Chapter 11

Specific Roles for Lipids in Virus Fusion and Exit
Examples from the Alphaviruses

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1. INTRODUCTION

Enveloped viruses infect cells by fusion of the viral membrane with a cellular membrane. Following replication, progeny virus particles are generally formed by budding through a cellular membrane, thus acquiring both the viral spike protein(s) and a lipid bilayer. The goals of current studies of virus membrane fusion and budding are two-fold: to use molecular information about these processes to develop specific and powerful anti-viral therapies, and to apply knowledge from the more accessible viral systems to help understand the identities and functions of proteins mediating cellular membrane fusion and budding processes. Alphaviruses such as Semliki Forest virus (SFV) have been particularly important for studies of membrane fusion. A key feature of the fusion reaction of this simple enveloped

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virus is that it requires the presence of cholesterol and sphingolipid in the target membrane. Cholesterol has also been shown to be required for the efficient exit of SFV from infected cells. To date, SFV is the first and best understood example of a virus with specific lipid requirements for fusion and exit. The mechanisms and wider implications of these lipid requirements are the central focus of this review.

In order to understand the interesting and important roles that cholesterol and sphingolipids play in the alphavirus lifecycle, the general features of alphavirus structure, replication, and assembly will be summarized, and our current molecular understanding of alphavirus membrane fusion will be discussed in some detail.The major emphasis of our review, however, is to describe what is known about the specific functions of cholesterol and sphingolipid in alphavirus infection, and their possible relevance to other virus and pathogen systems, rather than to comprehensively review the extensive body of research on alphaviruses. In each section, therefore, the reader will be referred to a number of excellent reviews for more in depth coverage of the areas that we do not describe in detail. The most extensive work on lipid requirements has used SFV as an experimental system, but more recent studies have characterized the alphavirus Sindbis virus (SIN) as well. We will review and compare results from the SFV and SIN systems as much as possible, which should help to highlight the key features of the lipid requirements that are likely to be general properties of the alphaviruses.

Alphaviruses have been critical in first demonstrating that particular lipids can play a key role in the virus lifecycle. At the end of this review, we will consider the available data suggesting a specific role of lipids in the entry and exit of other viruses and pathogens. While the evidence is as yet quite incomplete, there are already indications of the possible importance of lipids in a variety of systems. Hopefully the techniques, approaches, and information gained from the alphaviruses will help to point the way towards a better understanding of how lipids may act as important players in the membrane interactions of viruses and pathogens.

2. THE ALPHAVIRUS LIFECYCLE

Alphaviruses are a genus of the family Togaviridae containing about 26 viruses (see (Strauss and Strauss, 1994) for review).These viruses all have a lipid envelope and an RNA genome of positive polarity, and are transmitted in nature by arthropod vectors, primarily mosquitoes. Alphaviruses can infect a large range of host organisms including insects, small mammals, birds, and humans, and also readily infect tissue culture cells from a wide

variety of different hosts and tissues (Strauss and Strauss, 1994; Kielian, 1995). Some alphaviruses such as eastern equine encephalitis virus (EEE virus) and western equine encephalitis virus (WEE virus) can cause fatal encephalitis in humans and horses, while other alphaviruses such as Ross River virus can cause polyarthritis, fever, and rashes (Griffin, 1986). Both EEE virus and WEE virus are endemic to the United States and are responsible for periodic epidemics of encephalitis in humans (Anonymous., 1994; Anonymous., 1992). The alphaviruses SFV and SIN have low pathogenicity in humans, and have been widely used as experimental systems to study virus replication, assembly, the infectious entry pathway, and membrane fusion (Strauss and Strauss, 1994; Kielian, 1995). Such studies, particularly of viral entry and membrane fusion, take advantage of the fact that alphavirus growth, radiolabeling, and purification are well-characterized and extremely efficient, and that the virus as isolated has a high plaque forming unit/particle ratio (Kielian, 1995). These properties have made detailed biochemical analysis of alphavirus fusion both practical and meaningful. In addition, complete cDNA clones of SIN (Rice *et al.,* 1987), SFV (Liljeström *et al.,* 1991), and several other alphaviruses have made it possible to manipulate the virus sequence, transcribe infectious viral RNA *in vitro,* and introduce it into cells to generate virus infection. The highly symmetrical organization of alphavirus proteins, described below, has enabled the structural characterization of alphaviruses using cryo-electron microscopy (Strauss and Strauss, 1994; Cheng *et al.,* 1995; Fuller *et al.,* 1995). Thus the overall experimental potential and advantages of alphaviruses are high.

2.1. Virus Structure and Assembly

Alphaviruses are spherical viruses with a diameter of about 640–690 Å (Strauss and Strauss, 1994; Kielian, 1995; Fuller *et al.,* 1995; Cheng *et al.,* 1995; Paredes *et al.,* 1998). They contain one copy of the viral genome, a positive-stranded RNA of ~11.4kb that encodes the four subunits of the RNA replication complex and the structural proteins of the virus. The RNA is assembled with 240 copies of the capsid protein (~30kDa) to form the virus nucleocapsid, an icosahedrally symmetrical structure with triangulation number T = 4. The individual capsid proteins are arranged as pentamers and hexamers to form the spherical nucleocapsid.

The nucleocapsid is surrounded by a lipid bilayer derived from the host cell plasma membrane during virus budding. The lipid composition of the bilayer in general appears to reflect that of the host cell plasma membrane, and contains a cholesterol: phospholipid ratio of ~1 : 1 when the virus is

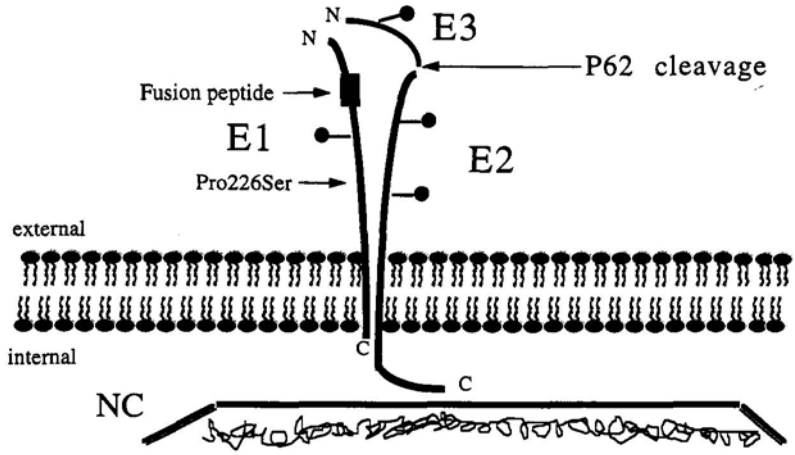

FIGURE 1. Schematic representation of the alphavirus spike glycoprotein and its interaction with the virus nucleocapsid. The ball and stick structures represent carbohydrate side chains. The location of the putative El fusion peptide, the p62 cleavage site, and the mutation (P226S) that affects *srf-3* cholesterol dependence are indicated. Figure is not to scale.

propagated in mammalian host cells such as BHK cells (Kielian, 1995; Strauss and Strauss, 1994).

The virus spike protein contains two type I transmembrane polypeptides, El and E2, which are each about 50 kDa and associate as a tight but non-covalent heterodimer (Figure 1). A peripheral spike polypeptide, E3, of ~10kDa is present in SFV particles but is released from the SIN spike protein and not associated with mature SIN particles. 240 copies of these spike polypeptides are present in each virus particle in a 1 : 1 ratio with the capsid protein, and are arranged in a T = 4 icosahedral protein shell. The virus spike protein structure is a trimer, $(E2\text{-}E1)_3$ in SIN or $(E3\text{-}E2\text{-}E1)_3$ in SFV, thus forming 80 spike proteins. The distal, tripartite projecting domain of the spike protein is triangularly-shaped and formed of the three E2-E1 pairs. More proximal to the membrane, this trimer separates to individual E2-E1 pairs. This separation forms a gap or cavity between the three spike stalks, which then traverse the membrane via the transmembrane domains of El and E2. Within the interior of the virus particle, each individual E2-E1 dimer interacts with a capsid monomer via the E2 internal domain. On the external surface of the virus, each trimeric spike also contacts adjacent spike proteins through lateral interactions to form a layer or "skirt" of spike protein that spreads over and covers most of the surface of the virus particle (Fuller *et al.,* 1995; Cheng *et al.,* 1995).

The capsid and spike polypeptides are synthesized as a polyprotein from a subgenomic RNA, in the order capsid-p62-6K-E1, and are post-translationally processed to produce the individual polypeptides (Strauss and Strauss, 1994; Kielian, 1995; Garoff *et al.,* 1994; Schlesinger and Schlesinger, 1986). p62 (termed PE2 in SIN) is a precursor to the mature E3 and E2 subunits. 6K is a small hydrophobic peptide that provides the signal sequence for El and is found at low levels in the mature virus particle. During virus infection, capsid protein is synthesized on free ribosomes, cleaves itself autoproteolytically from the nascent polypeptide chain, and assembles with viral RNA to form new nucleocapsids (Figure 2). A signal sequence on p62 then initiates translocation of the remainder of the polyprotein into the lumen of the rough endoplasmic reticulum (RER). The El and p62 subunits associate within the RER to form non-covalent heterodimers, and are transported as a complex through the RER and Golgi to the plasma membrane. Evidence suggests that this dimerization is required for proper spike protein folding and transport. Similar to cellular membrane proteins, El and p62 are glycosylated and fatty acylated during their transport through the RER and Golgi complex. The p62 precursor is cleaved after an ArgHisArgArg sequence to give E3 and E2. Cleavage probably occurs in or after the trans-Golgi network by the cellular protease furin (deCurtis and Simons, 1988; Strauss and Strauss, 1994). Following arrival at the plasma membrane, the spike proteins interact with the viral nucleocapsid via the E2 cytoplasmic domain to mediate the budding and release of progeny virus particles.

2.2. Virus Entry and Fusion

2.2.1. Endocytic Entry and Low pH-Triggered Fusion

SFV was the first virus demonstrated to infect cells via endocytic uptake and low pH-triggered fusion (Helenius *et al.,* 1980) (Figure 2), an overall pathway now known to be used by members of a number of virus families including orthomyxoviruses (Bentz, 1993; White, 1992; Wiley and Skehel, 1987), rhabdoviruses (Lenard, 1993), flaviviruses (Allison *et al.,* 1995), bunyaviruses (Gonzalez-Scarano, 1984), and adenoviruses (Greber *et al.,* 1993). The first step in alphavirus entry is the binding of virus to receptors on the plasma membrane. Receptor binding appears to occur primarily through the E2 subunit (Kielian, 1995; Strauss and Strauss, 1994), although there may be some involvement of the El subunit as well. Several molecules have been proposed as candidates for alphavirus receptors, including proteins such as the high affinity laminin receptor and class I major histocompatibility antigens (Wang *et al.,* 1992; Ubol and Griffin, 1991; Helenius

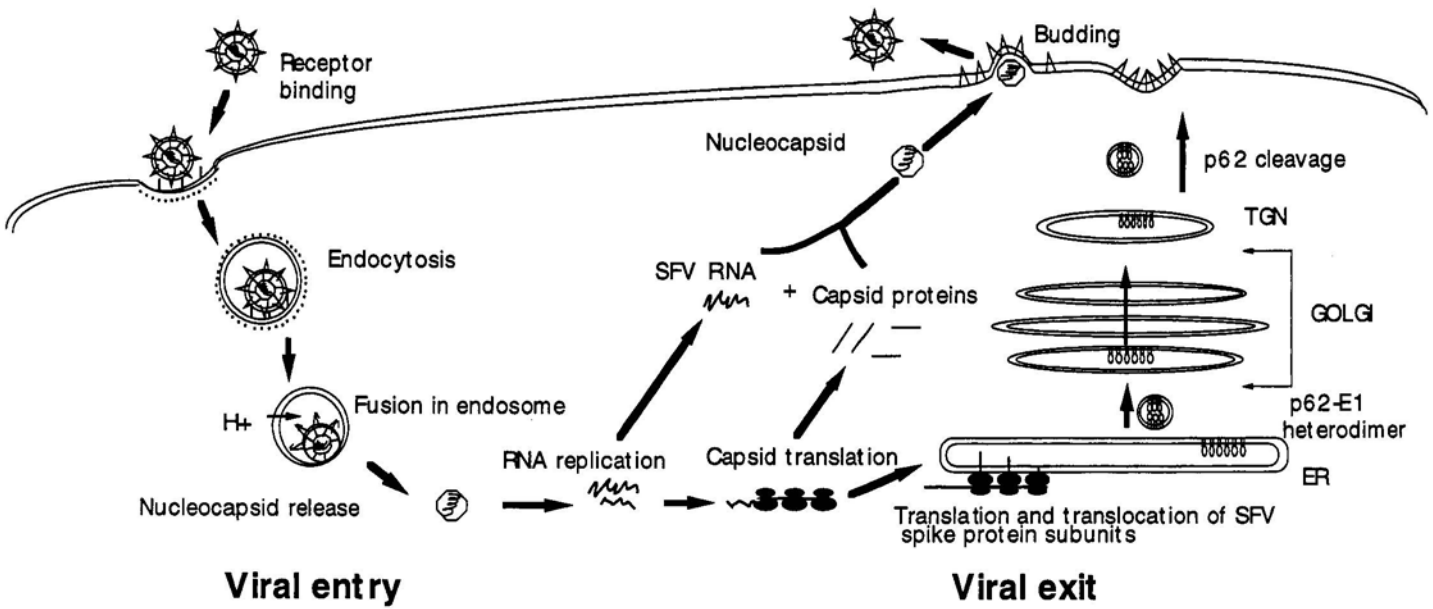

FIGURE 2. The alphavirus lifecycle. Figure taken from (Kielian, 1995) with permission.

et al., 1978), and more recently, cell surface heparan sulfate (Brynes and Griffin, 1998; Klimstra *et al.,* 1998).The available data suggest that a number of different molecules can act as alphavirus receptors, and this broad receptor usage is perhaps to be expected from the fact that alphaviruses infect such a range of different cell types and hosts.

Following receptor binding, the virus is internalized by receptor-mediated endocytosis, a constitutive pathway used by the cell to take up a variety of nutrients, growth factors, and hormones, and to control membrane homeostasis (Mellman *et al.,* 1986; Goldstein *et al.,* 1985) (see virus entry, Figure 2). Endocytic uptake of SFV has been demonstrated by a number of biochemical and morphological experiments (Helenius *et al.,* 1980; Kielian, 1995; Kielian, 1993). Uptake is rapid (half-time of ~10min in BHK cells or ~3–5min in CHO cells) and is inhibited by low temperature (such as incubation on ice). Electron microscopy has demonstrated that SFV endocytosis occurs via typical clathrin-coated endocytic vesicles (Helenius *et al.,* 1980), and delivery of anti-clathrin antibodies into the cytoplasm was shown to inhibit the ability of cells to internalize SFV and become infected (Doxsey *et al.,* 1987). Recently, the role of endocytosis in alphavirus infection was studied by using a dominant-negative dynamin mutant to block endocytic uptake (DeTulleo and Kirchhausen, 1998). Cells expressing this dynamin mutant were shown to be resistant to infection by both SIN and SFV.

Following endocytic uptake, the virus is transported to prelysosomal endosomes. The lumenal pH of endosomes ranges from ~6.5 to 5.0 due to a proton-translocating ATPase in the endosome membrane (Mellman *et al.,* 1986). Upon exposure to a pH of 6.2 or below, the SFV spike protein undergoes several characteristic changes in conformation and interacts with the target membrane (Kielian, 1995; Kielian, 1993, and discussed in section 2.2.3 below). The acid conformation of the spike protein then triggers the fusion of the virus membrane with the endosome membrane, releasing the viral nucleocapsid into the cytoplasm to initiate infection. Important evidence to support this pathway has been provided by studies with various agents that act by raising the pH of the endosome above the critical threshold required to trigger fusion (Glomb-Reinmund and Kielian, 1998b; Kielian, 1995; Kielian, 1993; Strauss and Strauss, 1994). Fusion and infection by both SFV and SIN can be blocked specifically during the early stages of virus uptake by adding weak bases such as ammonium chloride and chloroquine, ionophores such as monensin, or by treating cells with specific inhibitors of the endosomal ATPase such as bafilomycin A (Glomb-Reinmund and Kielian, 1998b; Pérez and Carrasco, 1994). Inhibition by these agents could be "bypassed" by binding the virus to the plasma membrane and brief low pH treatment to trigger direct fusion with the cell plasma membrane

(Helenius *et al.,* 1980; White *et al.,* 1980). Virus strains with a lower pH dependence for membrane fusion were shown to be more sensitive to inhibition by these agents (Kielian *et al.,* 1984; Glomb-Reinmund and Kielian, 1998b; Glomb-Reinmund and Kielian, 1998a), in keeping with the prediction that a lower concentration would be required to raise endosomal pH above the critical threshold level. While further discussion of the entry pathway for SFV and SIN infection is outside the scope of this review, the reader is referred to (DeTulleo and Kirchhausen, 1998; Glomb-Reinmund and Kielian, 1998b; Kielian, 1995; Kielian, 1993; Strauss and Strauss, 1994; Brown and Edwards, 1992) for additional information and perspectives.

2.2.2. *In Vitro* Fusion with Liposomes

SFV fusion has been extensively studied using protein-free liposomes as target membranes, and using either content mixing assays (White *et al.,* 1980; Kielian *et al.,* 1996) or membrane mixing assays based on dilution of a fluorescent probe such as pyrene or octadecylrhodamine (Wahlberg *et al.,* 1992; Bron *et al.,* 1993). Some key points about SFV fusion with liposomes are that it is rapid (at 37 °C, fusion is complete within ~5sec), receptor-independent, very efficient (50–95% of input virus), and highly pH-dependent, with a threshold pH of ~6.2 for wt SFV. Fusion is induced by low pH treatment and can be arrested before completion by shift to neutral pH. The *in vitro* pH dependence of virus-liposome fusion was found to correlate with the *in vivo* pH dependence of virus fusion with the endosome membrane (Kielian *et al.,* 1984; Glomb-Reinmund and Kielian, 1998b; Glomb-Reinmund and Kielian, 1998a). Unlike viruses such as HIV-1 (Berger, 1997) and ALSV (Hernandez *et al.,* 1997), SFV fusion can clearly occur without requiring receptor interaction. Thus the primary role of the SFV-receptor interaction *in vivo* is to carry the virus into the endosome where it is exposed to low pH. SFV fusion is temperature dependent, but occurs across a wide temperature range, 2°–37°C, with a uniform activation energy suggestive of a consistent fusion mechanism at each temperature (Bron *et al.,* 1993). Strikingly, while receptors, calcium, ion and pH gradients do not seem to be required for SFV-liposome fusion, fusion is highly dependent on the presence of cholesterol and sphingolipid in the liposome membrane, as described in detail below.

2.2.3. Conformational Changes in the Virus Spike during Membrane Fusion

One experimental advantage of the low pH-triggered alphavirus fusion reaction is the simplicity of exposing virus to either a fusion-active low pH

Table 1
Summary of events during SFV low pH-induced fusion

Order of events	Assay
1. Acid treatment	
2. E2-E1 dimer dissociation	sucrose gradient sedimentation, co-immunoprecipitation
3. E2 alterations	trypsin sensitivity
El alterations	Ab epitope exposure (enhanced by cholesterol and sphingolipid)
El homotrimerization	trypsin resistance, gradient sedimentation, SDS-PAGE (enhanced by cholesterol and sphingolipid)
4. Lipid bilayer interaction	liposome coflotation (cholesterol-dependent)
5. Other unknown steps	observed as lag time after virus-lipid binding
6. Fusion	lipid mixing, content mixing (requires cholesterol and sphingolipid)

Events are numbered in the probable sequence of their occurrence during the fusion reaction. The multiple events listed under some numbers have not yet been separated kinetically. Modified from (Kielian, 1995).

environment or a fusion-inactive neutral pH environment. The fusion reaction can be slowed by using sub-optimal conditions of pH and temperature, thus enabling careful dissection of the spike protein conformational changes involved. While the precise order of the conformational changes is not completely clear, a great deal of useful information has been gained by the analysis of these types of virus fusion reactions. Our current understanding of the spike protein conformational changes induced by low pH exposure and their relationship to fusion is summarized as follows and in Table 1. The lipid dependence of these conformational changes will be discussed in section 5.1.

The first spike protein alteration observed after low pH exposure is a change in the normally very stable but non-covalent E2-E1 dimer interaction. The acid-treated dimer dissociates upon solubilization in non-ionic detergent, and a loss of E2-E1 co-immunoprecipitation and co-flotation on sucrose gradients is observed. This dimer dissociation appears critical for virus fusion, since virus mutants that have a more acidic pH threshold for dimer dissociation also have a more acidic pH threshold for the subsequent conformational changes in El and for membrane fusion (Glomb-Reinmund and Kielian, 1998a). The p62-E1 dimer requires a considerably lower pH to trigger dissociation compared to the mature E2-E1 dimer (Wahlberg *et al.,* 1989), and this increased dimer stability is responsible for the more acidic pH required to trigger the fusion of virus or spike protein mutants con-

taining uncleaved p62 (Salminen *et al.,* 1992; Lobigs and Garoff, 1990; Jain *et al.,* 1991).

The evidence suggests that once the E2-E1 dimer is dissociated by exposure to low pH, the El subunit then undergoes several distinct conformational changes that are independent of further interactions with E2. Previously masked El epitopes for monoclonal antibody (mAb) binding become accessible upon low pH treatment. We have recently mapped one such acid-specific epitope to residue 157 of El, implying that this region of E1 becomes exposed during the conformational changes involved in fusion (Ahn *et al.,* 1999a). A second conformational change is the formation of a homotrimer of E1 subunits (Wahlberg and Garoff, 1992; Wahlberg *et al.,* 1992). While the SFV E1 homotrimer is not predicted to contain an □□ helical coiled-coil, it is clearly very stable, being resistant to SDS treatment at 30°C and highly resistant to trypsin digestion. With similar kinetics as these E1 conformational changes, the virus binds to target membranes (Bron *et al.,* 1993) via the E1 subunit, presumably due to insertion of the fusion peptide (Klimjack *et al.,* 1994). Photolabeling studies have demonstrated that the El region containing the putative fusion peptide is inserted into the lipid bilayer under fusion-active conditions (D. Mayer, J. Corver, J. Wilschut, M. Kielian, and J. Brunner, unpublished results). There is a lag period following membrane interaction, which is assumed to reflect additional conformational changes and/or reorganization of the El subunits in the membrane. Membrane fusion then ensues, as detected by both lipid and content mixing assays.

A useful form of E1 for studies of conformational changes has been a truncated ectodomain fragment termed El*. El* is produced by proteinase K cleavage at a site close to the El transmembrane domain (Kielian and Helenius, 1985). It contains most of the extraviral domain of E1 but lacks the hydrophobic transmembrane domain, and is a water-soluble monomer not associated with E2. Similar to full-length E1, El * efficiently undergoes acid-dependent conformational changes resulting in acid-specific mAb binding, El* trimerization, and El* membrane interaction (Klimjack *et al.,* 1994; Glomb-Reinmund and Kielian, 1998a). These studies indicate that the E1 ectodomain contains the E1 regions responsible for these conformational changes, and that further E2-E1 interactions are not involved in the fusogenic conformational changes in El once the dimer has dissociated.

Conflicting data exist on whether El undergoes its acid-dependent conformational changes before membrane insertion occurs. Analysis of the kinetics of these changes suggests that both epitope exposure and homotrimerization occur slightly before membrane binding (Bron *et al.,* 1993). In addition, both homotrimerization and epitope exposure can take

place in the absence of a target membrane (Wahlberg *et al.,* 1992; Kielian *et al.,* 1990). In contrast to these results, although both fusion and the El conformational changes are inhibited by the presence of Zn^{2+}, dimer dissociation and membrane binding are unimpaired (Corver *et al.,* 1997). Studies of a mutation in the putative El fusion peptide have shown that the mutation completely blocks both homotrimer formation and membrane fusion, strongly arguing that the El homotrimer is required for fusion (Kielian *et al.,* 1996). Results with this mutant suggest that El-membrane insertion and acid-epitope exposure are relatively independent of homotrimerization. Little information is available to indicate if the homotrimer formed in the absence of a target membrane, or the membrane binding seen in the absence of homotrimerization, represents fusion-active homotrimer or membrane binding, making it difficult to conclude whether blocks in one event can be used to establish the overall sequence of events. Thus, at this point the order of El-membrane association versus El conformational changes (or their co-incident occurrence) cannot be resolved.

2.3. Virus Exit Pathway and Requirements

The final steps in production of progeny virus involve the budding and release of completed virus particles at the plasma membrane of the host cell (Strauss and Strauss, 1994; Kielian, 1995; Garoff *et al.,* 1994; Strauss *et al.,* 1995). From mutagenesis studies of the SFV infectious clone, it is clear that SFV particle formation requires co-expression of both capsid and spike proteins (Suomalainen *et al.,* 1992). This is due to a specific interaction of the SFV E2 cytoplasmic tail with the nucleocapsid (Lee *et al.,* 1996; Skoging *et al.,* 1996), a reaction believed to drive budding. In contrast, the El cytoplasmic tail is dispensable for virus production (Barth *et al.,* 1992). Amino acid residues across the entire E2 31 amino acid cytoplasmic tail are probably involved in nucleocapsid binding (Strauss and Strauss, 1994), and in particular a key tyrosine residue in a pentapeptide repeat region appears to be critical for both binding and particle production (Zhao *et al.,* 1994). The spike-nucleocapsid interaction appears to stabilize both the nucleocapsid structure (Forsell *et al.,* 1996) and the spike protein (Zhao and Garoff, 1992). A number of studies have also implicated the alphavirus 6K polypeptide in virus exit (Kielian, 1995). Deletion analysis of 6K in an SFV infectious clone demonstrated that it was not essential for normal spike protein dimerization and transport to the cell surface, or for normal virus structure and infectivity, but seemed to play a role in the efficient budding of virus from the cell (Liljeström *et al.,* 1991). Both the E2 and 6K proteins are modified by fatty acylation within their transmembrane and cytoplasmic domains, and mutations that disrupt acylation can also affect budding

(Gaedigk-Nitschko and Schlesinger, 1991; Gaedigk-Nitschko *et al.,* 1990; Ivanova and Schlesinger, 1993).

It is apparent that lateral spike-spike interactions are also critical for SFV assembly at the plasma membrane (Kielian, 1995). Cross-linking analysis shows that the spike protomer is organized as a trimer on the surface of infected cells (Rice and Strauss, 1982). Although the cellular site of spike trimerization is not clear, trimer interactions are important in virus exit. A non-budding spike protein mutant with an E2 tail negative for capsid binding could be rescued by the formation of mixed trimers with the wt spike protein (Ekstrom *et al.,* 1994). In addition, several examples indicate that El-E2 dimer interactions are also critical for virus assembly and budding. The E2 cytoplasmic tail does not interact with the nucleocapsid unless the E2 subunit is complexed with El (Barth and Garoff, 1997). SFV spike proteins containing mutations within the putative El fusion peptide form highly unstable E1-E2 dimers (Duffus *et al.,* 1995). These mutant spike proteins are efficiently synthesized and transported to the plasma membrane where they associate with the nucleocapsid, but show a strong and thermoreversible defect in virus exit (Duffus *et al.,* 1995).

In summary, efficient alphavirus exit requires key viral components: the virus spike and nucleocapsid, the specific interaction of the E2 tail with nucleocapsid, the expression of 6K, and correct lateral spike protein interactions. The role of cholesterol in virus exit will be discussed in section 3.2 below.

3. THE ROLE OF CHOLESTEROL IN THE ALPHAVIRUS LIFECYCLE

3.1. Role of Cholesterol in Fusion

3.1.1. *In Vitro* Cholesterol Requirements

A role for cholesterol in alphavirus membrane interactions was first detected in studies of virus-liposome binding by Mooney *et al.* (Mooney *et al.,* 1975), who found that SIN bound to liposomes in a low pH and cholesterol-dependent reaction. As discussed above (section 2.2.3), such virus-liposome association is indicative of either El-membrane interaction prior to fusion and/or the actual fusion reaction itself. Once the analysis of SFV endocytic uptake indicated that virus fusion was triggered by low pH, the *in vitro* fusion of SFV with liposomes was characterized using content mixing assays (White and Helenius, 1980). Fusion with liposomes composed

of a variety of purified lipids revealed a striking requirement for cholesterol in SFV membrane fusion. Fusion is highly efficient (~90%) with "complete" liposomes containing phosphatidylcholine(PC) : phosphatidylethanolamine (PE) : sphinogomyelin(Sph) : cholesterol in a 1 : 1 : 1 : 1.5 ratio. In the absence of cholesterol, no fusion is observed, and maximal fusion requires a ratio of about 1 cholesterol per 2 phospholipid molecules. This cholesterol : phospholipid ratio is similar to those reported for the plasma membrane of animal cells (Dawidowicz, 1987), and thus these results suggested that the virus would show a similar cholesterol dependence during fusion with the host cell endosome membrane.

A later study used the SFV-liposome fusion system to examine the structural features of fusion-permissive sterols (Kielian and Helenius, 1984). Cholesterol causes a number of physical effects in membranes, including phospholipid condensation, increases in bilayer stability, decreased hydration, increases in the fluidity of the gel phase, and decreases in the fluidity of the liquid-crystalline phase (Nes and McKean, 1977a; Demel and DeKrijff, 1976; Kielian and Helenius, 1984). Three major features of cholesterol are believed to be important in its physical interactions with the membrane: the planar ring structure, the aliphatic side chain at C-17, and a free β-hydroxyl group at C-3 (Nes and McKean, 1977a; Demel and DeKrijff, 1976). SFV fusion occurred with coprostanol, a sterol containing a non-planar ring structure, and with androstanol, a sterol lacking the isooctyl side chain. Dihydrocholesterol, which lacks the 5,6 double bond, was also fully active. In contrast, any sterol with a modified 3b-hydroxyl group was inactive in fusion, including epicholesterol (3a-hydroxy), cholestanone, 5α-cholestane, cholesterol methyl ether, or cholesterol acetate (Kielian and Helenius, 1984), and chlorocholestene (Phalen, 1993). The sterol requirement for SFV fusion thus correlates with the sterol 3β-hydroxyl group rather than with the known effects of sterol on the physical properties of the lipid bilayer. Virus-liposome binding experiments demonstrated that binding is both low pH and sterol-dependent (Kielian and Helenius, 1984; Wahlberg *et al.,* 1992). Interestingly, although epicholesterol was inactive in fusion, it could support virus-liposome binding at about 30% the level observed for cholesterol (KieliaI and Helenius, 1984). The cholesterol dependence of both SFV-liposome fusion and binding is sigmoidal (White and Helenius, 1980; Nieva *et al.,* 1994), suggestive of some role of cooperativity such as formation of cholesterol-enriched domains (Nieva *et al.,* 1994).

Subsequent experiments using sensitive lipid mixing assays have corroborated the *in vitro* cholesterol requirement for SFV fusion and the importance of the 3β-hydroxyl group (Bron *et al.,* 1993; Wahlberg *et al.,* 1992). Both lipid and content mixing assays thus demonstrate strong cho-

lesterol dependence, indicating that a cholesterol-deficient membrane is not competent to carry out "hemifusion", the mixing of the two membrane outer leaflets, a step that is believed to occur prior to complete fusion or content mixing. In addition, recent experiments on SIN fusion with liposomes indicate that it is also cholesterol dependent (personal communication from J. Smit and J. Wilschut).

3.1.2. *In Vivo* Cholesterol Requirements

Although the phospholipid and fatty acyl chain composition of the liposomes used in the above fusion assays was selected to be similar to that of a mammalian host cell, the possibility remained that the strong cholesterol dependence observed *in vitro* might not be relevant to the productive infection pathway. In order to address the role of cholesterol in both fusion with biological membranes and infection of a cell, we took advantage of the fact that insect cells are cholesterol auxotrophs (Nes and McKean, 1977b) and mosquito cells are productive hosts for SFV. As demonstrated by (Silberkang *et al.,* 1983), insect cells in culture can be grown in low density lipoprotein (LDL)-depleted serum for multiple passages to achieve virtually complete cholesterol removal in the absence of changes in phospholipid composition or synthesis of replacement sterol. Unlike the situation in mammalian cells, cholesterol-depletion of insect cells causes no deleterious effects. We cultured mosquito cells (the C6/36 line) in LDL-depleted serum to deplete them to non-detectable levels of cholesterol (Phalen and Kielian, 1991). We then used the C6/36 cells to compare the virus infection pathway under control and sterol-depleted conditions. Virus binding, endocytic uptake, and endosome acidification are unaltered in the cholesterol depleted cells, but virus fusion and infection are blocked. Quantitation of infection of cholesterol-depleted cells indicates that it is reduced by ~4 logs compared with control cells (Vashishtha *et al.,* 1998; Marquardt *et al.,* 1993). The efficiency of virus fusion with the plasma membrane of sterol-depleted cells is reduced by ~5 logs (Vashishtha *et al.,* 1998). In contrast, depleted cells are readily infected either by direct transfection of SFV RNA, or by vesicular stomatitis virus (VSV), an unrelated virus that also enters cells via endocytosis and low pH-mediated fusion but is not cholesterol-dependent (Marquardt *et al.,* 1993). Repletion of cells with cholesterol reversed the block in SFV infection, while cholestenone, which lacks the critical 3β-hydroxyl group, was inactive (Phalen and Kielian, 1991). Recent work with SIN demonstrated that both SIN infection and fusion are highly cholesterol dependent, with a difference of about 5 logs between control and sterol-depleted cells (Lu *et al.,* 1999). Taken together, these results confirmed that, in keeping with the biochemical studies, cho-

lesterol plays an important role during *in vivo* fusion and infection by SFV and SIN.

3.2. Role of Cholesterol in Virus Exit

Results from the infectivity studies showed that cholesterol-depleted cells could be infected with SFV by infecting at high multiplicity or by direct transfection of the virus RNA.These methods were used to express the SFV genome in depleted cells and examine the cholesterol requirements for post-fusion steps in the virus life cycle (Marquardt *et al.,* 1993).The depleted cells transcribe virus RNA and translate the virus non-structural and structural proteins. The spike proteins are synthesized, p62 is cleaved, and the E1 and E2 spike subunits are transported to the plasma membrane. Thus most of the steps of the virus replication cycle appear largely unaffected by the absence of cholesterol. However, while the control cells efficiently release virus particles into the medium, the exit of SFV from depleted cells is dramatically inhibited, and is restored by repletion of the cells with cholesterol. The depleted cells are capable of budding virus particles, since VSV, which is cholesterol-independent for fusion, was efficiently produced in sterol-depleted cells (Marquardt *et al.,* 1993; Cleverley *et al.,* 1997).The cholesterol-dependence of SIN virus exit has recently been examined and was found to be dependent on sterol, analogous to the results with SFV (Lu *et al.,* 1999).Thus SFV and SIN are similar in their dependence on cholesterol for both entry (fusion) and exit. These two cholesterol requirements result in a strong inhibition of SFV and SIN growth in sterol-depleted cells compared to control cells, with final titers from 4–5 logs lower in the depleted cells (Vashishtha *et al.,* 1998; Lu *et al.,* 1999). As SIN and SFV are rather distantly related alphaviruses, their comparable cholesterol dependence suggests that sterol dependent fusion and exit are general properties of the alphavirus family. These data are in keeping with a previous paper suggesting that cells containing an increased level of cholesterol are resistant to inhibition of virus exit by low NaCl incubation (Garry *et al.,* 1985).

A partial exception to the cholesterol requirement *in vivo* has been described in mosquito cells depleted of cholesterol by prolonged passage in medium containing lipoprotein-deficient serum (Marquardt and Kielian, 1996). Following extended culture in the absence of lipoproteins, the cells "adapted" to sterol depleted conditions, and were then relatively more permissive for virus infection, fusion with the plasma membrane, exit, and growth. This adaptation is poorly characterized at present, but points out both the importance of limited passage of cells under sterol-depleting conditions, and the possibility that additional factors in the target membrane,

possibly lipids, may act to modulate the cholesterol dependence of SFV fusion and exit.

4. THE ROLE OF SPHINGOLIPID IN ALPHAVIRUS FUSION

4.1. *In Vitro* Requirement for Sphingolipid in Virus-Membrane Fusion

Studies from the laboratory of Jan Wilschut have demonstrated that the fusion of SFV with target liposomes is strikingly dependent on the presence of sphingolipid in the target membrane (Nieva *et al.,* 1994; Wilschut *et al.,* 1995; Corver *et al.,* 1995; Moesby *et al.,* 1995; He *et al.,* 1999). Both lipid and content mixing assays demonstrated strong sphingolipid dependence, indicating that sphingolipid is required for both hemifusion and complete fusion. About 2 mole percent sphingomyelin is sufficient to support maximal SFV liposome fusion (Nieva *et al.,* 1994), considerably lower than the ~30 mole percent cholesterol required for maximal SFV liposome fusion (White and Helenius, 1980). Sphingolipid does not seem to be required for the initial hydrophobic interaction of the virus with the target membrane (Nieva *et al.,* 1994; Moesby *et al.,* 1995), while efficient virus-liposome interaction does require cholesterol (Kielian and Helenius, 1984; Wahlberg *et al.,* 1992) (Table I). Recent experiments on SIN fusion with liposomes indicate that this virus is also strongly sphingolipid dependent (personal communication from J. Smit and J. Wilschut). Fusion studies with a wide variety of sphingolipids have demonstrated an interesting requirement for distinct structural features of the sphingolipid molecule, as detailed below. Thus both the cholesterol and sphingolipid requirements are suggestive of specific lipid-protein interactions during fusion.

4.2. Structural Features of Fusion-Permissive Sphingolipids

Sphingomyelin, ceramide, and galactosyl ceramide are all active in SFV fusion, indicating that the headgroup of the sphingolipid is not critical for fusion and can be either phosphocholine, a hydroxyl group, or a carbohydrate moiety (Nieva *et al.,* 1994). Ceramide is the minimal sphingolipid that can support fusion, and the sphingosine base is inactive (Nieva *et al.,* 1994). The reaction is stereospecific for the naturally occurring D-erythro stereoisomer while the three unnatural stereoisomers of ceramide are inactive (Moesby *et al.,* 1995). Acyl chain length does not appear critical since both C18-ceramide and C8-ceramide are active (Corver *et al.,* 1995; Moesby *et al.,* 1995). 3-deoxyceramide or 3-methoxyceramide do not support fusion, demonstrating the importance of the 3-hydroxyl group on the sphingosine

backbone (Wilschut *et al.,* 1995; Corver *et al.,* 1995). The 4,5-trans carbon-carbon double bond of the sphingosine backbone is also important, since dihydroceramide and Δ5-trans ceramide are inactive (Corver *et al*., 1995; He *et al.,* 1999). Although cholesterol and sphingomyelin are known to form complexes, complex formation does not appear to be required for fusion, since galactosyl ceramide is fusion-active but does not efficiently interact with cholesterol, at least in monolayer experiments (Nieva *et al.,* 1994).

Although sphingolipid synthesis inhibitors have been described (Rosenwald *et al.,* 1992), their prolonged use is toxic to tissue culture cells, and short-term use does not deplete cells below the level of sphingolipid required for fusion. As there are to date no viable sphingolipid-deficient cell lines, it has not yet been possible to examine the role of sphingolipids *in vivo* during alphavirus fusion and exit.

5. MECHANISMS OF CHOLESTEROL AND SPHINGOLIPID REQUIREMENTS IN ALPHAVIRUS FUSION AND EXIT

5.1. The Role of Cholesterol and Sphingolipid in Fusogenic Spike Protein Conformational Changes

The first evidence for a role of cholesterol in the SFV fusogenic conformational changes came from studies of the El* ectodomain (Kielian and Helenius, 1985). El * only converts to its trypsin-resistant form when treated at low pH in the presence of liposomes containing cholesterol (PC:PE: Sph : cholesterol). Epicholesterol-containing liposomes at either acid or neutral pH or cholesterol liposomes at neutral pH are inactive in supporting this conformational change. Subsequent experiments demonstrated that El* is similarly dependent on cholesterol for conversion to reactivity with acid-conformation-specific mAbs (Kielian *et al.,* 1990), and for El*-membrane binding and El* trimerization assayed by SDS-PAGE (Klimjack *et al.,* 1994). Thus these experiments are all consistent in their requirement for a fusion-active sterol in the conformational changes believed to reflect the fusion-active form of El. Studies of isolated lipid-free spike protein rosettes also show that acid epitope exposure and E1 trimerization are more efficient in the presence of cholesterol liposomes, although some conversion also occurs using liposomes without sterol (Justman *et al.,* 1993).

In contrast, studies of the low pH-dependent conformational changes in full length viral El did not reveal a requirement for cholesterol in these conformational changes (Kielian and Helenius, 1985; Wahlberg *et al.,* 1992; Bron *et al.,* 1993; Justman *et al.,* 1993; Kielian, 1995). Recent experiments have demonstrated that this apparent difference between El* and viral El

reflects the fact that although the conformational changes in full length El can occur in the absence of sterol, their kinetics are significantly enhanced when the virus is acid-treated in the presence of cholesterol liposomes (Corver, 1998). The early kinetics of the viral El conformational changes were examined using sub-optimal pH and/or temperature to slow the fusion reaction and allow careful kinetic measurements. These studies show that El homotrimerization (Corver, 1998; Chatterjee and Kielian, 1999b), and mAb epitope exposure (Chatterjee and Kielian, 1999b) are all strikingly potentiated by the addition of cholesterol-containing liposomes, and not by sterol-depleted liposomes. In contrast, dimer dissociation appears not to require the presence of a target membrane (Wahlberg *et al.,* 1989), in keeping with its being one of the first steps in the fusion pathway, prior to the subsequent E1 conformational changes (Justman *et al.,* 1993; Corver *et al.,*1997).

Similar to cholesterol, the initial suggestion that sphingolipids play a role in E1 conformational changes came from studies with El* (Klimjack *et al.,* 1994). Although not as absolute as the cholesterol dependence of El*, ectodomain homotrimerization, acid epitope exposure, and liposome binding are all greatly enhanced by the presence of sphingolipid in the target membrane. However, even examination of the early kinetics of the conformational changes in full length viral E1 using liposomes containing cholesterol, PC, PE with or without Sph found no effect of sphingolipid (Corver, 1998). Homotrimer formation was then evaluated using cholesterol-minus liposomes with or without sphingolipid (PC: PE : Sph vs. PC: PE) (Corver, 1998). This experiment was feasible since, as described above, cholesterol-depleted liposomes support El conformational changes although at a slower rate. Under these cholesterol-depleted conditions, a strong enhancement of E1 homotrimerization by sphingolipid is observed (Corver, 1998). This enhancement was confirmed for both E1 homotrimerization and acid epitope exposure (Chatterjee and Kielian, 1999b). Interestingly, the enhancement of El conformational changes by sphingolipid thus takes place using a sterol-depleted target membrane that is negative for both virus binding and fusion.

The above results indicate the importance of assaying the kinetics of spike protein conformational changes within the time frame of the fusion reaction, when rate-limiting steps in fusion can be differentiated (Bron *et al.,* 1993; Justman *et al.,* 1993). Taken together, these results suggest that E1 must interact with the target membrane at a step in fusion prior to El homotrimerization and epitope exposure, so that the presence or absence of the critical lipids is detected by E1 and reflected in the kinetics with which these conformational changes occur. Such a “lipid-sensing” interaction would presumably occur earlier and differ from the stable insertion

of the El fusion peptide into the membrane detected by liposome co-flotation. The existence of a discrete "lipid-sensing" step is supported by the fact that enhancement of El conformational changes by sphingolipid is observed even using cholesterol-deficient liposomes that are negative for El-fusion peptide insertion. A key question is to identify the domain(s) of El that are responsible for its interactions with cholesterol and sphingolipid during fusion.The suggestion from these results is that these domain(s) may be distinct from the viral fusion peptide.

5.2. Alphavirus Mutants with Reduced Cholesterol Requirements

To gain a better understanding of the role of cholesterol in SFV fusion and exit, and to identify specific spike protein domains involved in the cholesterol interaction, our laboratory has used several strategies to isolate SFV mutants that are significantly independent of cholesterol. One of these mutants, *srf-3,* has been extensively characterized and the results are summarized here. Further work focuses on the characterization of the region defined by this mutation and the identification of other regions and residues of the El spike protein that are important for determining the cholesterol requirement in the alphavirus lifecycle.

5.2.1. Sequences Involved in the Alphavirus Cholesterol Requirement

5.2.1.1. Sequences Involved in the SFV Cholesterol Requirement.

SFV cholesterol-independent mutants were derived by selecting for growth on cholesterol-depleted cells, thus simultaneously applying a selection pressure for decreased cholesterol dependence of both the fusion and exit steps of the virus lifecycle (Vashishtha *et al.,* 1998; Marquardt *et al.,* 1993; Chatterjee and Kielian, 1999a). Such selections have been performed using mutagenized or non-mutagenized virus stocks, and growth on either cholesterol-depleted mosquito cells or depleted cells repleted with a non-fusogenic cholesterol analogue, chlorocholestene. All of the resultant mutant viruses have a similarly increased ability to grow on cells lacking cholesterol. In all cases to date, however, the mutants still grow more efficiently on cells containing cholesterol than on cells without sterol. The first three mutant isolates were termed *srf-1, -2,* and *-3,* for sterol requirement in function (Vashishtha *et al.,* 1998; Marquardt *et al.,* 1993). Sequence analysis (discussed below) revealed that all three have the same genotype (Vashishtha *et al.,* 1998). Much of the subsequent characterization has been performed with *srf-3.*

Phenotypic analysis of the *srf-3* virus reveals that it has growth kinetics comparable to wt SFV when assayed on either BHK cells or on mos-

quito cells containing cholesterol (Vashishtha *et al.,* 1998). In contrast, although *srf-3* grows more slowly on cholesterol-depleted cells than control cells, it achieves the same final titer on both cell types, with an overall yield four to five logs higher than that produced by wt SFV in depleted cells (Vashishtha *et al.,* 1998). This enhanced growth on sterol-depleted cells is due to an increase in both the entry and exit of *srf-3.* Infection and fusion with cholesterol-depleted cells are increased at least 2 logs in *srf-3* compared with wt SFV. Pulse-chase experiments demonstrate that *srf-3* shows more efficient exit from sterol-depleted cells compared to wt SFV (Marquardt *et al.,* 1993; Vashishtha *et al.,* 1998; Marquardt and Kielian, 1996). Transmission electron microscopy indicates that *srf-3* virus buds from the plasma membrane of cholesterol-depleted cells and has an overall morphology similar to wt virus or *srf-3* produced in control cells (Marquardt *et al.,* 1993; Vashishtha *et al.,* 1998). The cholesterol-independent phenotype of *srf-3* is stable to passage in cholesterol-containing mosquito cells or BHK cells (Vashishtha *et al.,* 1998), or to passage in the mosquito vector (Ahn *et al.,* 1999b). Quantitation of primary infection demonstrates that the *srf-3* mutation does not change the host range of the virus on cholesterol-containing mosquito cells or BHK cells (Vashishtha *et al.,* 1998). Thus, the *srf-3* phenotype is similar to that of wt virus except for its increased infection, fusion, and exit in cholesterol-depleted cells.

Further *in vivo* analysis was performed with *srf-3* to see if its relative cholesterol-independence would affect its growth in mosquitoes, which like all insects are cholesterol auxotrophs (Ahn *et al.,* 1999b). *srf-3* was found to grow more efficiently than wt virus in mosquitoes. The difference is not as marked as the growth advantage of *srf-3* in sterol-depleted cells, and it is not clear if the increased *srf-3* virus production in the mosquito is related to the availability of cholesterol in the host.

Interestingly, an additional effect of cholesterol on virus particle stability has also been observed (Vashishtha *et al.,* 1998). *srf-3* virus propagated in cholesterol-depleted cells is less stable to centrifugal shear force than wt or *srf-3* propagated on cholesterol-containing cells. This result suggests a possible role of cholesterol in stabilizing spike protein interactions involved in the alphavirus particle.

Given what was known about SFV fusion and the effect of cholesterol on low pH-dependent conformational changes, the prediction was that the *srf-3* mutation would map to the El subunit. Mapping studies and sequence analysis revealed a single point mutation in the *srf-3* El spike protein, a change of proline at position 226 to serine (P226S) (Vashishtha *et al.,* 1998) (Figure 3). Introduction of this mutation de novo into the SFV infectious clone conferred the cholesterol-independent phenotype in SFV entry/fusion and exit, confirming the identity of the critical mutation. Our current understanding of the effect of this mutation on the conformational changes

Virus	224	Sterol independence
SFV	PS**P**GM VHVPY TQ	–
srf-3	--**S**-- ----- --	+
SIN	--AKN ----- --	–
SKN	--**S**KN ----- --	–
SGM	--**SGM** ----- --	+
SGN	--**SG**N ----- --	+
SKM	--**SKM** ----- --	–
AGM	--**AGM** ----- --	+
PGM	--**PGM** ----- --	–

FIGURE 3. The sequence of SFV, *srf-3,* SIN, and several SIN mutants in the El region from amino acids 224–235. The full sequence of SFV is listed, and the single amino acid change at residue 226 in *srf-3* is listed in bold while identical amino acids are indicated as dashes. The SIN sequence differences from SFV are listed while sequence identities are indicated as dashes. The changes from the SIN sequence in the SIN mutants are shown in bold in the sequence and underlined in the mutant name. Increased virus sterol indepdence for fusion is indicated as a +.

in *srf-3* is discussed below. It will now be important to determine if other regions of the SFV spike protein and other residues in the P226 region can similarly affect virus cholesterol dependence. Recently, two new *srf* mutants, *srf-4* and *srf-5,* have been isolated and shown to have increased infectivity, fusion, and growth on sterol-depleted cells, similar to *srf-3* (Chatterjee and Kielian, 1999a). The *srf-4* and *-5* mutations are located at position 44 and 178 on the El subunit, respectively. Thus it is already clear that additional regions of El can affect cholesterol dependence. These sequences and their potential interactions are currently being explored.

The El P226S mutation has been independently isolated 8 times using several different selection strategies, emphasizing the overall importance of this residue and region in SFV cholesterol dependence (Vashishtha et *al.,* 1998; Chatterjee and Kielian, 1999a). We have used *in vitro* mutagenesis of the SFV and SIN infectious clones to explore the role of the P226 region in virus cholesterol dependence. Results for SIN are discussed in (Lu *et al.,* 1999) and in section 5.2.1.2 below. Mutagenesis of the SFV infectious clone is being used to introduce various single point mutations into the El 226 region and test the progeny virus phenotype (Gibbons and Kielian, 1999). Because no other alphaviruses have a proline at position 226, our initial hypothesis was that the gain of the serine, rather than the loss of the proline, accounted for the mutant phenotype (Vashishtha *et al.,* 1998). Reasoning that the hydroxyl group on the side chain of serine might be acting to

somehow substitute for the 3β-OH of the cholesterol, the residue was mutated to a threonine. However, in changing the residue to a threonine (P226T) the wildtype phenotype was seen, whereas changing it to an alanine (P226A) produced a *srf-3* phenotype for both fusion and infection (Gibbons and Kielian, 1999). These results suggest that our initial hypothesis was probably an oversimplification, and that a hydroxyl-containing side chain is not specifically required at El position 226. Instead, our data in both the SFV and SIN systems (Lu *et al.,* 1999) indicate that the overall conformation of the El 226 region is important in modulating the virus cholesterol dependence.

5.2.1.2. Sequences Involved in the SIN Cholesterol Requirement. Our data indicate that the region around El residue 226 is involved in the cholesterol dependence of SFV entry and exit. This region of El is quite conserved among alphaviruses over a length of 12 residues (Figure 3), but position 226 itself is not conserved, and can be either proline (SFV and Ross River virus), valine (western equine encephalitis virus), or alanine (remaining alphaviruses) (Vashishtha *et al.,* 1998). In SIN, the sequence of El from residue 224-235 is identical to that of SFV except for ^{226}alanine-lysine-asparagine (AKN). In order to test the role of this region in SIN sterol-dependence, various SIN mutants were created (Figure 3), including a single point mutation changing SIN alanine to serine (SKN), and a triple mutation changing the wt SIN AKN sequence to SGM (SGM), thus making the sequence identical to that of *srf-3* from El 224–235 (Lu *et al.,* 1999). The SKN mutant is still strongly dependent on cellular cholesterol for its fusion, infection and growth. In contrast, the SGM mutant shows significantly enhanced infection and fusion with cholesterol-depleted cells compared to wt SIN, with increases of ~ 100 fold and 250 fold, respectively. Pulse-chase analysis of virus exit shows comparable kinetics for wt SIN and SGM in cholesterol-containing cells. In sterol-depleted cells, almost no wt virus is released, while the SGM mutant shows efficient virus exit. Thus, analogous to its role in SFV, the El 226 region acts in the control of the cholesterol dependence of both SIN entry and exit.

The importance of a specific amino acid or combination of amino acids in the control of SIN cholesterol dependence was investigated using several additional mutations in the El 226 region (Lu *et al.,* 1999) (Figure 3). Both the SGN and AGM mutants show increased cholesterol independence, indicating that serine^{226} and methionine^{228} are not specifically required. The SKM mutant is cholesterol-dependent for infection, indicating that a lysine at position 227 can abrogate the cholesterol independence of the SGM mutant. The PGM mutant is also strongly dependent on cholesterol, as might be predicted from the fact that it has the wt SFV sequence from position 224 to 235 on the SIN background. Thus, the overall conclusion from

these experiments is that the *srf-3* mutation of SFV predicts a region of E1 involved in the cholesterol dependence of SIN. This region appears to act by affecting the conformation of El, rather than by a simple role of specific amino acid side chains.

5.2.2. Mechanism of the *srf-3* Mutation

We hypothesized that the increased ability of the *srf-3* spike protein to fuse with cholesterol-depleted cell membranes is due to an alteration in some aspect of the E1 spike protein's interaction with cholesterol, resulting in the relative cholesterol-independence of a normally cholesterol-requiring step in fusion. To test this hypothesis, the initial kinetics of the El conformational changes were followed in the presence of either control or cholesterol-depleted liposomes using either wt or *srf-3* (Chatterjee and Kielian, 1999b). *srf-3* is strikingly less cholesterol-dependent than wt SFV for both acid-specific epitope exposure and El homotrimer formation. At time points when wt E1 conversion is greatly reduced in the absence of cholesterol, *srf-3* showed comparable levels of El conversion in the presence or absence of cholesterol. These E1 conformational changes were unaffected by the presence or absence of cholesterol in the virus membrane, as demonstrated by using *srf-3* stocks grown in either BHK cells or cholesterol-depleted mosquito cells.

In contrast, the *srf-3* El conformational changes are still dependent on the presence of sphingolipid in the target bilayer (Chatterjee and Kielian, 1999b). Acid-specific epitope exposure and homotrimer formation were evaluated using sterol-free liposomes, and neither wild type nor mutant virus support efficient El conversion in the absence of sphingolipid. Studies of virus infection in the presence of NH_4Cl demonstrate very similar NH_4Cl sensitivities for wt and *srf-3,* indicating that the two viruses have a comparable pH-dependence for fusion (Chatterjee and Kielian, 1999b). Thus, the *srf-3* mutant appears unaltered in its sphingolipid dependence and pH dependence, but is less dependent on cholesterol for the fusogenic conformational changes in E1.

The properties of wt and *srf-3* fusion were assayed *in vitro* in lipid mixing assays with pyrene-labeled wt or *srf-3* virus (Chatterjee and Kielian, 1999b). The initial rates of fusion were followed at different temperatures using complete liposomes (PC: PE : SPM : Chol. 1 : 1 : 1 : 1.5), and showed similar kinetics and extent of fusion for the two viruses. An Arrhenius diagram of initial fusion rates versus reciprocal temperature (Bron *et al.,* 1993) gave comparable plots for the two viruses, suggesting equivalent activation energies and general fusion characteristics between the two viruses. In contrast, when fusion with cholesterol-free liposomes (PC: PE : SPM 1 :

1 : 1) was analyzed, significantly higher levels of fusion were observed with *srf-3* compared to wt virus. Fusion of both wt and *srf-3* showed comparable sphingolipid dependence. Thus, studies of virus interactions with defined lipid bilayers indicated that both the membrane fusion activity of *srf-3* and the low pH-dependent conformational changes in its El protein are relatively independent of cholesterol while being otherwise similar to those of wt SFV.

5.3. Mechanism of Cholesterol in Virus Exit

From the data summarized above, it is clear that both SFV and SIN need cholesterol for virus fusion and exit, and that mutations within the El 226 region can modulate the cholesterol requirements for both viruses. Both the SFV mutant *srf-3* and the SIN mutant SGM which are less cholesterol dependent for virus entry are also less cholesterol dependent for virus exit, suggesting that the cholesterol requirement for alphavirus fusion may be tightly associated with its requirement for exit. Recent studies of SFV and *srf-3* indicate that the cholesterol dependent exit step(s) is late in the exit pathway, following arrival of the spike protein at the plasma membrane (Lu and Kielian, 1999). The exit pathway does not appear to require the virus fusion reaction per se, since the final virus assembly and budding steps involve membrane fission, a pinching-off reaction that initiates at the cytoplasmic surface of the plasma membrane and occurs at neutral pH (Marquardt *et al.,* 1993;Vashishtha *et al.,* 1998).Thus, while the data to date suggest a connection between the cholesterol dependence of alphavirus fusion and exit, the relationship between the cholesterol-dependent steps in fusion and exit, and the tightness of the linkage between them, is as yet unclear. One model is that the cholesterol requirements reflect a role of cholesterol in the optimal spike protein conformation for both fusion and exit. In the absence of cholesterol, mutations in the spike protein (e.g., within the El 226 region) may act to compensate for the suboptimal spike conformation and thus allow efficient fusion and exit. The same regions of the spike protein that require cholesterol to potentiate fusogenic conformational changes may also be involved in interactions between spike proteins during virus assembly and exit. Future studies must address the role of cholesterol in the spike protein conformation and interactions, and their importance to the exit pathway.

6. ROLE OF SPECIFIC LIPIDS IN THE ENTRY AND EXIT OF OTHER PATHOGENS

Specific lipid requirements for membrane fusion and exit were first described for alphaviruses. Evidence is accumulating that particular lipids

may be involved in the entry or exit of other pathogens from cells, or in other fusion reactions. We will discuss a number of possible examples of such specific lipid requirements. In cases such as the cholesterol-binding toxins involved in bacterial entry, the data are compelling and informative at the molecular level. In other cases, the information available to date is incomplete and less reliable. The examples presented are not comprehensive and certainly include a wide range of approaches, methods, and some conflicting results. Nevertheless, they serve to illustrate some of the diverse ways in which other viruses or pathogens may use specific lipids as critical features of their lifecycles.

6.1. The Role of Cholesterol in Bacterial Toxin-Membrane Interactions

Clear examples of organisms that exploit cholesterol-protein interactions in their lifecycles are bacteria that produce what are termed thiol-activated cytolysins. The thiol-activated cytolysins are an antigenically and structurally related family of more than 20 toxins (including perfringolysin O, listeriolysin O, pneumolysin, and streptolysin O) that are produced by Gram-positive bacteria and are important virulence factors in bacterial pathogenesis (reviewed in Alouf and Geoffroy, 1991; Cossart and Mengaud, 1989). The toxins are protein monomers of 50–80 kDa that bind to cholesterol-containing membranes and oligomerize to form pores responsible for the permeabilization of cell membranes. Pores are estimated to contain ~50 monomers and to have a diameter of approximately 150Å. It was shown many years ago that toxin binding to cell membranes (or artificial membranes) requires cholesterol or a structurally related sterol with a 3b-hydroxyl, that binding can be irreversibly inhibited by nM amounts of cholesterol in solution or by cholesterol-binding polyene antibiotics, and that prokaryotic or mycoplasma cells without cholesterol in their membranes are not susceptible to the lytic effects of the toxins (Alouf and Geoffroy, 1991; Watson and Kerr, 1974; Rottem *et al.,* 1976). The biological function of a thiol-activated cytolysin is probably best understood for the example of the facultative intracellular bacterium *Listeria monocytogenes* (reviewed in Portnoy *et al.,* 1992; Falkow *et al.,* 1992). *Listeria* are internalized by host cell phagocytosis, escape from the phagocytic vacuole, and replicate in the cytoplasm. Escape from the phagosome is mediated by bacterial secretion of the toxin listeriolysin O, which permeabilizes the vacuole by binding phagosome membrane cholesterol upon exposure to the acid pH of the phagocytic vacuole (Portnoy *et al.,* 1992). Listeriolysin-cholesterol binding has a pH optimum of 5.5, and requires the presence of a sterol with a 3β-hydroxyl group. Thus, both alphaviruses and *Listeria* use low pH-dependent lipid interaction as a strategy to breach the plasma membrane barrier. While other thiol-activated cytolysins are also specific for 3β-hydroxy sterols,

in general they do not have an acid pH optimum, suggesting that they act at the plasma membrane rather than within the endocytic system. This is in keeping with their role as lytic agents specific for cholesterol-containing cells.

The domain of the thiol-dependent cytolysins responsible for cholesterol binding is suggested to be localized to the most highly conserved sequence of the various proteins, the region containing a conserved cysteine from which this protein family derives its misplaced name. Although modification of the cysteine residue by various thiol-reacting reagents has long been known to result in loss of activity, this is probably due to steric hindrance of cholesterol binding, since site-directed mutagenesis shows that the cysteine is not necessary for toxin activity (904). A high resolution crystal structure of a perfringolysin O monomer (without bound cholesterol) was recently reported and was further used to build an homology model of pneumolysin (Rossjohn *et al.,* 1997; Rossjohn *et al.,* 1998). Sterol binding is believed to occur within domain 4 of the molecule, and to involve hydrogen bonds with the sterol 3β-hydroxyl group and non-contiguous aliphatic side chains that interact with the sterol ring structure (Rossjohn *et al.,* 1997). Domain 3 is suggested to transverse the membrane during pore formation by virtue of a two-stranded antiparallel β-sheet (Shepard *et al.,* 1998). It appears that insertion of domain 3 into the membrane can occur with oligomerization-negative mutants (Shepard *et al.,* 1998). Studies of pneumolysin in the absence of membranes show that the toxin has the ability to dimerize and oligomerize in solution without cholesterol (Gilbert *et al.,* 1998). This recent data suggests that cholesterol is not absolutely required for the oligomerization step, although it increases the efficiency of the reaction significantly. In support of this latter point, fluorescence measurements of the conformational changes in streptolysin O suggest that binding to cholesterol-containing membranes facilitates an allosteric transition which initiates the oligomerization reaction (Palmer *et al.,* 1998). Despite all of this recent molecular work it is still unclear exactly how toxin monomers interact with cholesterol, whether the membrane-bound oligomer requires cholesterol, and what the precise structural changes are that result in either oligomerization and/or membrane insertion. These data are also interesting in light of the data indicating that SFV fusion peptide insertion requires cholesterol (Klimjack *et al.,* 1994; D. Mayer, J. Corver, J. Wilschut, M. Kielian, and J. Brunner, unpublished results) and is independent of oligomerization (Kielian *et al.,* 1996; Corver *et al.,* 1997), and the finding that effects on the virus cholesterol requirement are mediated by mutations in E1 outside of the fusion peptide (Vashishtha *et al.,* 1998; Chatterjee and Kielian, 1999a). The potential parallels between the role of cholesterol in the actions of bacterial toxins and in the membrane binding

and homotrimerization reaction of SFV are striking. Studies of these two systems may provide useful insights into common general features of protein-cholesterol interaction, oligomerization, and membrane insertion. It is also interesting that a hemolytic toxin from earthworm was recently reported to specifically bind sphingomyelin (Yamaji *et al.,* 1998), suggesting that other lipid-binding proteins remain to be characterized.

6.2. Other Viruses that May Require Specific Lipids

The majority of enveloped viruses have not yet been tested for lipid requirements in fusion, infection, or exit. This reflects the fact that for many viruses, both fusion and budding are poorly understood. In addition, the trigger for fusion of many viruses is more complicated than the low pH involved in triggering alphavirus fusion, making it difficult at present to study fusion in an *in vitro* system resembling the fusion reaction *in vivo.* The low pH-dependent fusion reactions of both influenza virus, an orthomyxovirus, and vesicular stomatitis virus (VSV), a rhabdovirus, have been well characterized *in vitro,* and both appear to have fusion mechanisms independent of cholesterol, sphingolipid, or other specific lipid components (White *et al.,* 1982; Eidelman *et al.,* 1984; Phalen and Kielian, 1991; Cleverley *et al.,* 1997; Stegmann and Helenius, 1993). Here we will discuss several examples of viruses that may have specific lipid interactions during either fusion or exit.

6.2.1. Human Immunodeficiency Virus

Studies of the retrovirus human immunodeficiency virus (HIV-1 and HIV-2) have shown that the lipid composition of its envelope appears significantly different from that of the host cell plasma membrane, with the virus membrane containing a lower total lipid/protein ratio, a higher cholesterol/phospholipid ratio, and substantial changes in the phospholipid class ratios (Aloia *et al.,* 1993; Aloia *et al.,* 1988). Electron spin resonance studies suggest that the virus membrane lipid composition produces a more ordered membrane in the virus compared to the host cell plasma membrane from which it buds (Aloia *et al.,* 1988). Treatment of virus with cholesterol-'poor liposomes, cholesterol-binding drugs, or heating all increase the membrane fluidity and decrease virus infectivity (Pleskoff *et al.,* 1995; Aloia *et al.,* 1988; Mcdougal *et al.,* 1985). These data suggest that HIV may selectively exit from regions of the host cell plasma membrane enriched in cholesterol and sphingomyelin, and that the fluidity of the viral membrane may be important in virus stability and infectivity. However, the data are complicated by our current technical limitations in preparing purified plasma

membranes, which make it difficult to conclude what differences in lipid composition exist between HIV and host cell plasma membranes. The precise structural or functional requirements for cholesterol during HIV exit from the target cell, and the ability of other sterols to substitute thus are as yet unclear.

The role of target membrane cholesterol in HIV fusion has been tested by assaying the *in vitro* fusion of liposomes by peptides with sequences derived from the N-terminal fusion peptide of the HIV gp41 fusion protein. These studies demonstrated that the absence of cholesterol in the liposomes inhibits peptide-induced fusion by ~33%, while peptide-membrane binding is unaffected (Pereira *et al.,* 1997a; Pereira *et al.,* 1997b). Peptide-dependent fusion is inhibited by several physiologically relevant modulators of HIV-1 infectivity, such as inclusion of point mutations in the peptide that block HIV fusion, competition by hexapeptides derived from the N-terminal gp41 fusion peptide, and addition of several antiviral compounds. These results suggest that the peptide fusion system may accurately reflect an *in vivo* role for cholesterol in the target membrane during HIV fusion. However, it is important to note that such peptide-liposome fusion studies are prone to possible artifacts and may not reflect the mechanism of biological membrane fusion (Stegmann *et al.,* 1989a). For example, studies with influenza virus have demonstrated that the membrane insertion observed with a purified virus peptide from the spike coiled-coil region (Yu *et al.,* 1994) does not occur during the actual fusion of influenza virus with a target membrane (Durrer *et al.,* 1996). In addition to the potential problems with peptide-liposome studies, a group using an *in vitro* virus-liposome fusion system found that the presence or absence of cholesterol does not affect the kinetics or efficiency of fusion of HIV-1 or the closely related SIV-1 (Larsen *et al.,* 1990; Larsen *et al.,* 1993). The exact physiological relevance of the latter study is also not clear, since virus fusion in this system occurred with target membranes not containing the CD-4 receptor and co-receptor, the key molecules known to trigger HIV fusion during infection (Berger, 1997; Binley and Moore, 1997). Thus, a better understanding of the role of cholesterol in HIV entry and exit awaits further study.

The effects of cholesterol on HIV fusion are believed to be exerted via the virus fusion protein subunit gp41, which contains the putative fusion peptide at its N-terminus. The HIV gp120 spike protein subunit is responsible for the binding of virus to cells, usually by recognition of the CD4 receptor. However, several groups have identified the glycosphingolipid galactosylceramide (GalCer) as an alternative receptor to account for infection of CDCnegative cells such as colonic epithelial, cells, Schwann cells, and oligodendrocytes (Harouse *et al.,* 1991; Yahi *et al.,* 1992). Infection of cultured human colonic epithelial or neural cells is blocked by antibodies

against GalCer. In addition, the binding of recombinant gp120 to purified GalCer can be specifically inhibited by an anti-GalCer IgG. Binding of purified gp120 to GalCer in a liposome system is rapid, saturable, extremely stable, dependent on a critical GalCer concentration in the target membrane, and blocked by antibodies to GalCer (Long *et al.,* 1994). This interaction is also dependent on the conformation of the gp120. Various other structurally related glycosphingolipids (GSLs) such as lactosylceramide, glucosylceramide, and galactocerebroside sulfate can also bind purified gp120, but with decreased efficiency and stability. Several recent reports have also demonstrated that the addition of a GSL fraction to CD4 positive cells resistant to HIV-1 infection confers susceptibility to viral fusion, suggesting a role for GSLs as possible co-receptors for binding virus (Puri *et al.,* 1998a; Puri *et al.,* 1998b). The active component of the GSL fraction in conferring fusion activity in resistant cells is the neutral GSL, Gb3 (Puri *et al.,* 1998a). The relative *in vivo* importance of such GSL as alternative HIV receptors or coreceptors is currently under investigation. Taken together, the above results suggest that for HIV, as with alphaviruses, different target membrane lipid components may play a role at different stages of the virus lifecycle.

6.2.2. Mouse Hepatitis Virus

Mouse hepatitis virus (MHV) is a murine coronavirus that usually induces a persistent infection of the central nervous system, but many strains may also cause an acute, lytic infection marked by significant cell-cell fusion (Wege *et al.,* 1983).The choice between persistent and lytic infection is partially determined by the host cell and appears to be influenced by the cholesterol content of the cell membrane. Studies with cultured mouse fibroblast sublines, either susceptible or relatively resistant to virus-mediated cell fusion, showed that supplementation with cholesterol increases the membrane cholesterol/fatty acid ratio and concomitantly increases cell fusion in both cell types (Daya *et al.,* 1988; Cervin and Anderson, 1991). This effect is not due to enhanced MHV internalization, but rather due to some late event in infection, resulting in enhanced syncytia formation. In fact, it was shown that increased cholesterol in target membranes enhanced fusion between infected and neighboring uninfected cells. Further work demonstrated that changes in cholesterol content alone account for the enhancement of fusion because no alterations in the levels of fatty acids were detected. As previously discussed (Kielian, 1993; Kielian, 1995), virus-induced cell-cell fusion, although widely used and frequently useful, is a morphological assay that can be affected by numerous factors not directly relevant to the virus infection pathway, and thus effects

on cell-cell fusion must be validated using direct infection and/or fusion assays.

in vivo work using mice genetically resistant to infection showed that a hypercholesterolemic diet can make mice susceptible to MHV infection (an effect reversible with a normal diet) and that hepatocytes isolated from these hypercholesterolemic animals have increased virus adsorption and intracellular titer versus control animals (Braunwald *et al.,* 1991). To address the issue of whether or not this enhancement is due to changes in membrane fluidity, the genetically resistant A/J mice were fed one of two diets, either a hypercholesterolemic (HC) or a phosphatidylserine (PS) diet (Nonnenmacher *et al.,* 1994). The expected result is that the cholesterol would decrease membrane fluidity, while the PS would increase membrane fluidity. The HC diet increases hepatocyte susceptibility to MHV infection about 5 fold, while the PS diet has a significant dose-dependent inhibition. However, when fluorescence anisotropy measurements were performed on the cultured hepatocytes from these animals to characterize membrane fluidity, both the HC and PS diets produce an increase in fluidity, indicating that there is no correlation between fluidity and infection. These results are consistent with work by others showing that HC diets can be at least partially compensated by cellular homeostatic mechanisms, which tightly regulate the effects of increased dietary cholesterol on membrane fluidity (Larsen *et al.,* 1993). Taken together, these results suggest that cholesterol could play a role in both the initial MHV binding/fusion steps and the later stages of virus-induced cell-cell fusion. More work is necessary to fully understand the role of cholesterol in the lifecycle of MHV, but it is intriguing that dietary components may be able to modulate the genetically-determined susceptibility to virus infection.

6.2.3. Ebola Virus

Ebola virus is a member of the Filoviridae, filamentous enveloped RNA viruses that can cause severe hemorrhagic disease in humans. Similar to the work described earlier for HIV, recent work has focused on liposome fusion induced by the putative fusion peptide of the Ebola virus glycoprotein (Ruiz-Arguello *et al.,* 1998). The ability of synthetic peptides corresponding in sequence to the putative fusion peptide to induce fusion of liposomes is strictly dependent on the presence of Ca^{2+} in the reaction and phosphatidylinositol (PI) in the vesicle membranes. In fact, the best fusion rate and efficiency are achieved using large unilamellar vesicles with a lipid composition approximating that of the hepatocyte plasma membrane (PC : PE : Chol. : PI 2 : 1 : 1 : 0.5). The effect of PI is at least partially mediated by the initial interaction of the peptide and liposomes, but appears not to

be due only to the charge of the lipid because other anionic lipids (phosphatidylglycerol or phosphatidic acid) do not substitute. Additionally, PI from various animal and plant origins is interchangeable, suggesting that the acyl chain composition is unimportant. While these data suggest that PI may play a specific role in Ebola spike protein-mediated fusion, the previously mentioned caveats of work with isolated virus peptides apply here as well. Further *in vitro* and *in vivo* work will be needed to define the importance of PI in the context of Ebola virus fusion and infection.

6.2.4. African Swine Fever Virus

African swine fever virus (ASFV) is a unique animal DNA virus of an unnamed family. It has a complex structure with an overall icosahedral shape, formed by a DNA-containing viral core which is successively wrapped by two inner lipid bilayers derived from collapsed ER cisterna, a capsid protein with a hexagonal lattice structure, and an outer lipid membrane derived from the plasma membrane during virus budding (Andres *et al.,* 1997; Carrascosa *et al.,* 1984). ASFV is thought to infect cells via receptor-mediated endocytosis and low pH-triggered membrane fusion (Valdeira and Geraldes, 1985; Alcami *et al.,* 1989).

A recent report used several approaches to address the role of cholesterol in ASFV infection (Bernardes *et al.,* 1998). When cellular cholesterol synthesis is inhibited by cerulenin, W-7 and miconazole, the production of progeny ASFV is decreased by ~50%.When the cellular cholesterol level is decreased to ~50% of that in control cells by culturing Vero cells in delipidated serum, similar kinetics of endocytic uptake are observed for ASFV, while the kinetics of virus penetration to the cytoplasm are significantly slowed. Low pH-triggered fusion of ASFV with the plasma membrane of cholesterol-depleted cells is inhibited compared to fusion with control cells. In addition, similar to results previously published for SFV (Phalen and Kielian, 1991),ASFV fusion with the plasma membrane is inhibited by cholesterol oxidase pretreatment of the target cells. Since this enzyme is known to oxidize the sterol 3β-hydroxyl group, this result suggests that, similar to SFV, the sterol 3β-hydroxyl group may be important in the ASFV cholesterol requirement. The role of sphingolipid in ASFV infection and fusion has not been addressed. These experiments with ASFV, while provocative, are complicated by our limited ability to deplete cholesterol in mammalian cell lines and the potential wide-ranging side effects of such sterol depletion (Nes and McKean, 1977a). It will be important to compare the cholesterol-dependence of ASFV fusion and infection in parallel with that of a virus known to be cholesterol independent, such as VSV, to control for non-specific effects of inhibitors and depletion. It will also be important to

characterize the cholesterol-dependence of ASFV fusion with liposomes of defined and physiological lipid composition.

6.2.5. Sendai Virus

Sendai virus is a member of the paramyxovirus family that fuses with host cell plasma membranes at neutral pH upon binding to its receptor, sialic acid (see (Lamb, 1993; Baker *et al.,* 1999) for review). It has two spike glycoproteins, the fusion protein (F) that contains the putative fusion peptide, and the hemagglutinin-neuraminidase protein (HN) that mediates receptor binding and may potentiate the fusion reaction. Several early studies suggested that cholesterol is required for Sendai virus fusion. By measuring Sendai virus fusion *in vitro* with purified liposomes, Hsu and Choppin (Hsu *et al.,* 1983) demonstrated that the presence of cholesterol in the liposomal membrane is necessary for efficient fusion, and that the optimal concentration of cholesterol is 0.3–0.4 mole fraction, similar to the optimal concentration for SFV-liposome fusion (White and Helenius, 1980). Kundrot and MacDonald studied liposome lysis in response to Sendai virus and mild hypo-osmotic stress, a reaction presumably reflective of virus-membrane fusion, and found that cholesterol is required (Kundrot *et al.,* 1983). Both of these liposome studies were performed using PC : cholesterol liposomes. Fusion of Sendai with a more complex biological membrane also appeared to be cholesterol-dependent, as assayed by Sendai fusion with *Mycoplasma* membranes containing varying amounts of sterol (Citovsky *et al.,* 1988). Asano and Asano tested the ability of purified F protein to bind radioactive cholesterol *in vitro,* and demonstrated that 5–14% of intact F protein binds cholesterol in a filtration assay (Asano and Asano, 1988). Reduced binding is observed for F protein in which the N-terminal fusion peptide is removed by thermolysin digestion, suggesting that the fusion peptide is involved in cholesterol binding. Sterol binding appeared to require the A/B ring trans structure of cholesterol, rather than the 3β-hydroxyl group. It is important to note, however, that the purified F protein used for binding assays may not maintain its native conformation, and that the hydrophobic F protein transmembrane domain is present in the F protein preparations used. In addition, the binding assay may be over-simplified and does not take into account potential effects of the virus HN protein, receptor binding which is the presumed fusion trigger, or the normal target membrane lipid bilayer structure.

A cholesterol requirement has not been universally observed for Sendai virus fusion. Klappe *et al.* showed that Sendai virus could fuse with liposomes solely consisting of acidic phospholipids, particularly cardiolipin (Klappe *et al.,* 1986). Notably, however, virus fusion with cardiolipin-

containing liposomes is prone to aberrant effects from the charge and nature of this lipid (Stegmann *et al.,* 1989b). Cholesterol-independent fusion was also observed by Cheetham and Epand, using lipid-labeled Sendai virus with cholesterol-free liposomes containing PE and ganglioside (Cheetham *et al.,* 1990; Cheetham *et al.,* 1994). It is possible that the difference between PE and PC bilayer structure explains the differing results between this and the above liposome experiments. The incorporation of cholesterol sulfate into such PE liposomes was found to potently inhibit both the rate and extent of Sendai virus-mediated fusion (Cheetham *et al.,* 1990; Cheetham *et al.,* 1994). Such inhibition is also observed for human erythrocyte ghost target membranes that are pre-incubated with cholesterol sulfate. The mechanism of inhibition is not clear, but is presumed to be an effect of cholesterol sulfate on membrane stability and physical properties.

Clearly, conflicting data exist on the role of cholesterol in Sendai virus-induced fusion. Further systematic studies using physiological lipid mixtures and sterol-depleted biological membranes will hopefully resolve these issues.

6.2.6. Role of Cholesterol in Transport of Influenza Hemagglutinin

Influenza virus is a member of the orthomyxovirus family whose fusion reaction and hemagglutinin (HA) fusion protein have been exceptionally well-characterized (Wiley and Skehel, 1987; White, 1992; Hughson, 1995; Carr and Kim, 1993; Bullough *et al.,* 1994). Influenza virus infects cells via the endocytic pathway where the low pH in endosomes triggers fusogenic conformational changes in HA, leading to the formation of an extended coiled-coil that mediates viral membrane fusion with endosomal membranes. Fusion studies with liposomes excluded a critical role of cholesterol, sphingolipids or other specific lipids in influenza virus fusion (White *et al.,* 1982; Stegmann and Helenius, 1993). However, substantial evidence suggests a role for cholesterol and sphingolipids in the virus exit pathway.

Influenza virus buds from the plasma membrane of infected cells, and HA transport and budding occur selectively at the apical domain of polarized epithelial cells (Ikonen and Simons, 1998). In either polarized or non-polarized cells, HA associates selectively with cholesterol and sphingolipid-enriched membrane domains termed “raft” domains, which are characterized by their resistance to extraction by Triton X-100 at 4°C (Brown and Rose, 1992). HA association involves the protein’s transmembrane domain since replacement with a foreign transmembrane domain or point mutations in this region abolish HA-raft association (Scheiffele *et al.,* 1997). Depletion of cellular cholesterol results in dissociation of HA from raft domains, partial HA missorting to the basolateral domain, and a slower

transport rate of HA to the plasma membrane in both polarized and non-polarized cells (Scheiffele *et al.,* 1997; Keller and Simons, 1998). Analysis of purified fowl plague virus (FPV), an avian influenza virus, demonstrated that FPV particles contain a high percentage of detergent-resistant complexes enriched in cholesterol and sphingolipids (Scheiffele *et al.,* 1999). In contrast, purified VSV and SFV, both basolaterally-targeted viruses, were not similarly enriched in such complexes (Scheiffele *et al.,* 1999). These results suggest that influenza virus may select specialized membrane domains for both HA transport and virus budding, and that these domains may also be important in the virus's apical targeting pathway. Further studies will be necessary to demonstrate the importance of raft domains in *vivo* and to examine the effect of cholesterol or sphingolipid depletion on influenza virus budding. It is also important to note that transport to the apical domain most likely involves other mechanisms besides lipid rafts (Weimbs *et al.,* 1997; Brown and London, 1998). Although SFV budding from the plasma membrane is cholesterol-dependent, the fact that the SFV spike protein does not preferentially associate with lipid raft domains (Scheiffele *et al.,* 1999) suggests that influenza virus and SFV use different budding mechanisms, each of which may involve cholesterol.

6.3. Lipid Stalk Intermediates in Membrane Fusion Reactions

The previous discussion centers on the functions of various viral or bacterial proteins and the different ways in which they may interact with cholesterol or other lipids. We have focused on fusion proteins that may have specific interactions with particular lipids during fusion. In addition to these specialized lipid-requiring mechanisms, membrane fusion reactions may use a common, lipid-dependent structural intermediate during the actual fusion process. This has been termed the "stalk intermediate", referring to a strongly bent structure shaped like a stalk that mediates a local contact between the two lipid bilayers which are to fuse (Melikyan and Chernomordik, 1997; Chernomordik and Zimmerberg, 1995). Such an intermediate would involve primarily the lipids of the two fusing bilayers and would be similar regardless of the fusion proteins involved or the type of membrane fusion event under study (viral fusion, exocytosis, or intercellular fusion). The structure of this bent intermediate is strongly influenced by the types of lipids present in the membranes, and the respective geometries they impose on the membranes. The involvement of this stalk intermediate has been characterized primarily by observing the effects on fusion of lipids that favor a strongly bent stalk structure versus those that oppose it. (reviewed in Melikyan and Chernomordik, 1997; Chernomordik and Zimmerberg, 1995). For example, fusion is inhibited by lysophospholipids and

promoted by cis-unsaturated fatty acids. Available experimental and theoretical work suggests that a "stalk" fusion intermediate may be required in all fusion events (Kozlov and Markin, 1983; Siegel, 1993; Basanez *et al.,* 1998). The fusion proteins of viruses or cells could impose additional restraints on stalk formation or the subsequent fusion pore, but may be most important in the process of initiating lipid-lipid contact of the bilayers prior to formation of a stalk intermediate. While the role and importance of a lipidic stalk intermediate are currently an evolving area of investigation in the membrane fusion field, at the least these studies point out the potential importance of the effects of various lipids on the structural intermediates of bilayer fusion. It is possible that structural effects of differing lipid composition may alone account for some of the discrepancies reported for various virus-cell or virus-liposome experiments. The potential effect of lipid composition on bilayer structure and stalk formation should certainly be taken into account when trying to identify or ascribe a role for any particular lipids in viral or cellular fusion events.

6.4. Cellular Fusion Proteins

To date, viral spike proteins are the best understood fusion proteins and have significantly advanced both our general knowledge of fusion and our specialized understanding of the mechanisms by which changes in a protein's conformation lead to membrane fusion. All cells carry out fusion reactions as part of intracellular vesicular traffic during endocytosis and exocytosis (Hernandez *et al.,* 1996; Stegmann *et al.,* 1989a; Rothman and Warren, 1994). Many cells also fuse with other cells during a variety of developmental processes (Hernandez et *al.,* 1996; Stegmann *et al.,* 1989a; Podbilewicz and White, 1994). These cellular fusion events are numerous, highly specific, and tightly regulated. In many cases, the identity of the cellular fusion proteins is as yet unknown and the possible involvement of specific lipids is unexplored. Much of this remains to be addressed in future studies, but we will mention here several examples of proteins that may mediate cellular fusion events.

It is clear that most if not all intracellular vesicular fusion events involve proteins termed SNAREs which are found associated with either the vesicle (v-SNAREs) or target cell membrane (t-SNAREs) (Rothman and Warren, 1994; Bennett and Scheller, 1994). Recent structural information on v- and t-SNARE complexes is remarkable in that the structure of the SNARE complex has strong similarities to the extended α-helical coiled-coil believed to be operating in the fusogenic conformation of the influenza HA and other viral fusion proteins of this type (Sutton *et al.,* 1998). Thus these data suggest that viral and cellular fusion proteins may

act via similar mechanisms. While some studies indicate that the formation of the SNARE complex itself may drive membrane fusion (Weber *et al.,* 1998; Chen *et al.,* 1999), others suggest that the actual membrane fusion event does not require the SNARE complex (Ungermann *et al.,* 1998; Tahara *et al.,* 1998). This is an exciting and fast-moving area, and it will be important to determine how the SNARE proteins might be acting in membrane fusion, and whether such fusion events have any specific lipid requirements in the vesicle or target membranes.

During fertilization the sperm must first bind to the egg and then initiate a membrane fusion event so that the genetic material of the male can be deposited into the egg cytoplasm. The binding and fusion steps in this process have similarities to the other membrane fusion events discussed in this review. One of the proteins involved in sperm-egg fusion is the sperm surface protein fertilin, originally termed PH-30 (reviewed in Evans, 1999; Snell and White, 1996). This protein is a heterodimer, composed of a fertilin α and a fertilin β subunit, that may oligomerize into higher order structures. Both subunits are members of a family of proteins termed ADAMs (for A Disintegrin And Metalloprotease), and including other proteins implicated in fusion events, such as meltrin, which plays a role in myoblast fusion (Yagami-Hiromasa *et al.,* 1995). The fertilin α subunit contains a short stretch of residues (89–111) with similarities to and some homology to viral fusion peptides (Blobel *et al.,* 1992). Several studies with synthetic peptides corresponding to this putative fertilin fusion peptide have demonstrated its binding to liposomes and ability to fuse membranes under certain conditions (Martin *et al.,* 1998; Muga *et al.,* 1994; Niidome *et al.,* 1997; Martin and Ruysschaert, 1997). No specific lipid requirements have been observed to date. A number of genetic and biochemical experiments argue for the importance of ADAMs in both sperm-egg and myoblast fusion (Yagami-Hiromasa *et al.,* 1995; Wolfsberg and White, 1996; Cho *et al.,* 1998), and suggest that these interesting molecules play a significant role although it is too soon to conclude that they are themselves acting as fusion proteins.

A second protein implicated in sperm-egg fusion is bindin, a membrane-associated acrosomal sperm protein in sea urchin (Ulrich *et al.,* 1998; Glabe, 1985a; Glabe, 1985b). Bindin has been shown to bind to and fuse liposomes, and has particular affinity for gel phase membrane domains. In particular, bindin-mediated liposome fusion occurs rapidly when sphingomyelin/cholesterol liposomes are used (Glabe, 1985b). A minimal "fusion peptide" region of bindin has been characterized and shown to have fusion activity with sphingomyelin/cholesterol liposomes (Ulrich *et al.,* 1998). Such peptide and isolated protein studies require more extensive verification and experimentation in order to meaningfully interpret the importance of

bindin in fusion, but the lipid dependence of this reaction adds to the potential interest in bindin as a candidate fusion protein.

7. FUTURE DIRECTIONS

It is clear that while numerous lines of evidence point to potential roles for lipids in the function of fusion proteins and membrane budding reactions, in many cases this evidence remains at a very early stage, and much remains to be done to provide convincing proof that protein-lipid interactions are critical. In order to determine a definite role for lipid in a biological fusion reaction, it is clearly important to test the effect of lipid *in vitro* using a system as close to the biological fusion reaction as possible. This includes using the native virus fusion protein and the natural trigger for fusion, whether it be low pH or receptor interactions. The *in vivo* role of lipid in fusion or exit should be tested by using a lipid-modified cell system if this is possible, carefully controlling for the non-specific effects of lipid modification by evaluating cellular functions and by parallel experiments using viruses or proteins that are not lipid dependent. In particular, the interpretation of cell-cell fusion experiments must be carefully done, as this morphological assay can be affected secondarily by substances that do not affect the fusion step per se. Given that complete depletion of a specific lipid may be difficult or impossible, it may be necessary to correlate the amount of lipid depletion with the amount of inhibition observed. It is also important in all of these experimental approaches to determine the dependence of a lipid effect on the structural features of the lipid, and to evaluate any lipid effect in the context of membrane fluidity, charge, and bilayer stalk formation. While no experimental system is perfect and it is important that the role of lipids in fusion and exit be evaluated in spite of such shortcomings, at the least such guidelines provide a critical framework for the interpretation of experimental results.

Within the alphaviruses, both fusion and virus exit have well-defined and substantial lipid requirements. The *in vivo* role of sphingolipid has not been addressed, and it will be important to apply future advances in our ability to modify cellular sphinogolipids to test the importance of sphingolipid *in vivo.* The challenge in the alphavirus field is now to determine the molecular mechanisms by which cholesterol and sphingolipids interact with the viral proteins to exert their effects on the virus life cycle. We need to determine how these two lipids act to promote fusogenic conformational changes, and the individual and synergistic roles of each lipid in this process. How might the spike protein interact with the membrane to “sense” the presence of cholesterol and/or sphingolipid, even prior to the stable inser-

tion of the virus fusion peptide? A key question is the role of specific lipids and target membrane bilayer in the formation of the El homotrimer structure. As part of this, it will be important to determine the potential structural differences in the homotrimer formed in the presence of a fusion-permissive membrane versus a fusion-inactive membrane. Increased structural information on the monomeric and trimeric forms of the El fusion protein and the availability of virus mutants altered in their lipid requirements should point the way to understanding the sites and functions of lipid-protein interaction. Our understanding of the role of cholesterol in virus exit should become more mechanistic with advances in our knowledge of virus and spike protein structures, and more molecular analysis of the virus exit pathway. In addition, it is important for this and other virus systems to understand the relevance of such lipid requirements to infection of various types of cells in tissue culture, and to viral infection and pathogenesis in animal hosts. Ultimately, a better understanding of the function of particular lipids in virus lifecycles should enable us to design specific strategies to interfere with their function and thus limit virus replication.

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