding of these processes is incomplete and confused by the dual role of bundle formation as an energy supplier and mediator of membrane apposition. The situation is further confused by sometimes-conflicting results obtained with the variety of experimental systems used in the above studies, which include theoretical calculations, mutational analysis, artificial membranes and cells.

Similarly, models of SNARE-mediated fusion are beginning to define roles for associated factors that function after SNARE protein bundle formation and help regulate or execute the fusion reaction (509, 555). While the majority of SNARE-mediated fusion

events function according to the traditional model of SNAREs mediating membrane apposition and fusion, a few noteworthy exceptions appear. First, during calcium-triggered synaptic-vesicle exocytosis SNAREs are believed to participate in the targeting of vesicles to the plasma membrane and in mediating close apposition (509). Association of cellular factors with the SNARE complex hold the fusion reaction at a stalk-formation or hemifusion stage to ensure rapid completion of fusion when the calcium trigger is received (90, 138, 360, 581, 588). It is unclear if it is SNAREs or cellular factors that complete the fusion reaction. A second example of SNARE-mediated fusion with distributed fusion elements is vacuolar fusion (441, 442). In this example, SNAREs facilitate membrane apposition but it is believed that an associated ATPase component (V_0) executes the pore-forming steps of the fusion reaction (25, 231, 441, 442). Thus, it appears as though SNAREs have several functions within the fusion complex, and they can occasionally assign some components of the fusion reaction to other factors to increase the efficiency and regulation of the fusion reaction.

These examples of emerging themes should help to illustrate how the concepts of membrane fusion revealed by the unusual FAST protein fusion reaction can be applied to other fusion models to help expand our appreciation and understanding of these systems.

7.8. Implications of an uncoupled fusion reaction for physiological syncytium formation

Cell-cell fusion is a common physiological process; however, the proteins responsible for mediating physiological fusion remain elusive. Thus, the FAST proteins are the only proteins positively identified with the primary function of mediating cell-cell fusion. As such, knowledge gained from our understanding of these unusual fusion proteins may provide insights into the nature of cellular fusion proteins. There is an assumption in the literature that extends from the HA bias, which expects cellular fusion proteins to look and behave like viral fusion proteins. This bias tends to force those who study viral fusion proteins to place the "square peg" candidate cellular fusion proteins into to the "round hole" HA paradigm by looking for features common to viral fusion proteins.

Candidate cellular fusion proteins are as diverse and distinct a group as the FAST proteins, lacking many of the structural features common to well-characterized fusion systems (86). Whether these candidate proteins are *bona fide* fusion proteins or part of a cellular fusion complex remains to be seen. With the exception of syncytin, which is a proviral class I fusion protein (203, 368), none of these candidate cellular proteins contains helical domains or fusion peptides. Most of the candidate molecules have other cellular functions that include cell adhesion and cytoskeleton remodelling. These observations suggest that cell-cell fusion may proceed in a remarkably different fashion than vesicle trafficking or virus entry, or it may be that the actual fusion proteins are yet to be identified.

In light of the data presented here, which demonstrate how cellular adhesion machinery can allow decoupling of the binding and lipid-mixing steps of the fusion reaction, the lack of identified cellular fusion proteins may not be surprising. A proposed mechanism for *Drosophila* muscle development suggests uncoupling of binding and fusion. This mechanism proposes that adhesion molecules regulate binding and activate actin remodelling to promote membrane merger (86, 150). This is a particularly interesting model because it proposes that actin remodelling may promote fusion-inducing membrane-curvature strain, similar to the torsional strains believed to be supplied by transmembrane domains during HA-mediated fusion (153, 177, 343, 597). Here, the importance of actin remodelling in FAST protein-mediated fusion has been demonstrated (Chapter 6). While the evidence suggests that actin remodelling is needed for active adhesion, it may also contribute to the fusion mechanism. The involvement of the cytoskeleton in fusion has been largely overlooked despite the vital role it plays in membrane dynamics.

It is conceivable that physiological syncytium formation requires proteins that more closely resemble FAST proteins than SNAREs or HA. Syncytium formation differs from virus entry or vesicle trafficking in a number of ways. First, fusing metabolically active cells allows the *de novo* synthesis of a fusion protein as a regulatory element. This is not an option available to metabolically inert vesicles or virions that must incorporate regulatory mechanisms into the fusion machinery. Thus, expression of an unregulated fusion protein, like a FAST protein, could be upregulated in response to correct cell-cell

contact signalling. Further, as is the case with the FAST proteins, cell-cell contacts and junctions can fulfill the adhesion requirements for membrane fusion. Thus, cellular fusion machinery could eliminate bulky regulatory and coiled-coil domains in favour of minimalist fusion machinery.

The search parameters for cellular fusion machinery should be broadened to include proteins with FAST-like properties. Bioinformatics approaches can be used to search genome databases for small transmembrane proteins with hydrophobic and polybasic domains, and which are possibly acylated. Unfortunately, such a search is quite advanced for current computer programs but there is no practical reason why better such search engines cannot be developed. In addition, a second approach to identify cellular fusion proteins would be to analyze the protein profile of cells before, during and after physiological syncytium formation for small basic proteins. This can be accomplished with two-dimensional gel electrophoresis that separates proteins by size and charge. The protein profile can be greatly simplified by analyzing only integral membrane proteins and further refined if one could isolate plasma membrane fractions only. Mass-spectroscopy analysis of candidate proteins would provide sequences that could be searched in genomic databases. Once candidate proteins are identified, RNA silencing or genetic knockouts could be generated to ablate fusion activity and the responsible genes can be expressed in heterologous cell types or reconstituted into liposomes to confirm their fusion activity. Of course, the same methods can be applied to implicate non-FAST-like proteins as cellular fusion proteins as well.

7.9. Final thoughts

The FAST proteins, and the reoviruses that encode them, are scientific curiosities. They challenge scientific dogma concerning membrane fusion and non-enveloped-virus pathogenesis and force the re-evaluation of theories explaining these processes. It is becoming apparent that the success of each membrane-fusion reaction is governed not only by proteins that promote lipid mixing, but also by the surrounding environment composed of the lipid bilayer and the proteins associated with either side of it. Every fusion reaction might proceed through the same five steps, but the mechanism by which this occurs may not be universal. The diversity amongst the fusion proteins is reflective

of the multiple biological processes that require fusion and the various regulatory requirements needed to control when and where membranes fuse. The FAST proteins are just another example of form fitting function in nature. Their ability to exploit cellular adhesion machinery and other host factors to facilitate untriggered and unregulated fusion is consistent with their role as cell-cell fusion proteins that promote virus spread.

7.10. Figures

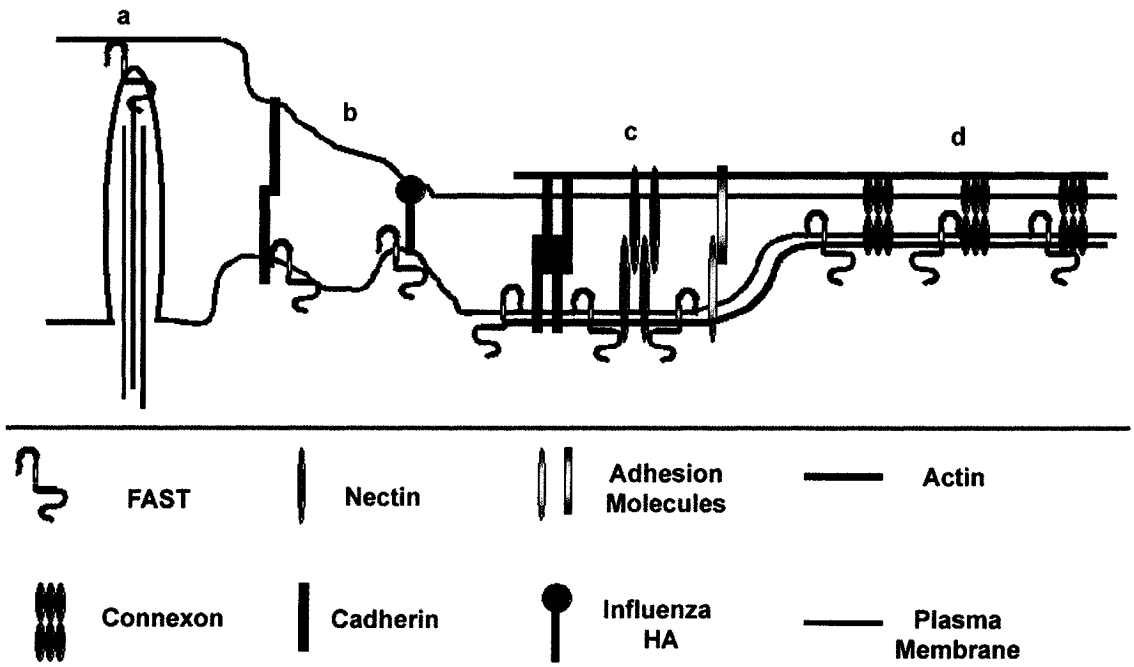

Figure 7.1 The FAST proteins exploit cellular adhesion machinery for fusion. The plasma membrane is a heterogeneous environment that presents multiple opportunities for the FAST proteins to exploit cellular machinery to promote membrane fusion. Motile cells extend actin-filled projections (a) into the environment in search for other cells. The high membrane curvature and the presence of adhesion molecules at the tips of these protrusions could promote fusion. The initial interactions of adhesion molecules with their receptors results in passive adhesion conditions (b), which may support FAST protein-mediated fusion in a distance-dependent manner (Chapter 4). Actin assembly at the plasma membrane stabilizes cell-cell contacts (active adhesion), which promotes formation of adherens junctions (c) and gap junctions (d). These structures can enhance FAST protein-mediated fusion by holding membranes together over larger areas and for long periods of time.

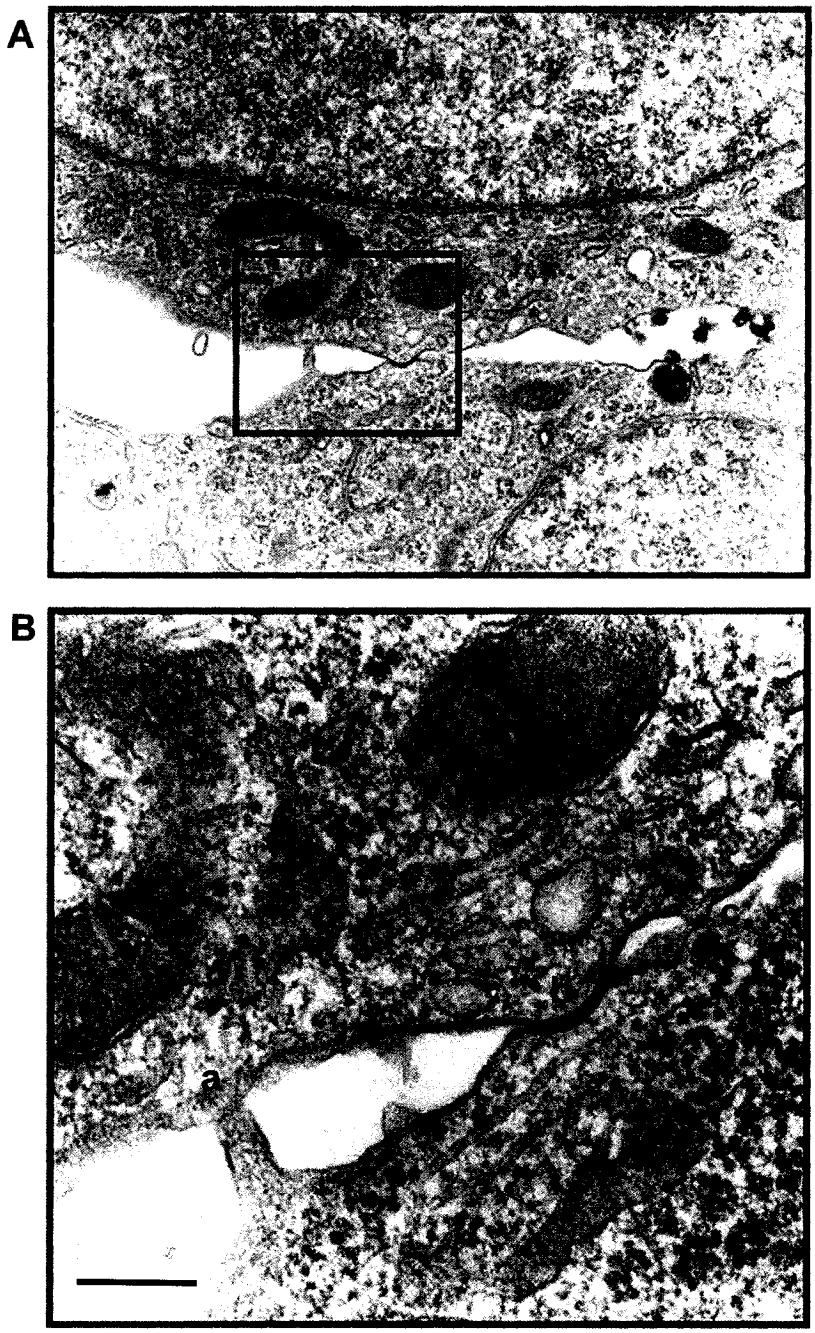

Figure 7.2 The different types of adhesion in QM5 fibroblasts. **(A)** RRV-p14-transfected QM5 fibroblasts have undulating membranes that form multiple protrusions and points of contact. **(B)** Enlargement of cells outlined in **(A)** showing membranes contacting at a protrusion (a), a gap junction (b) and a smaller membrane protrusion mediating membrane contact near gap junctions (c). Scale bar = 100 nm.

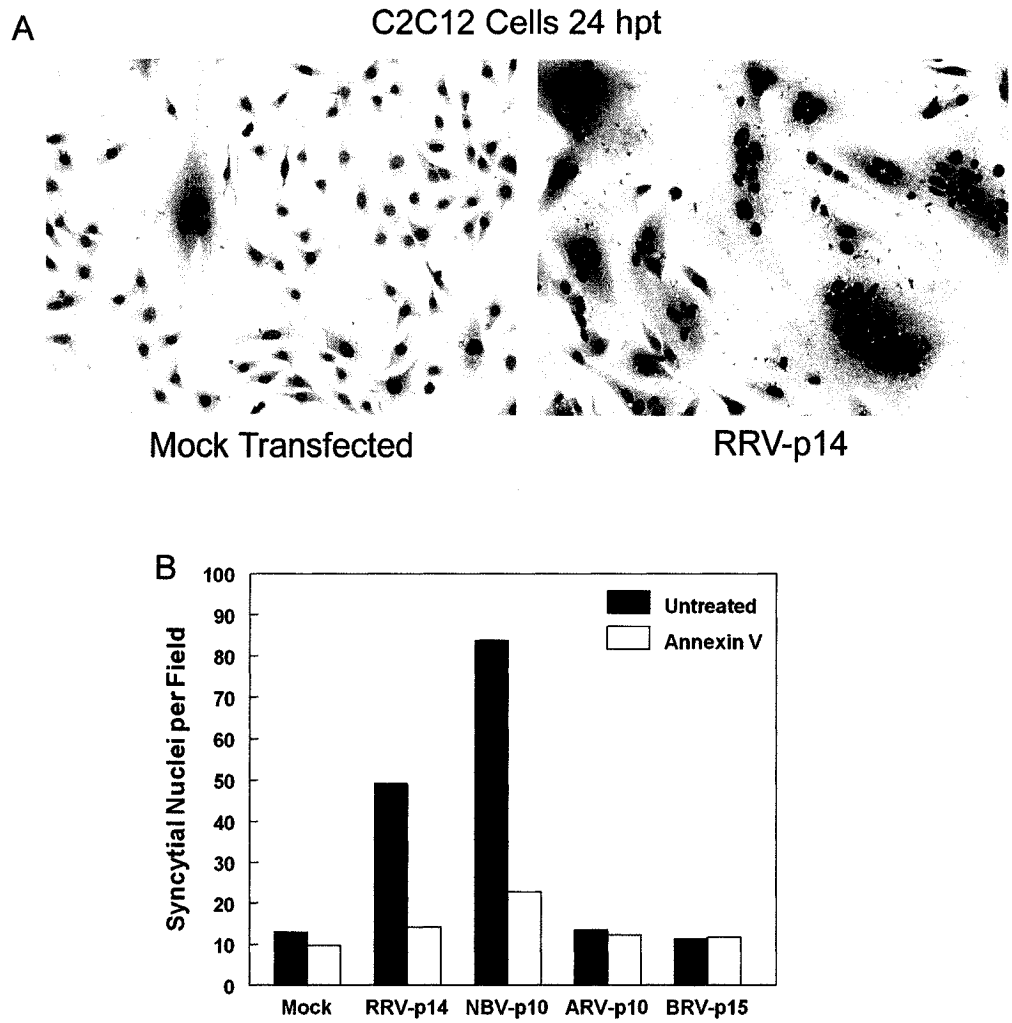

Figure 7.3 Annexin V inhibits FAST protein-mediated fusion in C2C12 cells. **(A)** C2C12 mouse muscle fibroblasts were transfected with RRV-p14, fixed at 24 h post-transfection (hpt) and Giemsa stained. **(B)** FAST protein-transfected C2C12 cells were incubated in the presence of 100 μg/ml annexin V, fixed and Giemsa stained. The average number of syncytial nuclei per field was quantified. Data represent the mean from a single experiment.

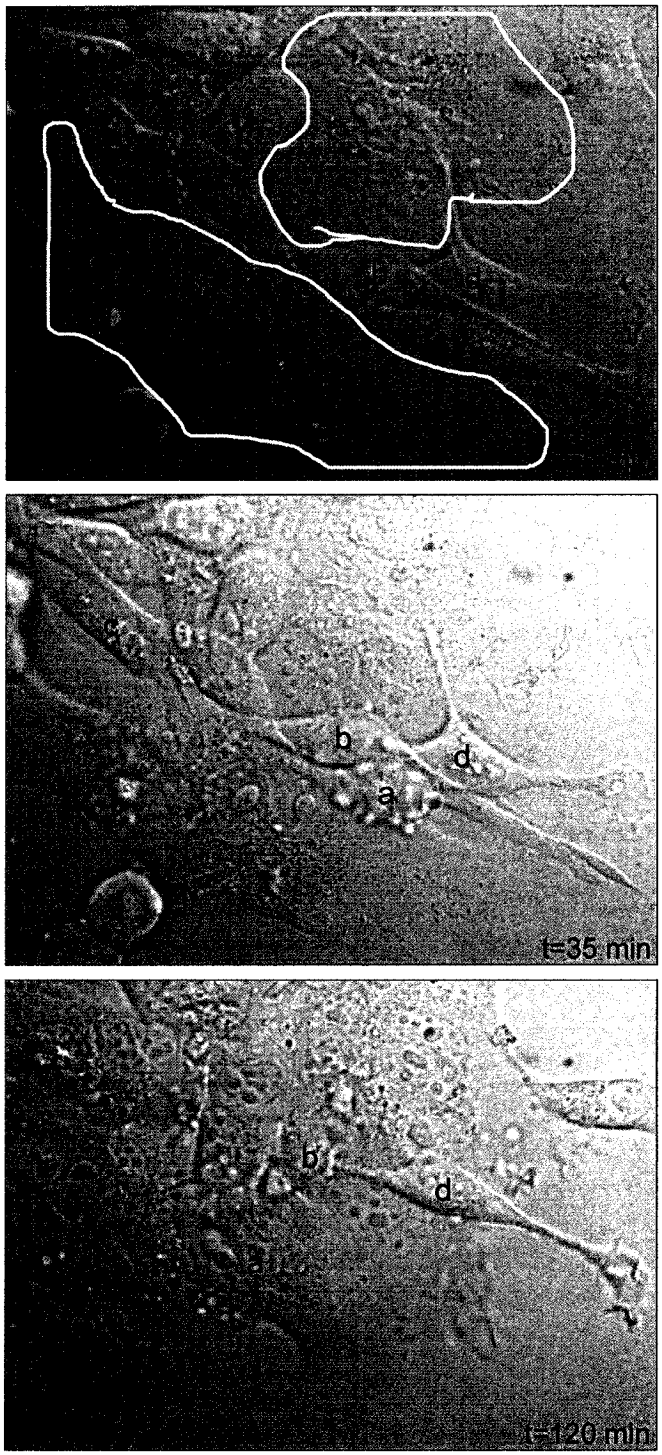

Figure 7.4 Time-lapse footage of RRV-p14-mediated fusion between QM5 cells. QM5 cells were transfected with RRV-p14 and DIC images were captured every 20 s for 2 h. Individual frames were compiled into an avi movie format (provided on CD as "fusion movie.avi") with a playback rate of 1 s = 4.8 min elapsed time = 14.4 frames. Syncytia are denoted with white borders in the top panel (t=0 min). Cells labelled a, b, c, and d are referred to in the text.

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APPENDIX B
Description of electronic supplements

The compact disk associated with this document contains a time-lapse movie of RRV-p14-mediated fusion in QM5 cells (fusion movie.avi) to be viewed as part of Figure 7.4 and interpreted as described in the text.