Chapter 1

General Introduction
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1.1 The Alphaviruses

The Alphavirus genus belongs to the family of the Togaviridae (reviewed in Strauss and Strauss, 1994; Griffin, 2001). The current 25 members of the alphavirus genus are closely related in structure and molecular characteristics but the diseases they cause have a wide variety of clinical manifestations ranging from mild disease, such as acute arthropathy and systemic febrile illness, to fatal encephalitis. For example, the alphavirus Ross River virus generally causes a relatively mild arthritis, whereas Eastern equine encephalitis virus (EEE) and Western equine encephalitis virus (WEE), which not only infect horses but occasionally also humans, may cause an often fatal encephalitis. However, the number of fatalities due to alphavirus infections is low and often alphavirus infections of humans remain subclinical.

Alphaviruses are usually transmitted by mosquitoes, but Sindbis virus (SIN) has also been isolated from mites and ticks and EEE from chicken mites and lice (Strauss and Strauss, 1994; Scott and Weaver, 1989). Once a mosquito has been infected, it becomes chronically and lifelong infected with the alphavirus, without showing significant pathology (DeFoliart et al., 1987). The mosquitoes are an essential element of the arthropod-vertebrate cycle in which the alphaviruses are maintained. The vertebrates in this cycle function as amplifiers for the virus. Common amplifier hosts are birds and small rodents because they are the preferred feeding hosts for the mosquito and the virus is able to induce a significant viremia in these animals. Humans are not a major amplifier host, but rather represent an accidental target for the virus. The frequency of human infections depends on the human activity in the area where the mosquito-vertebrate cycle is manifesting and on the preference of the mosquito to feed on humans (Johnston and Peters, 1996). Therefore, the isolation of EEE from pools of Aedes albopictus in Florida (Mitchell et al., 1992) is worrisome because this mosquito species is capable of efficiently transmitting alphaviruses and prefers to feed on humans among other hosts (Johnston and Peters, 1996). The distribution of the mosquitoes reflects, at least in part, the distribution of the viruses. Birds are postulated to be responsible for the global spread of alphaviruses. This has resulted in a wide geographic distribution to all continents except Antarctica (Strauss and Strauss, 1994). Humans may also have contributed to the global spread of alphaviruses. For example, Ross River virus, an alphavirus primarily found in Australia, was most likely introduced to Fiji by a single infected traveler and caused an explosive epidemic of polyarthritis (Strauss and Strauss, 1994; Marshall and Miles, 1984; Burness et al., 1988; Faragher et al., 1985).

Semliki Forest virus (SFV) and SIN represent prototype members of the alphavirus genus (Strauss and Strauss, 1994; Schlesinger and Schlesinger, 1996). These two viruses are widely used in laboratories to study the life cycle of alphaviruses, in part because of their low pathogenicity in humans. The latter property, together with the fact that alphaviruses grow to high titers in cell culture makes it possible to study these viruses both biochemically and structurally in detail. Site-specific mutagenesis of the viral genome has been made possible by the generation of full-length clones of both viruses (Rice et al., 1987; Liljestrom et al., 1991).
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1.2 Structure and Biosynthesis of Alphaviruses

Structure of the Virion

Alphaviruses are homogeneously sized spherical viruses of about 70 nm in diameter (Strauss and Strauss, 1994; Fuller et al., 1995; Cheng et al., 1995; Paredes et al., 1993). A cryo-EM image reconstruction of SIN is depicted in Figure 1. Each particle contains one copy of a positive-sense RNA genome of about 11.7 kb in size. The RNA molecule is complexed with 240 copies of the capsid protein (~30 kD) together forming the nucleocapsid, the core of the virus. The nucleocapsid has a spherical configuration because of the arrangement of the individual capsid proteins as pentamers and hexamers in icosahedral symmetry (Cheng et al., 1995; Paredes et al., 1993).

A lipid membrane surrounds the nucleocapsid. The membrane of viruses grown in baby hamster kidney (BHK) cells has a phospholipid composition of 25% sphingomyelin (SPM), 27% phosphatidylcholine (PC), 19% phosphatidylserine (PS), and 26% phosphatidylethanolamine (PE) with an asymmetric distribution of the lipids between the two leaflets (van Meer et al., 1981; Allan and Quinn, 1989). In general the phospholipid composition of the viral membrane resembles that of the membrane of the host cell. However, the ratio of cholesterol to phospholipid is 1:1, which is much higher than for cellular membranes (Laine et al., 1973; Renkonen et al., 1971). Virus grown in mosquito cells appear to have a different composition with more ethanolamine-based phospholipids and a cholesterol:phospholipid ratio of 1:6 (Luukonen et al., 1976, 1977).

The spike proteins of the virus are inserted in the lipid bilayer and together they form the viral envelope (Strauss and Strauss, 1994). The virus contains 80 spikes, each spike consisting of two or three subunits, two type I transmembrane glycoproteins E1 and E2 (each about 50kD) which are noncovalently but stably associated, and, in some alphavirus species, a peripheral protein E3 (10kD). The latter subunit is a product of the cleavage of the precursor protein for E2 late in the spike maturation process. E3
remains associated with the SFV spike but is released in the case of SIN (see below; Welsh and Stefton, 1979; Mayne et al., 1984). Like the glycoprotein shell, the nucleocapsid is organized in an icosahedral lattice (Cheng et al., 1995; Paredes et al., 1993). The amino-termini of E1 and E2 face outward from the membrane and a membrane-spanning anchor is located near the carboxy-terminus. In SIN, E2 is 423 amino acids long and has a 33 amino-acid cytoplasmic tail. E1 is 439 residues long and has a very short cytoplasmic tail of 2 amino acids. Both E1 and E2 are glycosylated but the number of oligosaccharide chains varies among alphaviruses from one to two for E1, and two to three for E2. At least for SFV and SIN, both glycoproteins are also palmitoylated in or near the membrane-spanning domains. E1 is acylated with a single palmitic acid chain, while acylation of E2 involves three to six palmitic acid chains (Strauss and Strauss, 1994; Ryan et al., 1998). E1 contains a hydrophobic domain, located from residues 75 to 97, which is conserved among alphaviruses and is the putative fusion peptide of the virus (Garoff et al., 1980; Levy-Mintz and Kielian, 1991). This fusion peptide domain inserts into membranes and thus plays an important role in the membrane fusion process (see Section 1.4). Besides E1, E2 and E3, a small hydrophobic peptide called the 6K protein is present. While 6K is expressed in a stoichiometric fashion with E1, E2, and E3, it is associated with the virus only in low quantities from 7 to 30 molecules per virus particle (Gaedigk-Nitschko and Schlesinger, 1990; Lusa et al., 1991). The function of 6K in the alphavirus life cycle is not fully understood. Site-directed mutagenesis of 6K has demonstrated that the protein is involved in glycoprotein processing and trafficking (Gaedigk-Nitschko and Schlesinger, 1991; Sanz and Carrasco, 2001; Schlesinger et al., 1993), and virus budding (Sanz and Carrasco, 2001; Loewy et al., 1995). A study using chimeric viruses of SIN and Ross River virus, in which SIN 6K was replaced by Ross River virus 6K, severely inhibited virus assembly and budding, and caused a different conformation of E1 in the E1/E2 heterodimer expressed on the cell surface (Yao et al., 1996). Recently, it was shown that 6K from the alphaviruses Ross River virus and Barmah Forest virus form ion channels in planar lipid bilayers (Melton et al., 2002). Because early events in infection do not require the 6K protein (Liljeström et al., 1991; Dick et al., 1996), it was proposed that it is unlikely that the ion channel activity is involved in membrane fusion but that the activity would be located in the ER, Golgi, TGN, or plasma membrane.

Structurally, each spike is composed of a trimer of three hetero-trimers (E1/E2/E3), or hetero-dimers (E1/E2). Thus to build 80 spikes, 240 copies of each subunit are required (Strauss and Strauss, 1994; Kielian 1995). Recent crystallographic and cryo-EM studies have shown that the folding of the E1 glycoprotein of SFV resembles that of the flavivirus E protein (Lescar et al., 2001). The latter study showed that SFV E1 can be divided into three domains, a β-barrel domain at the amino-terminus, a finger-like projecting domain containing the putative fusion peptide, and an Ig-like domain at the carboxy-terminus (Figure 2A). The amino-terminal domain is, in fact, located in the center of E1 and is flanked by the projecting domain and the carboxy-terminal domain. The above study together with a study of SIN glycosylation mutants (Pletnev et al., 2001) localized E1 to lie almost parallel to the viral surface with E2 forming the protruding densities of the viral spike (Figure 2B). An earlier study had localized E3 midway between the center and the tips of the spike of a mutant SIN virus containing E3 (Paredes et al., 1998).
Figure 2. Structure of SFV E1 and positioning of the protein on the virus surface at neutral pH. A, 3D diagram of the folded structure of SFV E1. Below the structure, a schematic diagram is given of the location of the three domains and the fusion peptide in E1. B, the positioning of E1 and E2 proteins in a cryo-EM reconstruction of the viral spike viewed from the side. For clarity, the three domains of the E1 protein in the front are outlined with white solid lines. The visible domains of a second E1 protein in the back are outlined with white dashed lines. Black arrows indicate the outer and inner leaflets of the viral membrane. (Structure of E1 and cryo-EM reconstruction are taken from Lescar et al., 2001, with permission from Dr. F. A. Rey and Elsevier.)
RNA Replication and Protein Synthesis

Once inside the cell, the positive-sense RNA of alphaviruses directly serves as the messenger RNA for protein synthesis and as a template for the generation of minus-sense RNA strands. A schematic overview of the genome organization is shown in Figure 3. The genomic RNA is translated to a polyprotein, p1234, which is autoproteolytically cleaved, by function of nsP2, into the nonstructural proteins nsP1, nsP2, nsP3, and nsP4 (Ding and Schlesinger, 1989; Hardy and Strauss, 1989; Strauss et al., 1992). These nonstructural proteins are responsible for the replication of the plus-sense genomic RNA (Schlesinger and Schlesinger, 1996). Like all RNA viruses, SFV and SIN have a low fidelity of viral RNA replication and lack error-correcting mechanisms, causing an error frequency of about 1 base substitution in 10,000 bases, which corresponds roughly with 1 error per alphavirus genome (Strauss et al., 1996). Generally, the substitutions will go unnoticed. However, when a substitution confers a selective advantage to the virus mutant over the parental virus, the mutant will outgrow the parental virus and become the predominant species (Hahn et al., 1987). For example, selective pressure on the parental virus by antibodies, antiviral compounds, or limited quantities of the cell surface receptor will inhibit the growth of the parental virus and will give rise to escape mutants which are not...
susceptible to the antibodies or antiviral compounds or use other abundantly present receptors.

The minus-sense RNA molecule serves as a template for the synthesis of 26S subgenomic mRNA. The viral structural proteins are translated from this 26S subgenomic mRNA as a polyprotein consisting of capsid-P62-6K-E1. The aminoterminal region of the polyprotein contains a serine protease, which autoproteolytically cleaves the capsid protein from the polyprotein (Barth et al., 1992; Liljestrom and Garoff, 1991). The cleavage takes place in the cytosol of the host cell, revealing a signal sequence on the remaining polyprotein which results in the cotranslational translocation of the polyprotein to the lumen of the rough endoplasmic reticulum (RER) (Garoff et al., 1990). Figure 4 shows a schematic representation of the location of protein synthesis and transport, as well as the assembly of new virions in the host cell. The details will be discussed below. In the RER, carbohydrates are covalently attached to the polyprotein immediately after synthesis (Sefton, 1977) and the protein is further cleaved by signalases into p62 (for SIN this precursor of E2 is usually referred to as PE2), 6K, and E1 (Liljestrom and Garoff, 1991). Folding of the proteins into the native conformation takes place in the RER. The carbohydrate side chains of p62 and E1 are thought to play an important role in protein folding as well as in preventing aggregation and increasing the solubility of the proteins (Gibson et al., 1980; Hsieh et al., 1983). When correctly folded, p62 and E1, originating from the same polyprotein, interact to form a heterodimer (Barth et al., 1995) and are transported to the Golgi complex.

During their journey, after leaving the RER but probably before entering the Golgi complex, the proteins are palmitoylated at cysteine residues (Bonatti et al., 1989; Ryan et al., 1998). The function of this addition of fatty acids to the proteins is currently unknown. Site-specific mutagenesis has shown that deacylation of SIN E1 or E2 slows down virus growth and makes the virus more sensitive to detergent treatment (Ivanova and Schlesinger, 1993; Ryan et al., 1998). However, membrane fusion of SIN was not affected by deacylation of the glycoproteins E1 and E2 (Smit et al., 2001a). After transport through the Golgi complex the proteins are transported via the transGolgi network (TGN) to the plasma membrane.

The heterodimers appear at the plasma membrane already oligomerized into trimers (Ekstrom et al., 1994). Where this oligomerization takes place is unknown. Just before arrival at the plasma membrane, p62 is cleaved by a furin-like host protease into E2 and E3 (Mayne et al., 1984; Klenk and Garten, 1994). The uncleaved p62 presumably functions as a chaperone for protection of the viral spike from premature destabilization by the slightly acidic lumen of the TGN. Low pH is the trigger for spike rearrangement and membrane fusion (see section 1.4). The p62-E1 heterodimers are quite stable and more resistant to low pH than E2-E1 heterodimers (Wahlberg et al., 1989). SFV and SIN mutants impaired in P62 (or PE2, for SIN) cleavage are also impaired in spike rearrangement and membrane fusion when exposed to the mildly acidic pH which triggers spike rearrangement and fusion in the wild-type viruses. However, exposure to very low pH values overrides the impairments resulting in spike rearrangement and fusion (Glomb-Reinmund and Kielian, 1998a; Smit et al., 2001b).
Assembly and Budding

The assembly of the nucleocapsids from capsid proteins and viral genomic RNA occurs in the cytosol (Brown and Smith, 1975; Strauss et al., 1977; see Figure 4). Packaging of viral RNA involves an encapsidation signal only present on the genomic RNA and thereby selecting the genomic RNA out of the RNA pool in the host cell cytosol (Weiss et al., 1989). The encapsidation signal lies within the nsP1 gene for SIN, while for SFV the encapsidation signal has been proposed to be located in the nsP2 gene (Lehtovaara et al., 1981, 1982). A region of 32 amino acids (amino acids 76 to 107) of the capsid protein binds to the encapsidation signal on the RNA (Geigenmüller-Gnirke et al., 1993) and a stretch of 18 amino acid residues in the amino-terminal region has also been proposed to be of importance for nucleocapsid formation (Perea et al., 2001). The binding of one capsid protein initiates the binding of more capsid proteins by capsid-capsid and, presumably, non-specific but charge-related, capsid-RNA interactions resulting in a T=4 icosahedral nucleocapsid particle (Strauss and Strauss, 1994; Garoff et al., 1994).

The nucleocapsids are believed to diffuse freely to the site where the glycoproteins have accumulated, which is the plasma membrane in vertebrate cells.
There, the nucleocapsids bind to the glycoproteins, resulting in a bending of the glycoprotein-containing membrane around the nucleocapsid until the whole particle is surrounded by the membrane and finally buds off from the plasma membrane (Strauss and Strauss, 1994; Schlesinger and Schlesinger, 1996). Budding of alphaviruses requires the presence of both capsid proteins and the glycoproteins (Suomalainen et al., 1992; Zhao and Garoff, 1992). The capsid proteins do not need to be pre-assembled into nucleocapsids for virus budding to occur (Forsell et al., 1996), although pre-assembly into nucleocapsids occurs in wild-type virus budding. The budding process at the plasma membrane has been found to involve several protein-protein interactions. The interactions take place between the capsid protein and tyrosine and leucine residues in a conserved region of the E2 cytoplasmic tail (Owen and Kuhn, 1997; Zhao et al., 1994). Aromatic residues on the capsid protein of SFV and SIN are able to interact with these tyrosine and the leucine residues of E2 (Skoging et al., 1996; Lee et al., 1996; Owen and Kuhn, 1997; Hernandez et al., 2000; Zhao et al., 1994). Another conserved region of the E2 cytoplasmic tail flanks the tyrosine-leucine region and contains palmitoylated cysteine residues. This region is also involved in budding, since mutations disrupting palmitoylation appear to affect virus budding (Gaedigk-Nitschko and Schlesinger, 1991; Ivanova and Schlesinger, 1993).

The interactions between the cytoplasmic carboxy-terminal tail of E2 and the nucleocapsids occurring in virus budding have long been thought to drive virus budding (Cheng et al., 1995; Garoff et al., 1994; Lee and Brown, 1994). However, interactions between E2 and E1 have also been shown to be involved in virus budding in that E1 appeared to be required for the interaction of E2 with the nucleocapsid (Barth and Garoff, 1997). Recently, it has become clear that lateral spike-spike interactions are critically involved in alphavirus budding. It has even been proposed that the role of E2-nucleocapsid interactions is only to trigger the spikes to interact laterally with each other, because the lateral interactions were found to be required for virus budding as virus was formed with an SFV mutant unable to assemble nucleocapsids (Forsell et al., 2000). Spike-spike interactions are now proposed to be responsible for the viral envelope formation (Garoff and Cheng, 2001).

An absolute requirement for Chol in efficient budding of wild type SFV and SIN was demonstrated by Kielian and co-workers (Lu et al., 1999; Lu and Kielian, 2000; Marquardt et al., 1993; Vashishtha et al., 1998). However, the precise role of Chol in virus exit remains to be elucidated.

### 1.3 Cell Entry of Alphaviruses

**Receptor Interaction**

The first step in cell entry of all viruses is the interaction with a receptor on the surface of the target cell. The receptors on the cell surface generally represent molecules that are used by the cell for other purposes, and viruses take advantage of their presence. Since SFV, SIN, and other alphaviruses have many hosts, ranging from mammals to insects, they are able to infect many different cell types. This suggests that alphaviruses use a variety of different surface molecules or one ubiquitous molecule
(Schlesinger and Schlesinger, 1996). For SFV, the major histocompatibility antigens were found to act as receptors (Helenius et al., 1978), but their presence on the cell surface is not absolutely required for infection (Oldstone et al., 1980). For SIN, the high-affinity laminin receptor has been recognized as a receptor (Wang et al., 1992). This receptor is highly conserved on mammalian cells and a homolog is present on mosquito cells as shown by partial inhibition of SIN binding to these cells by an antibody against the high-affinity laminin receptor (Wang et al., 1992). Studies using anti-idiotypic antibodies against E2-specific antibodies revealed that SIN infection of chicken cells is not mediated by the high-affinity laminin receptor but by a 63 kD protein (Wang et al., 1991). A 74 kD protein and 110 kD protein acted as SIN receptors on mouse neuroblastoma cells (Ubol and Griffin, 1991). It is concluded that alphaviruses in general use multiple receptors on host cells.

There is strong evidence to indicate that the E2 glycoprotein of alphaviruses is responsible for receptor binding. This is based on studies involving anti-idiotypic antibodies, which were directed against SIN E2-specific antibodies, as discussed above, and studies showing that mutations in E2 altered the virus binding efficiency to cells (Dubuisson and Rice, 1993; Salminen et al., 1992; Tucker and Griffin, 1991). A study using Fab fragments from monoclonal antibodies, which were implicated to be directed against the cell receptor recognition site on E2 of SIN and Ross River virus, demonstrated that the Fab fragments bound to the outermost tips of the viral spike densities (Smith et al., 1995). Recently it was shown that SIN and other alphaviruses utilize cell-surface heparan-sulfate (HS) as an attachment receptor as a consequence of cell-culture adaptation (Bernard et al., 2000; Heil et al., 2001; Klimstra et al., 1998). For SIN, it was found that the E2 subunit was mutated during the adaptation to growth in BHK cells and three loci in E2 were identified as responsible mutation sites, namely, E2:1; E2:70, and E2:114 (Klimstra et al., 1998). The cell-culture adaptation occurred very rapidly, already after 2 passages. As mentioned in Section 1.2, this rapid adaptation to the environmental changes arises from the low fidelity of viral RNA replication and results in the generation of mutants that outgrow the non-adapted parental virus. Selective pressure most probably has played a role in the establishment of the wide host range of alphaviruses. The characteristics of HS-adapted SIN variants and an SFV strain, passaged frequently over BHK cells, are presented in Chapter 5. In Chapter 6, HS-adapted SIN and SFV strains are utilized as tools to study the antiviral effect of human lactoferrin (hLF).

Receptor-Mediated Endocytosis

In the early 1980s, Helenius and coworkers demonstrated that after receptor binding, SFV is internalized by receptor-mediated endocytosis (RME) and is delivered intact into endosomes (Helenius et al., 1980; Marsh and Helenius, 1980; Marsh et al., 1983). Figure 5 shows a schematic representation of the entry of SFV. This pioneering work also revealed that productive infection was dependent on the acidic lumen of the endosomes. The authors demonstrated that lysosomotropic weak bases and carboxylic ionophores that neutralize acidic organelles, such as endosomes and lysosomes, inhibit SFV infection and that virions fuse with the plasma membrane by brief exposure to acidic medium (Helenius et al., 1980 and 1982; Marsh et al., 1982; White et al., 1980).
Figure 5. Cell entry of SFV via receptor-mediated endocytosis. See text for details.

RME involves the formation of clathrin-coated pits, in which the receptor and its ligand, or a virus, are recruited and eventually internalized. Clathrin is a trimeric scaffold protein, which organizes itself into cagelike lattices. In RME, the formation of a clathrin lattice on the cytosolic side of the plasma membrane captures a section of the plasma membrane into a clathrin-coated vesicle (Kirchhausen, 1993). The clathrin coat curves the membrane into a vesicle which eventually buds off into the cytosol by membrane fission mediated by ATPase and GTPase (Schmid and Smythe, 1991; Carter et al., 1993). The detached clathrin-coated vesicle becomes uncoated by an uncoating ATPase, and the clathrin molecules are recycled (Schlossman et al., 1984; Greene and Eisenberg, 1990). The uncoated vesicle then fuses with an early endosome (Schmid et al., 1989).

The lumen of early endosomes is mildly acidic because ATP-dependent proton pumps are present in the endosomal membrane (Mellman et al., 1986). Early or peripheral endosomes have a pH between 6.2 and 5.3, and late, perinuclear endosomes have a pH of 5.3 or lower (Tanasugarn et al., 1984; Schmid et al., 1989). The low pH is a trigger for conformational changes within the viral spike protein, which eventually
leads to fusion of the viral envelope with the endosomal membrane (Figure 5). The
details of this low-pH-triggered fusion process are discussed in Section 1.4.

It has long been thought that SIN enters the cell by a different pathway than SFV.
One line of evidence suggested that SIN enters the cell by direct plasma membrane
fusion (Abell and Brown, 1993; Cassell et al., 1984; Coombs et al., 1981; Edwards and
Brown, 1991; Flynn et al., 1990). The interaction of SIN with its receptor has been
proposed to trigger the conformational changes in the spike protein required for fusion
(Abell and Brown, 1993). However, more recent evidence with cells (Glomb-Reinmund
and Kielian, 1998a, 1998b; DeTulleo and Kirchhausen, 1998) and model membranes
(Smit et al., 1999, 2001b) showed that SIN entry requires budding of clathrin-coated
pits and low pH, both indicative of virus cell entry via RME. It must be mentioned that
penetration into mosquito cells appears not to require low pH (Hernandez et al., 2001).
In Chapter 5, more evidence is presented arguing against the requirement for a receptor
in SIN membrane fusion in agreement with the evidence of SIN fusion with receptor-
free liposomes (Smit et al., 1999).

Nucleocapsid Disassembly

The nucleocapsid gains access to the cytosol of the host cell by fusion of the viral
and the endosomal membrane (Figure 5). Subsequently, the nucleocapsid is
disassembled (Figure 4). The capsid proteins are released into the cytosol, while the
viral RNA remains associated with cellular membranes (Singh and Helenius, 1992),
which are presumably endosomal membranes. An intriguing question is how is it
possible that a nucleocapsid is unstable when it first enters the cytosol of a previously
uninfected cell while newly assembled nucleocapsids are stable later on in the infection
process (Strauss and Strauss, 1994)? Although the answer is not clear, several
possibilities have been put forward. The capsid protein of SFV and SIN was found to
bind to ribosomes (Glanville and Ulmanen, 1976; Wengler and Wengler, 1984;
Wengler et al., 1984, Ulmanen et al., 1976; Ulmanen et al., 1979). In a model proposed
by Wengler and Wengler (Wengler, 1987; Wengler and Wengler, 1984; Wengler et al.,
1984) the ribosome competes with the viral RNA for capsid protein. Upon infection of
the cell, the large excess of unoccupied ribosome-binding sites leads to disassembly of
the nucleocapsid and later, during virus replication, saturation of the ribosomes with
newly synthesized capsid protein leads to a switch from disassembly to assembly of
nucleocapsids. In support of this model, it was found later that the ribosome-binding
site mapped to residues 94 to 105 of the SIN capsid protein (Wengler et al., 1992), and
that this region is also involved in the binding of viral RNA during nucleocapsid
assembly (Geigenmüller-Gnirke et al., 1993). Although confirming several aspects of
the above model, Singh and Helenius (1992) also found that ribosomes in infected cells
were not saturated with capsid protein and were still able to disassemble nucleocapsids
in vitro. As the above model was not sufficient, other proposals have been made. The
first is a possible maturation event of nucleocapsids, based on the observed difference
in size between newly synthesized nucleocapsids and mature nucleocapsids in virions,
which would trigger disassembly in the cytosol (Strauss and Strauss, 1994). The second
is that during infection changes in Na\(^+\) and K\(^+\) concentrations in the cytosol stabilize the
nucleocapsids (Strauss and Strauss, 1994). The third is that exposure of the virus to low
pH in the endosome leads to the formation of ion channels in the virus allowing the
entry of protons inside the virus, which facilitates nucleocapsid uncoating (Lanzrein, 1993).

1.4 Alphavirus Membrane Fusion Activity

Low-pH Trigger of Fusion

As mentioned in Section 1.3, early work of Helenius and coworkers demonstrated that SFV membrane fusion can be induced by a low-pH trigger (White et al., 1980) and that infection of cells by SFV is dependent on acidic endosomes (Helenius et al., 1980, 1982; Marsh et al., 1982). Others have confirmed this low-pH dependence of SFV fusion in cells (Izurun et al., 1997; Glomb-Reinmund and Kielian, 1998a, 1998b). In addition to the cellular assays, the low-pH requirement became evident in virus-liposome systems (White and Helenius, 1980; Wahlberg et al., 1992; Bron et al., 1993). The pH dependence for SFV fusion with liposomes, with a pH threshold for fusion activation of pH 6.2 and optimal fusion at pH 5.5 (Bron et al., 1993), was shown to closely correlate with the in vivo pH dependence of fusion (Glomb-Reinmund and Kielian, 1998a, 1998b). This pH dependence suggests that the alphaviruses fuse from within early endosomes, since the lumen of early endosomes is mildly acidic (having a pH between 6.2 and 5.3, see above). Studies involving liposomes as target membranes revealed, additional to the pH-dependence, that SFV and SIN fusion was receptor independent, rapid, and efficient (White and Helenius, 1980; Wahlberg et al., 1992; Bron et al., 1993; Smit et al., 1999).

Low pH triggers conformational changes in the viral spike protein. The current understanding in the literature of the conformational changes and their relationship to fusion is discussed below (for a recent review see Kielian et al., 2000). For a hypothetical model of the fusion process, including a proposed sequence of conformational changes based on recent data presented in this thesis, see Chapter 7.

Conformational Changes of the Viral Spike

The first spike rearrangement occurring after the low-pH trigger is the dissociation of the E2/E1 heterodimer. E2 and E1 are no longer co-immunoprecipitated or do not co-float on sucrose gradients (Wahlberg et al., 1992; Wahlberg and Garoff, 1992; Bron et al., 1993; Justman et al., 1993; Ferlenghi et al., 1998). This dissociation is important for fusion as shown by SFV and SIN cleavage mutants, which have a more acidic threshold for dissociation, resulting in a more acidic threshold for fusion (Glomb-Reinmund and Kielian, 1998a; Smit et al., 2001b).

After dissociation, further rearrangements occur independently of E2. E1 regions, masked at neutral pH, are exposed and become accessible to monoclonal antibodies (mAbs) (Schmaljohn et al., 1983, Ahn et al., 1999). One acid-specific epitope was mapped to E1:157 (Ahn et al., 1999). Most probably, after the exposure of this epitope, E1 monomers rearrange into a homotrimeric configuration (Wahlberg et al., 1992; Wahlberg and Garoff, 1992). The E1 homotrimer is very stable. It is resistant to SDS at 30°C, urea, and trypsin digestion and its formation is strictly dependent on low pH and
could not be induced by heat or urea (Wahlberg et al., 1992; Gibbons et al., 2000; Meyer et al., 1992).

The E1 homotrimer was shown to be involved in membrane fusion of SFV (Wahlberg et al., 1992). However, it is not clear if the E1 homotrimer is formed before or after membrane binding. Kinetic analysis of virus-liposome binding, fusion, epitope exposure and homotrimer formation suggested that both epitope exposure and homotrimer formation occur slightly before membrane binding and fusion (Bron et al., 1993). However, inhibition of fusion by Zn\(^{2+}\) also inhibited homotrimer formation but permitted heterodimer dissociation and virus binding to liposomes (Corver et al., 1997). In addition, an SFV mutant with a mutation in the fusion peptide, E1:Gly91Asp, showed similar results (Kielian et al., 1996). Based on new results we propose that E1 homotrimer formation and stable membrane binding are separate processes, and both are required to facilitate membrane fusion (Chapter 7).

Very recently, a cryo-EM and crystallization study of the low pH and membrane-inserted conformation of a homotrimer of E1 ectodomains revealed that, compared to the neutral form of the E1 ectodomain, the Ig-like domain (domain III) moves 37 Å toward the fusion loop and interacts with the finger-like domain (domain II), which redirects the polypeptide chain so that its C-terminus points toward the fusion loop at the tip of domain II (Gibbons et al., 2004). At neutral pH, domains II and III lie in a region of extended conformation (Gibbons et al., 2004; Lescar et al., 2001). The homotrimer is essentially formed by the central interactions between the bottom β-sheets (domain I) of three E1 subunits, and is continued in domain II interacting with the proximal half of domain I of an adjacent E1 subunit. The tips of domain II, the fusion loops, do not display intra-trimer contacts, but are involved in inter-trimer contacts (Gibbons et al., 2004).

The Fusion Process

Membrane fusion is the key event in the entry process of enveloped viruses. Fusion delivers the viral genome to the cytosol of the host cell. Membrane fusion is facilitated by viral surface proteins which are triggered either by receptor interaction or by low pH. Influenza hemaglutinin (HA) is the best studied viral fusion protein thusfar and is the prototype of a class of viral fusion proteins that are structurally similar (reviewed in Skehel and Wiley, 2000 and Colman and Lawrence, 2003). Examples of other viruses having viral fusion proteins similar to influenza are measles virus, human immunodeficiency virus (HIV), and Ebola virus. The fusion proteins of this class are all synthesized as a single-chain precursor, which form trimers. The precursor proteins are cleaved to generate a new amino-terminal region that contains a hydrophobic sequence denoted as the fusion peptide (Wilson et al., 1981). A three-chain, α-helical, coiled-coil, which is assembled upon the trigger for fusion, is responsible for projecting the fusion peptide toward the target membrane and is the main structural element of the fusion machinery of the fusion protein (Bullough et al., 1994; Baker et al., 1999; Chen et al., 1999; Melikyan et al., 2000; Russell et al., 2001).

The fusion protein of SFV has been shown to be structurally different from the above mentioned class of fusion proteins (Lescar et al., 2001; see Section 1.2). The alphavirus fusion protein is similar to the fusion protein of flaviviruses, such as tick borne encephalitis virus and dengue virus (Rey et al., 1995; Modis et al., 2003). The
fusion protein of alpha- and flaviviruses does not form α-helical coiled-coils and associates with a second “protector” protein, which is E2 in alphaviruses. The structural difference of the fusion proteins of these viruses with the fusion protein of influenza has led to the classification of the influenza-like fusion proteins as class I fusion proteins and the alpha- and flavivirus fusion proteins as class II fusion proteins (Lescar et al., 2001).

The study of Gibbons et al. (2004) and two other very recent studies on the fusion proteins of flaviviruses dengue virus (DEN) (Modis et al., 2004) and tick borne encephalitis virus (TBE) (Bressanelli et al., 2004) suggested that alpha- and flaviviruses, having class II fusion proteins, fuse with their target membrane in a roughly similar fashion as the viruses with class I fusion proteins. For influenza, the fusion proteins are already arranged in trimers at neutral pH, whereas for alphaviruses, as mentioned above, trimers are involved in the fusion process (although it is not exactly clear when they are formed). The suggested mechanistic similarities between both fusion protein classes give rise to the following simplified model for membrane fusion of alphaviruses, based on the model for influenza (reviewed in Skehel and Wiley, 2000 and Colman and Lawrence, 2003) and the above mentioned recent studies on the alpha- and flavivirus fusion proteins. Figure 6 shows a cartoon of this model, as viewed from the side, with the E1 subunits positioned as in Figure 2B. At neutral pH, the projecting domains of the E2 subunits are proposed to interact with domain II of E1 subunits (Figure 6a, light gray domain). The transmembrane region of a E2 subunit is proposed to interact with the transmembrane region of another E1 subunit (Lescar et al., 2001), which is not shown for clarity. Upon the low-pH trigger, the E2/E1 heterodimer dissociates (Wahlberg and Garoff, 1992) and E2 moves away, unmasking the fusion peptide at the tip of the finger-like domain II. The mechanism of the E2 movement is not clear, but it is proposed to involve a reciprocal movement of the E1 and E2 domains in the protein shell region (Haag et al., 2002). E1 most probably moves from its parallel position to the viral membrane to a more upright position, allowing the fusion protein to bind to the opposing target membrane via its fusion peptide (Figure 6b). After heterodimer dissociation E1 forms homotrimers (Wahlberg et al., 1992; see Figure 6c).

Evidence suggests that this homotrimerization occurs after membrane binding (Corver et al., 1997; Kielian et al., 1996), but binding and trimerization can also be independent processes (see Chapter 7). A group of five trimers forming a “volcano-like” structure has been proposed to bring the two opposed membranes together (Gibbons et al., 2003 and 2004): for clarity only two are shown in Figure 6. The subunits of the trimers fold back like a jackknife into a post-fusion hairpin conformation (Figure 6d-f). In this conformation the fusion peptides and C-terminal transmembrane regions of the trimer subunits are located at the same end of the structure (Gibbons et al., 2004). The hairpin formation drives fusion as it forces the target membrane in close proximity of the viral membrane, resulting in merging of the two membranes and creating an aqueous fusion pore.
1.5 The Involvement of Sphingolipids and Cholesterol in Alphavirus Membrane Fusion

Alphaviruses are dependent on the presence of specific lipid components in the target membrane for fusion to occur. In liposomal model systems it has been established that SFV fusion requires the presence of cholesterol (White and Helenius, 1980; Kielian and Helenius, 1984; Bron et al., 1993) and sphingolipids (Nieva et al., 1994; Wilschut et al., 1995; Corver et al., 1995; Moesby et al., 1995). The cholesterol dependence of SFV fusion has also been demonstrated in cells (Phalen and Kielian, 1991). Recently, it was shown that SIN, like SFV, requires the presence of cholesterol and sphingolipids in the target membrane for fusion (Smit et al., 1999). In this section, the features of sphingolipids and cholesterol that are or can be of importance for alphavirus fusion are discussed.

Structure of Sphingomyelin

Sphingolipids, together with glycerophospholipids, are important components of the plasma membranes of eukaryotic cells (Koval and Pagano, 1991; Lange et al., 1989; van Meer and Holthuis, 2000). Moreover, they are essential for eukaryotic life (Hanada
et al., 1992). All of the sphingolipids have the same backbone, which is called “sphingosine.” This term was coined by Johann L. W. Thudichum for the lipid compound he isolated from brain tissue. Because of its enigmatic chemical properties (the molecule contains both amine and alcohol groups, but is insoluble in water) he named the compound after the Sphinx, who posed riddles in Greek mythology (Thudichum, 1884). In 1927, the structure of SPM was revealed to be N-acylsphingosine-1-phosphorylcholine (Pick and Bielschowsky, 1927). The molecule consists of a long-chain (typically C18) sphingoid base, sphingosine, containing a double bond between C4 and C5, an amide-linked acyl chain which is generally 16 to 24 carbon atoms long, and the hydrophilic phosphorylcholine head group (Figure 7). The configuration of the sphingoid base in natural SPMs is always D-erythro (2S,3R) (Shapiro and Flowers, 1962). Without the phosphorylcholine head group, sphingosine together with the amide-linked acyl chain, form the precursor lipid ceramide, which in cells is used for the biosynthesis of SPM and glycosphingolipids (Merril and Jones, 1990) and also is a well-known lipid second messenger that mediates a wide range of cellular responses to external stimuli. Ceramide has been shown to have the minimally required molecular characteristics of a sphingolipid in order to promote SFV fusion, as sphingosine alone did not support fusion (Nieva et al., 1994; see below).

The structure of SPM has three features that are unique to sphingolipids. These are the 3-hydroxyl group, the 4,5-trans double bond and the amide group at carbon 2 which links the fatty acyl-chain to the sphingoid base. In contrast to other phospholipids such as PC, SPM has both hydrogen-bond donating and accepting groups, whereas PC only has hydrogen-bond accepting groups. These features, together with the predominantly long and saturated acyl chain (Barenholz and Thompson, 1980), give SPM unique properties in cell membranes (reviewed by Ramstedt and Slotte, 2002). SPM is able to form both intra- and intermolecular hydrogen bonds. Intramolecular hydrogen bonding between the 3-hydroxyl group and the phosphate ester oxygen has been reported recently and is unique for the D-erythro enantiomeric configuration of SPM (Talbott et al., 2000). The intra- and intermolecular hydrogen bonding seems to be influenced by the 4,5-trans double bond because without the double bond, as in membranes with dihydrosphingomyelin (dihydro-SPM), intramolecular hydrogen bonds were weaker, but intermolecular bonds, as with cholesterol, were stronger (Talbott et al., 2000; Ferguson-Yankey, et al., 2000; Kuikka et al., 2001). The different hydrogen bonding behavior between SPM and dihydro-SPM could be of importance for SFV fusion as 4,5-dihydroceramide was not able to promote SFV fusion with liposomes (Corver et al., 1995; see below).

Structure of Cholesterol

Like sphingolipids, cholesterol is indispensable for eukaryotic life as it is required for cell viability and proliferation. In membranes of mammalian cells, the only sterol present is cholesterol. Cholesterol is mainly localized in the plasma membrane. In membranes, cholesterol interacts with phospholipids and influences membrane permeability, phospholipid acyl-chain mobility, rotational ordering, and lateral packing density (Bittman, 1997; Brown, 1998; Slotte, 1999; Ohvo-Rekilä et al., 2002).

Cholesterol (cholest-5-en-3β-ol) has a tetracyclic hydrocarbon ring skeleton in which the rings are fused together in the trans configuration resulting in a planar and
rigid molecule. It contains a hydroxyl group at carbon 3, a double bond between carbons 5 and 6, an iso-octyl hydrocarbon side chain attached to carbon 17, and methyl groups at carbon 18 and 19 (Figure 7). The hydrocarbon side chain, as opposed to the ring structure, is rather flexible and the hydroxyl group gives the otherwise hydrophobic molecule an amphiphilic character. Therefore, cholesterol is located in membranes with its hydroxyl group facing toward the aqueous interface. Furthermore, the three-dimensional structure has the β-configuration, which means that the hydroxyl group, the side chain, and the two methyl groups are all on the same side of the molecule (Bittman, 1997; Brown, 1998; Ohvo-Rekilä et al., 2002).

Figure 7. The chemical structure of sphingomyelin and cholesterol.
Interactions between Sphingomyelin and Cholesterol in Membranes

In membranes cholesterol appears to interact more favorably with SPM than with other naturally occurring phospholipids, probably because of the hydrogen-bond donating and accepting capacity of SPM and the enhanced attractive van der Waals interactions arising from the generally long saturated N-linked acyl chain and the stretched configuration of the sphingoid base. Glycerolipids only have hydrogen bond accepting groups and most natural glycerolipids have predominantly polyunsaturation with cis-double bonds; therefore, they are rich in kinked acyl chains, which will interfere with close molecular packing with cholesterol (Bittman, 1997; Ohvo-Rekilä et al., 2002; Slotte, 1999; Ramstedt and Slotte, 2002; Rietveld and Simons, 1998). It was concluded from model membrane studies that the position of the cis unsaturation greatly influences the ability of lipids to interact with cholesterol (McIntosh et al., 1992; Smaby et al., 1994). A cis double bond within the first 10 carbon atoms of the fatty acyl chain of a phospholipid interferes significantly with the interaction with cholesterol. In Chapter 2, this background is used to deliberately abolish SPM-cholesterol interactions required for the formation of lipid rafts, as discussed in more detail below. Thus, long saturated acyl chains are favorable for the interaction of a phospholipid with cholesterol. The saturated fatty acyl chains align with the planar steroid ring structure and interact with each other by van der Waals interactions (Brown, 1998). McMullen et al. (1993) used differential scanning calorimetry studies with PC/cholesterol mixtures to show that the hydrophobic length of cholesterol is equivalent to the length of a PC molecule with a 17-carbon saturated chain. Another study showed that the rate of cholesterol oxidation is lowest in PC monolayers having fatty acyl chains between 14 and 17 carbon atoms in length, suggesting that cholesterol interacts more favorably with PC molecules having chain lengths between 14 and 17 carbon atoms than with PC molecules having longer or shorter chains (Mattjus et al., 1994). However, the interaction of SPM with cholesterol has been shown to be less sensitive to the hydrophobic length of the N-acyl chain; also, the interaction of cholesterol with SPM was stronger than with acyl chain matched PCs (Ramstedt and Slotte, 1999). The authors conclude that SPM possibly forms hydrogen bonds with cholesterol in addition to the van der Waals forces between the two molecules. The hydrogen bond could be formed between the hydroxyl group of cholesterol and the amide group of SPM. Indeed, the amide linkage of SPM may be mainly responsible for the intermolecular hydrogen bonding of SPM with cholesterol. The 3-hydroxyl group of SPM was not critical for the SPM-cholesterol interaction since 3-deoxy-SPM interacts with cholesterol. However, replacement of the amide group weakened the SPM-cholesterol interaction significantly (Kan et al., 1991; Gronberg et al, 1991; Bittman et al., 1994).

Since the 3-hydroxyl group of SPM was important for SFV fusion with liposomes (Corver et al., 1995), it can be suggested that SPM-cholesterol hydrogen bonding behavior does not play an essential role in SFV fusion, although the importance of the amide group of SPM for SFV fusion has not yet been investigated.

Sphingolipid-Cholesterol Lipid Domains in the Plasma Membrane: “Rafts”

During the last decade, extensive evidence has become available for the existence of SPM-cholesterol microdomains or “rafts” in biological membranes. The raft concept was proposed by Simons and Ikonen (1997). In this model rafts are
(glyco)sphingolipid/cholesterol microdomains floating in a glycerolipid-rich environment. A schematic representation of a raft is depicted in Figure 8. Because of their ordered lipid packing, rafts are thought to recruit proteins that favor such a packing environment. For example, glycosylphosphatidylinositol (GPI)-anchored proteins localize in rafts. This feature of rafts makes them important players in a myriad of cellular functions such as protein sorting events and cell signalling cascades. The formation of lipid rafts is most likely driven by lipid-lipid interactions between saturated lipids, which are greatly facilitated by cholesterol for the reasons discussed in the previous section (for reviews, see Harder and Simons, 1997; Brown and London, 1998a; Rietveld and Simons, 1998; Brown and London, 2000). The lipids in rafts are probably in the liquid-ordered phase \( (L_0) \) phase, which is characterized by tight acyl chain packing but high lateral mobility (Brown and London, 1998b). This phase lies between the gel phase, in which the acyl chains are highly ordered but there is no lateral movement, and the liquid-disordered phase, in which there is high lateral movement and little ordering. Although clustering of sphingolipids in model membranes also occurs without cholesterol, the formation of the \( L_0 \) phase requires the presence of cholesterol. The tight packing of lipids in the \( L_0 \) phase apparently makes them detergent-insoluble in Triton X-100, a non-ionic detergent, in the cold. Because lipids in the fluid state are soluble in Triton X-100 the \( L_0 \) phase lipids can be isolated as detergent-resistant membranes (DRMs) from cells (Brown and Rose, 1992; Schroeder et al., 1994). The DRMs isolated from cells are thought to be derived from rafts because they are rich in cholesterol and sphingolipids and contain GPI-anchored proteins. Therefore, the DRM extraction method is widely used to study rafts, although it is recognized to be less than an ideal assay of raft formation. The use of Triton X-100 in the cold to study rafts, however, raised the possibility that rafts are an artifact of temperature or the detergent use. To date, however, there is extensive evidence for the existence of sphingolipid-cholesterol domains in the absence of detergent and at physiological temperatures in both model and cell membranes (Pralle et al., 2000; Samsonov et al., 2001; Xu and London, 2000; Xu et al., 2001; Rinia et al., 2001; Dietrich et al., 2001).

Figure 8. Schematic representation of a sphingomyelin-cholesterol raft.
The Role of Sphingolipids and Cholesterol in Alphavirus Fusion

Specific molecular characteristics of cholesterol and sphingolipids were found to have an influence on SFV fusion. The 3-hydroxylation group and the 4,5-trans double bond need to be present in the sphingolipid in order for the fusion of SFV with liposomes to take place (Corver et al., 1995). Furthermore, a 5,6-trans double bond in the sphingoid backbone did not replace the 4,5-trans double bond in its function to support SFV fusion (He et al., 1999). SFV fusion also been shown to be strictly dependent on the presence of the D-erythro stereoisomer of sphingolipid (Moesby et al., 1995). Ceramide possesses the minimally required structural characteristics of a sphingolipid for promoting SFV fusion (Nieva et al., 1994). The length of the N-acyl chain was not important for SFV fusion since N-stearoylceramide and N-octanoylceramide were equally active (Corver et al., 1995; Moesby et al., 1995). Recently, it was demonstrated that N-acetylcylceramide also promoted SFV fusion (Samsonov et al., 2002).

The only molecular feature of cholesterol that is strictly required for SFV fusion is the 3β-hydroxylation group. It has been demonstrated that sterols with a modified 3β-hydroxyl group, such as epicholesterol, cholestan-3-one, 5α-cholestan, cholesterol methyl ether, cholesteryl acetate, and 3-chlorocholestene, are inactive in fusion (Kielian and Helenius, 1984, Kielian et al., 2000). Mutants of SFV, which are relatively cholesterol independent in cell entry and exit, have been isolated by selection for growth on cholesterol-depleted cells (Kielian et al., 2000). These mutants are called srf mutants for their modification in sterol requirement in function. The first and best characterized mutant is srf-3. This mutant has comparable growth kinetics to wild-type SFV on cholesterol-containing BHK or mosquito cells. In contrast, on cholesterol-depleted cells srf-3 has a yield of four to five logs higher than wild-type SFV. Infection and fusion of cholesterol-depleted cells is at least 2 logs higher with srf-3 than with wild-type SFV (Vashishtha et al., 1998). Experiments with liposomes prepared with or without cholesterol showed that epitope exposure, E1 homotrimer formation, and fusion of srf-3 compared to wild-type SFV are less cholesterol-dependent. The pH dependence and sphingolipid dependence for fusion of srf-3 are similar to that of wild-type SFV (Chatterjee et al., 2000). Recently, two other mutants, srf-4 and srf-5, were isolated and shown to have an increased cholesterol-independence for growth, infection, fusion, and exit (Chatterjee et al., 2002). However, unlike the srf-3 mutation, the srf-4 and srf-5 mutations make the virus sphingolipid independent in fusion. The srf mutations all map to domain II of E1. The srf-3 mutation lies within a loop adjacent to and associated with the fusion peptide at the tip and the srf-4 and srf-5 mutations lie more central in domain II. Interestingly, the srf-4 and srf-5 mutations make the homotrimer structure less stable than that of the wild type as demonstrated by SDS sensitivity, suggesting that the mutations affect the function of the homotrimer in fusion by uncoupling its normal sphingolipid dependence (Chatterjee et al., 2002).

Cholesterol has been proposed to mediate the binding of alphaviruses to the target membranes (Nieva et al., 1994). The findings that SFV fusion is dependent on specific molecular features of sphingolipids, and that only a very low concentration of sphingolipid is sufficient to ensure maximal fusion, led to the hypothesis that sphingolipids act as a cofactor in the actual fusion event, possibly mediating additional conformational changes in E1 needed for fusion to occur (Nieva et al., 1994, Corver et al., 1995). Samsonov et al. (2002) proposed that the 3-hydroxyl group and the amide
group of sphingolipids form a hydrogen bond with E1 and that the molecular features of sphingolipids required for fusion, other than the 3-hydroxyl group, participate in constraining the molecule in a position most favorable for establishing the hydrogen bonds with E1.

1.6 Aims and Approaches

Aims of the Research Project Presented in this Thesis

Receptor interaction and membrane fusion are key events in the entry pathway, which is an important part of the alphavirus life cycle. Alphavirus membrane fusion has been shown to be dependent on cholesterol and sphingolipids. Cholesterol has been found to function in virus-membrane binding, and sphingolipids have been suggested to function as a cofactor in the membrane fusion event. However, the exact role of these two lipids in alphavirus membrane fusion remains to be clarified. Therefore, the first aim of the research project described in this thesis is unraveling the role of these two lipids in alphavirus membrane fusion.

The receptor functions as a cell-surface attachment device for the virus. However, it is not clear whether the receptor has more functions in the entry pathway. It has been proposed that the receptor is able to trigger membrane fusion. Determining the role of receptor interaction in alphavirus membrane fusion is the second aim of the research project described in this thesis.

Experimental Approaches

Fusion between viruses and their target membranes has been studied in both cellular and model systems. Virus-cell fusion can be evaluated in an indirect manner by polykaryon formation, hemolysis, or infection (Kielian, 1995). However, to obtain a more detailed insight into the molecular mechanisms involved in the fusion process of alphaviruses, fusion assays using liposomes as artificial target membranes have been developed (White and Helenius, 1980; Kielian and Helenius, 1984; Wahlberg et al., 1992; Bron et al., 1993). Among the advantages of virus-liposome systems over virus-cell systems are a higher sensitivity, the possibility of obtaining quantitative data, and the ability to vary the target membrane lipid composition (Hoekstra and Klappe, 1993; Smit et al., 2003). Alphavirus fusion has been studied in liposomal fusion assays based on (a) content mixing, in which the delivery of internal content of the virus to the aqueous lumen of the liposome is measured, and (b) lipid mixing, in which fluorescent probes are used to monitor the merging of the viral membrane with the liposomal membrane (reviewed by Smit et al., 2003). The latter assay is the most sensitive and provides information about the kinetics and the extent of the fusion reaction.

The lipid mixing assay used in the studies described in this thesis relies on the capacity of a fluorescent probe, pyrenehexadecanoic acid (which is biosynthetically converted into glycerophospholipids), to form excited state dimers, also called excimers (Galla and Sackmann, 1974; Galla and Hartmann, 1980). Excimers consist of a pyrene probe molecule in the excited state and a probe molecule in the ground state. The formation of excimers is dependent on the distance between the probe molecules, and
the emission of pyrene excimers is shifted to longer wavelengths relative to the emission of pyrene monomers. Within membrane, the intensity of excimer fluorescence is proportional to the surface density of the pyrene probe molecules. Fusion of a membrane containing the pyrene probe with a membrane lacking pyrene probe molecules causes the surface density of the pyrene probe to decrease. As a result, there is a decrease in the excimer fluorescence intensity, which is monitored in a continuous fashion (Wahlberg et al., 1992; Bron et al., 1993). For determining fusion of SFV or SIN with liposomes the virus is usually labeled with the pyrene-probe. For this, the probe is added to cells, which are later infected by the virus (Smit et al., 2003). The pyrene-probe becomes incorporated in the plasma membrane of the cells and will be incorporated in the viral membrane of newly synthesized viruses when they bud from the plasma membrane.

The content mixing assay is based on the exposure of the viral nucleocapsid to RNase or trypsin, encapsulated in the liposomal lumen, after membrane fusion. Fusion is observed as a degradation of the radiolabeled RNA or capsid protein (White and Helenius, 1980; Helenius et al., 1980).

Liposomes can be also be used to determine the extent of target membrane binding by the virus (Kielian and Helenius, 1984; Bron et al., 1993; Smit et al., 1999; Smit et al., 2003). Liposomes float to low density on density gradients. Virus binding to liposomes can be measured after mixing of the virus-liposome suspension with high-density sucrose, and subsequently overlaying the mixture with a sucrose-density gradient. Liposome-bound virus is separated from unbound virus by coflotation with the liposomes to low density during ultracentrifugation.

As mentioned above, one of the advantages of the use of model membranes is that the lipid composition can be controlled experimentally. This advantage is exploited in the research described in this thesis to gain insight into the role of sphingolipids in alphavirus fusion. The role of the receptor in the entry of alphaviruses is studied by incorporating a receptor, known to be used by some alphaviruses, into the liposomal membrane. This is a novel tool to study interactions of viruses with this specific receptor, and is another advantage of the use of model membranes in virus research.

1.7 The Scope of this Thesis

The first three research chapters of this thesis describe studies on unraveling the role of target membrane lipids in alphavirus fusion activity. The last two research chapters deal with the virus-receptor interaction.

In Chapter 2, the question as to whether lipid rafts are involved in alphavirus fusion is addressed. The effect of sphingolipid and cholesterol analogs on the degree of microdomain formation in liposomes was investigated. The effect of the presence of these lipid analogs in the liposomes on SFV and SIN membrane fusion was determined and compared with the degree of microdomain formation.

In Chapter 3, conditions are revealed at which sphingolipid-independent fusion of SFV with liposomes occurred. The basis for this surprising sphingolipid-independent fusion was investigated by quantitative measurements of SFV fusion and binding with liposomes of different lipid compositions at different temperatures.
In Chapter 4, the effect of low-pH treatment and subsequent neutralization on SFV infection, membrane binding, membrane fusion, and spike protein conformational changes were investigated. Furthermore, the effect of the presence of liposomes as artificial target membranes during the acidification-neutralization treatment of SFV was investigated.

In Chapter 5, a comparative study is described of the receptor binding properties of heparan sulfate (HS) adapted SIN and non-adapted SIN using liposomes containing lipid-conjugated heparin as a receptor analog. The role of virus-receptor interaction in SIN membrane fusion activity was investigated. In addition, the potential HS-adaptation of a SFV laboratory strain was further examined.

In Chapter 6, the novel liposomal model for virus-receptor interaction described in Chapter 5 is applied to investigate the effect of the antiviral compound lactoferrin on SIN-receptor interaction. Furthermore, the effect of this compound on SIN binding and fusion was also investigated using the heparin containing liposomes.

In Chapter 7, the results and conclusions of the preceding chapters are summarized, discussed, and reconciled in a model of alphavirus fusion.

References

Chapter 1


General Introduction
