Chapter 1

General Introduction
The alphavirus genus

Sindbis virus (SIN) and Semliki Forest virus (SFV) are positive-strand RNA viruses belonging to the genus Alphavirus of the family Togaviridae (41). The alphavirus genus consists of about 25 different viruses. Alphaviruses are transmitted in nature by anthropod vectors, primarily mosquitoes. SIN has also been isolated from mites and ticks (124). Alphaviruses can infect a wide range of host organisms, including birds, insects, mammals, and mosquitoes. The skeletal muscle and fibroblasts are the initial sites of infection, after which the viruses spread to the central nervous system (41). The alphaviruses Venezuelan equine encephalitis virus (VEE), Western equine encephalitis virus (WEE), and Eastern equine encephalitis (EEE) are known to cause fatal encephalitis in horses and humans, but the incidence particularly in humans is low (124). These viruses are endemic in North and South America and cause periodic outbreaks of encephalitis in horses. Other alphaviruses, like Ross River virus (RR), SFV, and SIN can cause fever, rash, arthralgia, and polyarthritis in humans, but again the incidence is low (41). SFV and SIN are among the least pathogenic alphaviruses for humans, and therefore have been widely used as “prototypes” to study the life cycle of these viruses. These studies take advantage of the fact that alphaviruses grow to high titers in tissue culture cells with high PFU-to-particle ratios, properties which enable a detailed biochemical and structural characterization of the virus particles. In addition, full-length cDNA clones of SFV (77), SIN (107), or VEE (21) have made it possible to study the viral life cycle by site-specific mutagenesis of the viral genome. In this thesis, we primarily used SIN to unravel the molecular mechanisms involved in the cell entry process of alphaviruses.

Structure and characteristics of Sindbis virus

Alphaviruses have a uniform structure with a diameter of about 70 nm (16, 45, 100, 124). The viral genome consists of a single-stranded RNA molecule of positive polarity. The viral RNA is assembled with the capsid protein to form the nucleocapsid (35). The nucleocapsid is surrounded by a lipid bilayer, in which the viral spike proteins are inserted (45).

**Nucleocapsid.** The nucleocapsid contains the single-stranded RNA molecule of approximately 11.7 kb, assembled with 240 copies of the capsid protein (~30 kD). The entire genome of SIN has been sequenced (123). Cryo-electron microscopy (cryo-EM) and image reconstruction analysis have revealed that the nucleocapsid has a symmetrical icosahedral structure with a triangulation number \( T=4 \) (16, 100). The individual capsid proteins are arranged as pentamers and hexamers to form a roughly spherical nucleocapsid.

**Envelope.** The SIN envelope consists of a lipid bilayer in which 240 copies each of two transmembrane glycoproteins E1 and E2 are inserted (124). The lipid
bilayer is composed of lipids derived from the plasma membrane of the host cell during budding of the virus. The viral membrane consists of about 25% sphingomyelin, 27% phosphatidylycholine, 19% phosphatidylserine, and 26% phosphatidylethanolamine (4, 132). The cholesterol to phospholipid ratio in the viral membrane is approximately 1:1 (70, 106).

Figure 1. Cryo-EM picture of Sindbis virus. The protruding spikes are clearly visible. Each spike consists of a trimer of E2/E1 heterodimers. (courtesy of Dr. S. Mukhopadhyay, Purdue University).

The 240 copies of the glycoproteins E2 and E1, both with a molecular mass of about 50 kD, extend from the lipid bilayer and are organized in a T=4 lattice (16, 45). The glycoproteins form 80 hetero-oligomeric spikes, a single spike consisting of a trimer of E2/E1 heterodimers. The E2 and E1 glycoproteins are classified as type I membrane proteins, with the amino-terminus facing outward from the membrane. E2 is 423 amino acid residues long, with a carboxy-terminal tail of 33 amino acids. E1 has 439 amino acid residues and a carboxy-terminal tail of only 2 amino acids. Both E2 and E1 are acylated. E2 has three acylation sites in the carboxy-terminal tail and two in the transmembrane region (124). E1 is acylated at one position in the transmembrane region (109). Furthermore, both E2 and E1 are each glycosylated at two positions in the ectodomain of the protein (124). Sequence analysis of the E1 glycoprotein has revealed a hydrophobic domain that extends from residues 75 to 97; this region has been proposed to represent the fusion peptide of the virus (35, 75).

Crystallographic and cryo-EM studies revealed that the E1 glycoprotein is folded to an elongated molecule that in many respects resembles the flavivirus E protein (74). SFV E1 is composed of a central (amino-terminal) β-barrel domain that is flanked by a finger-like projecting domain, containing the fusion peptide,
and a carboxy-terminal Ig-like domain. In the protein crystal E1 forms a head-to-tail linked dimer, which can be fitted in the cryo-EM density map of SFV. In another elegant study, SIN glycosylation mutants were used to localize the carbohydrate moieties on the virus using cryo-EM imaging (104). Both studies revealed that the E1 protein lies approximately parallel to the viral surface, whereas the E2 glycoprotein forms the protruding spikes. These data indicate that the E1 glycoprotein is responsible for the icosahedral scaffold, that organizes the T=4 architecture of the mature virus particle.

The E2 glycoprotein is synthesized in the infected cell as a PE2 (PE2 is called p62 in SFV) precursor that is cleaved into E2 and a periphal polypeptide E3 (~11 kDa). E3 is lost in mature SIN particles, but remains associated to the E2/E1 heterodimer of SFV (88). Cryo-EM and image reconstruction analysis of a mutant PE2-containing SIN virus revealed that E3 is positioned as a bulge in the heterodimer localized midway between the center of the spike complex and the tips (100).

There is also a small hydrophobic peptide, produced as a linker between E2 and E1, called the 6K protein, that has been found to be associated with the virus in low quantity (7-30 molecules per particle; 33, 82). The 6K polypeptide is palmitoylated at positions 35, 36, 39 (124).

**Replication and assembly of Sindbis virus**

**RNA replication of the viral genome.** A schematic representation of the genomic RNA and the translation of the viral proteins is depicted in Figure 2. The genomic RNA of SIN can be divided into two major regions: a non-structural domain encoding the replicase proteins and a structural domain encoding the three major viral proteins. The non-structural proteins are translated as a polyprotein directly from the genomic RNA. This polyprotein is cleaved in an autoproteolytic fashion, resulting in the release of nsP1, nsP2, nsP3, and nsP4. The protease responsible for these cleavages is encoded in the sequences at the C-terminal domain of nsP2 (24, 44, 122). nsP1 appears to be involved in the association of the alphavirus replicase to membranes (3). nsP2 is involved in the regulation of minus-strand RNA synthesis (112) and in the initiation of subgenomic 26S RNA synthesis (126). The function of nsP3 is unknown. nsP4 has been identified as the viral polymerase (7, 43, 73, 111). It appears that the function of the viral polymerase is dependent on an aromatic or histidine residue at the amino-terminal end of nsP4 (115). In summary, the alphavirus non-structural proteins are responsible for replication of both positive-strand genomic RNA and minus-strand RNA, the latter serving as a template for production of the 26S subgenomic mRNA and new positive-strand RNA.
Figure 2. RNA replication of the SIN genome. See text, for details.

The structural proteins are synthesized as a polyprotein from the 26S subgenomic mRNA, in the order NH$_2$-capsid-PE2-6K-E1-COOH, and are post-translationally processed to produce the individual polypeptides. Figure 3 gives a schematic representation of the translation of the structural proteins, assembly and budding of alphavirus particles.

**Capsid protein synthesis and nucleocapsid assembly.** The capsid protein is autoproteolytically released from the polyprotein precursor by its serine protease activity (6, 76). Once the capsid protein has cleaved itself from the polyprotein chain, the proteinase is no longer active because the active site remains occupied by the C-terminal tryptophan residue (17). Newly synthesized capsid proteins then bind to one or more encapsidation sequences of the genomic RNA, and this
binding is believed to be important for the initiation of nucleocapsid formation (139). For SIN, a signal sequence in the encoding region of nsP1 (from nucleotide 945 to 1076) has been identified as the encapsidation signal (17). Moreover, it was found that a stretch of 18 amino acids, called helix I, in the amino-terminal region of the capsid protein is important for nucleocapsid formation (101). After the binding of a capsid subunit to the RNA, more capsid proteins associate with the complex, until a T=4 symmetry is obtained (36).

**Glycoprotein synthesis and folding of the viral spike proteins.** Once the capsid protein is cleaved off, a signal sequence at the amino-terminus of PE2 targets the remainder of the polyprotein for cotranslational translocation to the rough endoplasmic reticulum (ER). In the ER the polyprotein is cotranslationally cleaved by signal peptidases into PE2, 6K, and E1 (113).

Addition of carbohydrates. Mannose groups are added to the PE2 and E1 polypeptides in the lumen of the ER, and these carbohydrate chains are modified during transit of the proteins through the Golgi apparatus (113). The asparagine residues in a consensus sequence Asn-X-Ser/Thr in the ectodomains of the alphavirus glycoproteins are glycosylated and carry either simple or complex oligosaccharide chains. It has been postulated that a glycosylation site remains
simple when the folding of the protein renders the chain inaccessible to cellular processing enzymes during transit through the Golgi apparatus, whereas if the chain is accessible to cellular enzymes it is further modified to a complex type (57). For SIN, the oligosaccharide at E1:139 may be either a complex or a simple type of carbohydrate, while the oligosaccharide at E1:245 appears to be modified to a complex-type. E2 is glycosylated at positions 196 and 318, the oligosaccharide at position 318 being processed to a complex-type of carbohydrate. It is believed that the function of carbohydrate chains at the initial stage of glycosylation is to increase the solubility of the protein and prevent aggregation or side reactions from occurring. However, it appears that glycosylation of the ectodomains of SIN glycoproteins has other distinct functions in the life cycle of alphaviruses (Chapter 6).

A ddition of fatty acids. Fatty acid acylation of the structural proteins of SIN in vertebrate cells occurs after exit of the ER but probably before arrival in the cis Golgi (10). In most cases the fatty acid, usually palmitic acid, is covalently linked in a thiol ester bond to the cysteine residues of the protein. Mutational analysis of the transmembranal cysteine residues of the glycoproteins E1 (at position 430) and E2 (at positions 388, 390) revealed that these residues are palmitoylated (109). Moreover, it has been shown that the cytoplasmic domain of E2 is palmitoylated at positions 396, 416, and 417 (33, 58). Site-specific mutagenesis of the 6K protein revealed that the cysteines at positions 35, 36, and 39 are palmitoylated (32, 33). The role of acylation of the SIN glycoproteins is not quite clear, although it has been shown that deacylation of the carboxy-terminal domain of E2 or the 6K protein affects virus budding. For influenza virus, conflicting reports have been published with regard to the role of envelope glycoprotein acylation in viral fusion (28, 60, 90, 93, 103, 121). In Chapter 7, the influence of acylation of SIN glycoproteins E2 and E1 on the membrane fusion process is investigated.

Folding, transport, and PE2 cleavage of the glycoproteins. The folding of the viral glycoproteins begins immediately upon entry of the proteins into the ER. It requires molecular chaperones, folding enzymes, energy, and the formation of disulfide bridges (124). After folding of the glycoproteins, heterodimerization of PE2 and E1 occurs in the ER. The PE2/E1 heterodimer matures further by passing through the Golgi and trans-Golgi network (TGN). During transport of the PE2/E1 heterodimer through the slightly acidic TGN, the uncleaved PE2 is presumed to function as a chaperone, protecting the spike from premature destabilization. The influence of PE2 on the stability of the PE2/E1 heterodimer is further discussed in Chapter 8. In the TGN or in a post-TGN compartment, PE2 is cleaved to form E2 and E3 (88). PE2 cleavage is mediated by a furin-like host protease at the consensus sequence XBXBBX, where X is a hydrophobic and B a basic amino acid (67). After PE2 cleavage the E2/E1 heterodimers are transported to the plasma membrane of the cell, where they are used to assemble
new virus particles. The spikes appear on the cell surface as trimers of E2/ E1 heterodimers; it is unclear when exactly these trimers are formed.

**Spike-nucleocapsid interactions and budding of virus particles.** The preformed nucleocapsids in the cytoplasm of the cell interact with the viral spike proteins, located on the plasma membrane of the cell, which results in the budding and release of new progeny virus particles. SFV mutagenesis studies have revealed that particle formation requires co-expression of both the capsid and the spike proteins (127). It has been proposed that alphavirus budding is driven by specific interactions of the carboxy-terminal tail of E2 with the nucleocapsids at the plasma membrane of the cell (16, 36, 71). The carboxy-terminal domain of E2 contains two conserved regions. The first conserved region contains a tyrosine and a leucine residue, and this region is shown to interact with aromatic residues in the capsid protein (54, 72, 98, 119, 143). The second region consists of palmitoylated cysteine residues, flanking the tyrosine-leucine motif, and mutations that disrupt palmitoylation affect virus budding (33, 58). In addition, E2-E1 dimer interactions are important for virus assembly and budding. It was found that the E2 carboxy-terminal tail does not interact with the capsid protein unless the E2 protein is dimerized with E1 (5).

Interestingly, it has been observed that pre-assembly of nucleocapsids in the cell cytoplasm is not a prerequisite for budding (30, 118). Moreover, Forsell and co-workers (31) showed that lateral spike-spike interactions are required for virus assembly. It was observed that a capsid mutant with a large deletion (amino acid 40 to 118), which is unable to assemble into nucleocapsids in the cell cytosol, does assemble at the plasma membrane and leads to the release of new virus particles. It was proposed that the role of the capsid proteins is restricted to triggering the spike proteins to undergo lateral spike-spike interactions. This, in contrast to the assumption that spike-capsid interactions would drive virus assembly, suggests that lateral spike-spike interactions are responsible for the envelope formation. Moreover, based on the recent information on the positioning of E1 in the viral envelope of alphaviruses (74, 104), one could argue that lateral spike-spike interactions between the E1 glycoproteins are responsible for envelope formation (34).

It has also been shown that the 6K polypeptide is involved in virus budding (32). Deletion analysis of 6K demonstrated that the budding efficiency of the D6K virus was down to 10% when compared to wild-type SFV (77, 79). These observations demonstrate that the 6K polypeptide facilitates virus assembly but that it is not absolutely required.

Furthermore, efficient budding of SFV and SIN requires the presence of cholesterol in the cellular membrane (80, 81, 87, 133). Transfection of viral RNA into cholesterol-depleted cells demonstrated that, while RNA replication, spike protein dimerization, and transport to the cell surface were normal, virus budding was dramatically inhibited.
Cell entry of Sindbis virus

Entry of enveloped viruses into a host cell requires fusion of the viral envelope with a cell membrane after attachment of the virus to the cell. Fusion of a viral membrane with a cellular membrane is mediated by the viral spike proteins and occurs either at the plasma membrane of the cell, or from within acidic endosomes after cellular uptake of virus particles through a process of receptor-mediated endocytosis, as depicted in Figure 4. In the process of plasma membrane fusion, the interaction of a virus particle with a cellular receptor triggers conformational changes in the viral spike protein which subsequently results in fusion of the viral membrane with the plasma membrane of the cell. On the other hand, when the virus is taken up by the cell through receptor-mediated endocytosis, the mildly acidic pH in the lumen of the endosomal compartment triggers conformational changes in the viral proteins, that are required for fusion of the viral membrane with the endosomal membrane. There is considerable controversy as to whether SIN infects its host cell by receptor-mediated endocytosis or plasma membrane fusion. Before discussing the evidence for both pathways in detail, we will first describe the receptor binding properties of alphaviruses.

**Receptor binding.** The first step in the entry process of alphaviruses is the interaction of the virus particle with a receptor on the plasma membrane of the cell. It was found that the E2 glycoprotein is primarily responsible for virus-receptor interaction, as evidenced by the ability of anti-E2 antibodies to inhibit binding to cells (12, 84). Anti-idiotypic antibodies, directed against E2-specific antibodies, have been used in attempts to identify putative virus receptors on target cells (131, 137). Furthermore, cryo-EM and image reconstruction analysis of SIN and RR, using Fab fragments of monoclonal antibodies, revealed that the outermost tips of the viral spike protein are involved in interaction with a cellular receptor (120).

Identification of specific alphavirus receptors has been difficult and, in retrospect, may have been complicated by the use of virus strains that are adapted to tissue cell culture (69). Because alphaviruses have a wide host range and replicate in a variety of different species as well as in many different cell types, the viruses must use a ubiquitous cell surface molecule or a variety of different molecules as receptors. The first putative receptor identified for SFV was the major histocompatibility complex class I molecule on mouse and humans cells (51). However, cells that do not express class I molecules can still be infected by SFV (95). For SIN, a high-affinity laminin receptor has been proposed as a potential receptor on baby hamster kidney (BHK-21) cells (136).
Recently, it has been shown that SIN, RR, and VEE interact with heparan sulfate (HS), a ubiquitously expressed glycosaminoglycan on the plasma membrane of the cell, as a cell culture adaptation (9, 12, 13, 49, 69). Passage of non-HS-adapted SIN TR339 on BHK-21 cells results in the generation of virus mutants which bind with high affinity to BHK-21 cells and interact with heparin (69). Positive-charge amino acid substitutions have been identified in the mutant E2 proteins of SIN, RR, and VEE, substitutions which appear to be responsible for interaction with HS (9, 49, 69). With regard to SIN, three loci in E2 have been identified (E2:1; E2:70; E2:114) that mutate during the adaptation of SIN in BHK-21 cells and can independently confer the ability to the virus to bind to cell-surface HS (69).

**SIN entry via plasma membrane fusion.** A mechanism of SIN entry via plasma membrane fusion has been first suggested on the basis of studies which indicated that weak bases, such as chloroquine and ammonium chloride (which are commonly used to raise the pH of intracellular compartments) have little effect on the formation of SIN infectious centers, as detected by translation of viral RNA in the cell cytosol (14, 18). Accordingly, it was found that SIN was able to infect a mutant Chinese hamster ovary cell line, temperature sensitive for endosome...
acidification, at either permissive or non-permissive temperatures (26). Furthermore, it was shown that attachment of SIN to cells leads to the exposure of new transitional epitopes on both glycoproteins, using E1- and E2-specific antibodies (29). In the context of these experiments, Abel and Brown (1) proposed a model for SIN entry in which virus-receptor interaction reduces critical disulfide bridges in the E1 glycoprotein through thiol-disulfide exchange reactions. The reduction of disulfide bridges would reorganize the viral spike protein of SIN, subsequently resulting in fusion of the viral membrane with the plasma membrane of the cell. Consistent with this model, SIN-induced polykaryon formation was enhanced by the presence of 2-mercaptoethanol, which reduces disulfide bonds, while SIN infection was partially inhibited by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), a thiol-alkylating agent and exchange inhibitor (1). Moreover, it has been observed that the infection of mosquito cells by SIN is not inhibited by chloroquine, under conditions such that the drug prevented endosome acidification (55).

**SIN entry via receptor-mediated endocytosis.** There is also evidence that SIN infects its host cell by receptor-mediated endocytosis and fusion from within acidic endosomes. Glomb-Reinmund and Kielian (40) investigated the role of low pH and disulfide shuffling in the entry process of SIN. These authors made a direct comparison between SIN and SFV, a virus which is known to infect cells via receptor-mediated endocytosis and low-pH-induced fusion from within acidic endosomes (50, 52, 53, 59, 64, 85, 86). It was found that both SIN and SFV infection are inhibited in cells treated with ammonium chloride, during entry of the virus, as evidenced by viral RNA translation and infection. Inhibition of viral infection was concentration dependent and correlated with the pH threshold of virus-induced cell-cell fusion. Bafilomycin and concanamycin, two reagents that prevent endosome acidification by a different mechanism than the weak bases, also inhibited cellular infection of SIN and SFV. Furthermore, the authors were unable to detect a specific role for the reduction of disulfide bonds in SIN and SFV infection. In another study, alphavirus infection was studied using cells expressing a dominant-negative mutant of dynamin, which blocks the budding of clathrin-coated pits, and it was found that SIN and SFV infection was inhibited (23). On the basis of these studies it would appear as though SIN, like SFV, infects its host cells by receptor-mediated endocytosis, and that exposure of the virus to the low pH, within the lumen of the endosome, is physiologically important for activation of the viral membrane fusion reaction. The controversy about the route of cell entry of SIN represents the basis for the studies presented in Chapter 2, 3 and 8 of this thesis.

**Nucleocapsid uncoating.** Through fusion of the viral membrane with the cellular target membrane the viral nucleocapsid gains access to the cytoplasm of the cell. It has been demonstrated that after the fusion reaction, the nucleocapsid remains associated with the cytosolic leaflet of the endosomal membrane (117).
The nucleocapsid is uncoated such that the RNA becomes accessible to ribosomes. It has been proposed that the binding of the nucleocapsid to the ribosomes triggers the uncoating process (124). The region from amino acid residue 94 to 106 of the capsid protein appears to be involved in the binding of the protein to the ribosomes (140). This was found to be the same region of the protein that interacts with viral RNA during encapsidation (37, 99). Once the RNA is accessible to the ribosomes, RNA replication and translation of the viral proteins are initiated. This will eventually lead to the production of new virus particles.

Membrane fusion activity of Semliki Forest virus

The basic features underlying the membrane fusion properties of enveloped viruses have been studied extensively in virus-cell and virus-liposome systems. In virus-cell systems, fusion is detected directly, measuring fusion of the virus with the plasma membrane of the cell or, indirectly, on the basis of polykaryon formation. These assays might give a qualitative insight in the membrane fusion reaction, but do not allow kinetic or quantitative analysis of the process. To obtain detailed insight in the membrane fusion process of enveloped viruses, it is necessary to follow the process in a sensitive and continuous fashion. To this end, virus-liposome systems have been developed, which offer a number of advantages over virus-cell assays, including the simplicity of the technique, high sensitivity, possibility to obtain quantitative data, and the opportunity to identify target membrane components required for viral fusion (56). Virus-liposome systems are either based on lipid mixing between the interacting membranes or on intermixing of the interior of the virus and the liposomal lumen. Particularly, the lipid mixing assays provide a kinetic and quantitative insight in the membrane fusion process, while contents mixing meets a very stringent criterion for fusion. Virus-liposome systems have been used extensively to unravel the molecular mechanisms involved in the membrane fusion process of SFV, as discussed in more detail below.

General features of SFV-liposome fusion. It has been demonstrated that fusion of SFV with liposomes lacking a protein receptor is strictly dependent on the exposure of the virus to low pH (11, 134, 141). Optimal fusion kinetics were observed at pH 5.5. The pH threshold for fusion activation of SFV was found to be pH 6.2 (11). The in vitro pH dependence of fusion (11) closely correlates with the in vivo pH dependence of virus fusion with the endosomal membrane (40). The efficient fusion kinetics of SFV with protein- and carbohydrate-free liposomes suggests that virus-receptor interaction is not a mechanistic requirement for membrane fusion. SFV fusion is temperature dependent, but fusion can be measured in a temperature range of 4 to 37 °C. The fusion-active conformation of SFV is short-lived, pre-incubation of the virus alone for 30 s at pH 5.5 resulting in a complete loss of membrane fusion activity (11). Fusion of SFV with liposomes
requires cholesterol and sphingolipid in the target membrane, as described in detail below.

**Lipid dependence of SFV fusion.** Fusion of SFV with liposomes composed of a variety of purified lipids revealed that fusion is strictly dependent on the presence of cholesterol (11, 134, 141) and sphingolipid (94, 142) in the target membrane. Optimal fusion kinetics were observed with liposomes containing 35 mol% cholesterol and 2 mol% sphingolipid in the target membrane, with a cholesterol to phospholipid ratio of 1:2. It was found that cholesterol is required for low-pH-induced binding of the virus to the liposomal membrane, whereas sphingolipid is important for the subsequent fusion process (94). Cholesterol has also been found to be required for efficient cellular infection, SFV fusion and infection being dramatically reduced on cholesterol-depleted mosquito cells (see below). To date it has not yet been possible to study the role of sphingolipid during cellular infection, since there are no viable sphingolipid-deficient cell lines available.

To unravel the structural features of cholesterol in supporting membrane fusion, SFV-liposome fusion studies were conducted using liposomes composed of a variety of sterol analogs. Three major features of cholesterol are believed to be important in its physical interactions with a membrane: the planar ring structure, the aliphatic side chain at C-17, and the 3β-hydroxyl group. It was observed that the planar ring structure, and the aliphatic side chain are not required for membrane fusion activity of SFV (63). In contrast, the 3β-hydroxyl group of cholesterol was found to be essential (63, 102, 134). For example, sterols with a modified 3β-hydroxyl group such as 3-α-hydroxy cholesterol, cholestanone, 5α-cholestane, cholesterol methyl ether, cholesterol acetate, and chlorocholestene, were inactive in supporting membrane fusion of SFV.

The structural features required for sphingolipids to mediate fusion of SFV have been examined in a similar fashion, using liposomes containing a variety of sphingolipid analogs. Efficient fusion of SFV was observed with liposomes containing either sphingomyelin, ceramide, or galactosyl ceramide, demonstrating that the nature of the headgroup of the sphingolipid is not a crucial factor (94). Ceramide is the minimally required sphingolipid still supporting fusion, the sphingosine base being inactive. The length of the acyl chain has no prominent effect on the capacity of ceramide to induce membrane fusion of SFV, since both C8-ceramide and C18-ceramide are active (19). In contrast, the 3β-hydroxyl group and the 4,5-trans double bond of the sphingosine backbone were found to be critical (19, 46). The sphingolipid requirement is stereospecific, D-threo, L-threo, and L-erythro ceramides or sphingolipids being inactive (92). In conclusion, fusion studies with a wide variety of sphingolipids have demonstrated that the action of sphingolipid in the membrane fusion process of SFV exhibits a remarkable molecular specificity. In **Chapter 2**, it is demonstrated that fusion of SIN, like that of SFV, also requires sphingolipid in the target membrane. The molecular
specificity of this sphingolipid requirement differs from that of SFV fusion (unpublished observations) and is currently under detailed investigation.

**E1 conformational changes.** Upon exposure of SFV to low pH, conformational changes take place in the viral spike protein, which result in fusion of the viral membrane with the target membrane. The fusion reaction of SFV with liposomes can be slowed down by raising the pH of the medium or by lowering the temperature, through which one can differentiate between different stages in the series of conformational rearrangements that occur. Upon acidification, the E2/E1 spike heterodimer dissociates, as evidenced by the loss of E2-E1 co-immunoprecipitation and co-flotation on sucrose-density gradients (11, 27, 61). After E2/E1 heterodimer dissociation, the E1 glycoprotein undergoes several distinct conformational changes that are independent of E2. Monoclonal antibody binding experiments showed that previously hidden epitopes of E1 become accessible (2, 114). Using these Mabs, it was found that residue E1:157 becomes exposed after low pH treatment of the virus (2). Then, the E1 glycoproteins rearrange to form an E1 homotrimeric structure (134, 135). Treatment of alphaviruses with heat or urea also induces conformational changes within the spike proteins, but there is no formation of an E1 homotrimer under these conditions (38, 91). The formation of an E1 homotrimer is strictly dependent on the incubation of the virus at low pH (134). Analysis of the low-pH-dependent fusion reaction in presence of Zn\(^{2+}\) suggests that the E1 glycoprotein inserts into the target membrane before E1 trimerization (20). Similar results were obtained with the SFV mutant (E1:Gly91Asp; 66). The E1 homotrimer has a very stable configuration: it is found to be resistant to SDS treatment at 30 °C, urea, and trypsin digestion (38, 134). It has been proposed that the energy derived from the formation of the E1 homotrimer could drive the merging of the membranes. This would imply that the formation of the E1 homotrimer occurs concomitantly with membrane fusion. However, kinetic data suggest that E1 trimerization occurs before the onset of membrane fusion (11). Therefore, it appears that additional rearrangements within or between E1 homotrimers are required to establish fusion of the viral membrane with the target membrane. The influence of low pH on the conformation of the spike proteins of SIN is presented in Chapter 2.

**Alphavirus mutants and the effect on virus entry and membrane fusion**

**Adaptation.** RNA viruses are known to adapt rapidly to new growth environments as a result of the facile generation and selection of virus mutants. For example, rapid adaptation is the basis for the emergence of new influenza virus variants that have the ability to escape from the immunological response generated by the host against a prior virus variant. Also, adaptation is the basis for
the development of resistance against antiretroviral drugs in HIV-infected individuals. Furthermore, the emergence of new virus variants makes it virtually impossible to design a vaccine against HIV that would control the infection by induction of virus-neutralizing antibodies.

The rapid generation of RNA virus mutants is directly related to the relatively low fidelity of viral RNA replication, the error frequency being approximately 1 in 10,000 bases (125). Furthermore, viral RNA polymerases generally lack error-correcting mechanisms. This implies for alphavirus RNA replication, that on average each newly synthesized viral genome will contain a base substitution. As these substitutions are inserted randomly throughout the genome, they will generally go unnoticed, either because the substitution remains silent or because it may be lethal. Only when the substitution confers a selective advantage to the corresponding virus mutant, relative to the parent virus, it may become visible. In this case, selective pressure will allow the virus mutant to outgrow and develop into the predominant species (42). These selective conditions may involve immunological pressure within the host, the presence of drugs interfering with replication of the parent virus, changes in culture conditions, etc.

The facile emergence of adapted virus mutants has also contributed significantly to our current understanding of the life cycle of RNA viruses. For example, to gain a better insight in the requirement of cholesterol in the fusion and the budding process of SFV particles, cholesterol-independent SFV mutants have been selected by serial passage of the virus on cholesterol-depleted mosquito cells (15, 87, 133). Likewise, Chapter 3 presents a characterization of SIN variants adapted to efficient interaction with HS after passaging of wild-type virus on tissue culture cells. Also, Chapter 8 deals with infectious second-site mutant SIN viruses generated from a poorly infectious PE2 cleavage mutant virus by passaging of the original virus on BHK-21 cells. At the same time, however, one should be aware of the fact that propagation of virus particles in cell culture can lead to unrecognized adaptive mutations, which may result in misinterpretation of results and erroneous conclusions regarding the life cycle of the virus concerned (8). In order to avoid the generation of adaptive mutations, viruses can be used derived from an infectious cDNA clone. Infectious cDNA clones have been developed for SFV (77), SIN (107), and VEE (22). In these clones, the viral genome has been positioned downstream of a promotor encoding an SP6 RNA polymerase. The viral RNA is transcribed in vitro, and transfected into susceptible cells, which subsequently leads to the production of progeny virus. Thus, with this procedure, no additional passage of the virus is required, thereby minimizing the possibility of generating mutant viruses.

In the following paragraphs, several alphavirus mutants are described, generated by passage of the virus under selective pressure or by site-specific mutagenesis of the full-length cDNA clone encoding the genome of SFV or SIN.
**PE2 cleavage mutants.** In SFV, cleavage of p62 (p62 is called PE2 in SIN) was prevented by mutating the p62 cleavage site from Arg-His-Arg-Arg to Arg-His-Arg-Leu (110). The resultant p62-mutant SFV was found to be non-infectious in BHK-21 cells because of impairment of receptor interaction and fusion. Viral infectivity of the p62-containing mutant virus could be restored by in vitro cleavage of p62 with trypsin or by exposure of the virus to extremely low pH (78, 110, 135). Analysis of the conformational changes of the viral spike proteins revealed that the pH threshold for p62/E1 heterodimer dissociation was pH 5.0. This is considerably lower than that for wild-type virus, in which E2/E1 heterodimer dissociation is already observed at pH 6.2. Therefore, it appears that the block in membrane fusion lies at the level of the dissociation of the p62/E1 heterodimer.

The role of PE2 cleavage in viral infection has also been investigated for SIN. A Chinese hamster ovary cell, RPE.40, defective in PE2 processing, was used to generate PE2-containing SIN particles (138). The produced PE2-containing virus particles were found to be non-infectious on RPE.40 and other cells. Viral infectivity was regained by in vitro cleavage of PE2 by trypsin.

In contrast, a PE2-containing mutant of the SIN strain SAAR86 was found to be as infectious as the PE2-cleaving parental virus on RPE.40 cells (108, 138). The PE2 cleavage defect resulted from a single amino acid mutation at E2:1 in which a Ser-to-Asn substitution created a signal for N-linked glycosylation. In a later study, a similar amino acid substitution was introduced in the infectious clones of TRSB (corresponding to a laboratory strain of SIN) and TR339 (containing the consensus sequence of SIN AR339), and it was demonstrated that these mutant viruses were non-infectious on RPE.40 cells and BHK-21 (47, 48, 68). The authors found that infection was blocked downstream of binding of the virus to the cell surface, but before RNA replication. This suggests that these viruses are impaired in their capacity to induce membrane fusion. In Chapter 8, we investigated the infectivity and fusogenic properties of these and other PE2 cleavage mutant SIN viruses with liposomes.

**Cholesterol-independent SFV and SIN mutants.** The presence of cholesterol in the host cell membranes has been found to be required for efficient infection, fusion, and budding of SFV and SIN, as discussed earlier. To gain a better insight in the cholesterol dependence of these viruses, Kielian and co-workers have generated cholesterol-independent mutants of SFV and SIN. Cholesterol-independent mutants of SFV were obtained by passaging wild-type SFV on cholesterol-depleted mosquito cells (15, 87, 133). The resultant mutant, called srf-3 (sterol requirement in function), was found to be more infectious, by about 4 to 5 logs, on cholesterol-depleted cells, when compared to wild-type SFV (133). It was observed that the enhanced growth of the srf-3 mutant virus on sterol-depleted mosquito cells was due to an increased efficiency of viral entry and exit. Analysis of the membrane fusion properties of the srf-3 virus with liposomes revealed that both the E1 conformational changes and the membrane fusion
activity of the mutant virus were relatively independent of cholesterol (15). Furthermore, it was found that the srf3 virus, propagated in cholesterol-depleted cells, was less stable to centrifugal force than wild-type SFV, which suggests a possible role for cholesterol in stabilizing the virus particles (133). Sequence analysis of the E1 protein of srf-3 mutant virus revealed a single point mutation from Pro to Ser at position 226. Introduction of this mutation into the full-length clone of SFV resulted in a cholesterol-independent mutant, indicating that the cholesterol dependence of SFV is indeed located at E1:226 (133). The cholesterol dependence of SIN is also located in the E1:226 region, as evidenced by site-specific mutagenesis of the full-length clone of SIN (80). However, other cholesterol-independent mutants of SFV, srf4 and srf5, have been identified which were found to have mutations at E1:44 or E1:178, respectively (62). Thus, it appears that several regions in E1 may be involved in the cholesterol dependence of alphavirus cell entry and membrane fusion.

E2 virulence mutants. Sequence analysis of neurovirulent strains of SIN, and the corresponding construction of recombinant viruses, have revealed that E2 and E1 are important determinants of virulence (21, 83, 105). It was found that amino acid changes in E2 which are important for virulence in mice, often affect an early step in viral entry. For one of these mutants (E2:Gly172Arg), the attenuated virulence in mice has been shown to correlate with a decrease in binding to neural cells (128). Other SIN mutants, with a reduced virulence in mice, exhibit an increased penetration rate in BHK-21 cells (21, 89, 96, 97, 108). These mutants were generated by passaging of wild-type SIN virus on tissue culture cells. Sequence analysis revealed that these mutants had positive-charge amino acid substitutions in E2 (E2:Ser114Arg or E2:Glu70Lys). In later studies, it was observed that these amino acid changes confer the ability to the variants to bind to cell-surface HS (69). Indeed, several mutants of SIN, VEE, and RR that bind to HS have been found to have a reduced virulence in mice (9, 49, 68, 69). It has been proposed that in vivo HS-adapted mutants bind non-productively to extracellular membranes and basal laminae, and therefore may be cleared from the blood more rapidly than wild-type viruses (9, 13, 49).

Passaging of SIN strain AR339 through neonatal and adult mouse brain resulted in a virus mutant with a profound increase in neurovirulence. Sequence analysis and construction of recombinant viruses revealed that the increased virulence of this mutant is due to a single amino acid substitution, from Glu to His, at E2 position 55 (130). This amino acid change alters viral entry and affects early steps in replication more profoundly in neural cells than in non-neural cells (25, 129). Since the E2 glycoprotein is found to be responsible for virus-receptor interaction, one could argue that the amino acid change in TE influences the virus-receptor interaction at the cell surface of neural cells. This question represents the basis for the study presented in Chapter 5 of this thesis.
**SFV fusion mutants.** The amino acids 90 to 92 (Gly-Gly-Ala) in E1 are strongly conserved among alphaviruses. These residues lie within the conserved hydrophobic domain (amino acid 79 to 97) of E1, a domain which has been identified as the putative fusion peptide of E1. Mutation from the Gly residue at E1 position 91 to Ala or Asp resulted in virus mutants that were affected in viral fusion and assembly (75, 66, 116). The E1:Gly91Ala mutant was found to have a decreased efficiency in virus-liposome association, E1 trimerization, and fusion when compared to wild-type SFV. Both cell-cell fusion and virus-liposome fusion studies revealed that the pH threshold for fusion of the E1:Gly91Ala mutant is shifted to a more acidic pH. The E1:Gly91Asp mutant is completely blocked in membrane fusion, as evidenced by lipid mixing, content mixing, and polykaryon formation assays. It appears that the block in membrane fusion activity is after binding of the viral to the target membrane and epitope exposure but prior to E1 trimerization. This indicates that the E1 glycoprotein inserts as an E1 monomer in the target membrane and that E1 trimerization occurs upon interaction with the target membrane, as indicated above. Other amino acid substitutions in the fusion peptide of SFV revealed that the membrane fusion activity of the virus is dependent on the length of the fusion peptide, its overall hydrophobicity, and its specific sequence (75, 116).

Another SFV fusion mutant, called fus-1, with a lower pH threshold for membrane fusion was isolated by selecting viruses resistant to in vitro fusion with RNase-containing liposomes (65). The acid shift in the membrane fusion activity of fus-1 appears to be a consequence of the more acidic pH threshold for the initial dissociation of the E2/E1 heterodimer (39). Sequences analysis revealed that a single amino acid substitution at E2 position 12 from Thr to Ile is responsible for this fusion phenotype (39). This indicates that the amino-terminal end of E2 stabilizes the E2/E1 heterodimer interaction and regulates the pH dependence of E1-catalyzed fusion by controlling the dissociation of the E1/E2 heterodimer.

**Scope of this thesis**

Mutational analysis of the viral spike proteins has greatly contributed to our current understanding of the molecular mechanisms involved in the membrane fusion process of alphaviruses. The studies described in this thesis attempt to further unravel the molecular basis of the membrane fusion activity of SIN, by site-specific mutagenesis of amino acids in the E2 and E1 glycoprotein sequences. These mutations are generated in full-length clones of SIN. The receptor interaction and the membrane fusion characteristics of the mutants are analyzed by cell binding assays, on-line lipid mixing assays, content mixing assays, and analysis of the viral spike conformational changes. The first chapters deal with the general characteristics of the membrane fusion process of SIN with liposomes. Subsequently, the influence of post-translational modifications, including
glycosylation, palmitoylation, PE2 cleavage, of the viral glycoproteins on the membrane fusion process of SIN is investigated.

In Chapter 2, the basic membrane fusion characteristics of SIN with target liposomes are investigated with emphasis on the issue as to whether receptor interaction or low pH is the crucial factor in triggering the fusion process. Furthermore, the role of cholesterol and sphingolipid in the membrane fusion process of SIN is investigated. Sub-optimal conditions for fusion were used to differentiate between different stages in the virus-liposome fusion process.

In Chapter 3, the receptor binding properties of HS-adapted mutant SIN versus non-adapted SIN TR339 are investigated using liposomes in which a lipid-conjugated heparin is incorporated as a specific attachment receptor for HS-adapted SIN. The effect of virus-receptor interaction on membrane fusion activity is analyzed in further depth, using lipid mixing and content mixing assays. Also, the potential adaptation of SFV to cell-surface HS is examined.

In Chapter 4, the leakiness of SIN and SFV fusion is investigated by examining the redistribution of fluorescent dyes or radiolabeled contents, entrapped in the liposomal lumen during fusion. It is demonstrated that the fusion reactions are largely non-leaky, supporting the notion that a hemifusion intermediate is involved.

In Chapter 5, the cell entry properties of SIN mutants TE and 633 are investigated. A distinction is made between binding and fusion. It is shown that the TE virus is more infectious on neural cells than SIN mutant 633. The enhanced infection efficiency of TE in neural cells is found to be related to an increased ability of TE to bind to these cells rather than to an enhanced membrane fusion capacity of this virus.

In Chapter 6, single deglycosylated SIN mutants are used to identify the function of the carbohydrate chains in infectivity and membrane fusion activity of the virus. It is shown that SIN glycosylation mutants have a reduced infectivity on BHK-21 cells. Moreover, the deglycosylated SIN mutants are impaired in their capacity to induce membrane fusion, indicating that the reduced infectivity of the mutant viruses on cells lies at the level of the fusion process.

In Chapter 7, the effect of deacylation of the transmembrane domains of SIN glycoproteins E2 and E1 on the viral membrane fusion capacity is investigated. The mutants were generated by site-specific mutagenesis of the infectious cDNA clone Toto1101 of SIN. It appeared that deacylation of the transmembrane regions of E2 and/or E1 has no effect on the membrane fusion characteristics of the virus.

In Chapter 8, the role of PE2 in the membrane fusion process of SIN particles is investigated. It is demonstrated that PE2 cleavage mutant viruses are non-infectious on BHK-21 cells because these mutants are impaired in viral fusion. Furthermore, it is shown that second-site resuscitating mutations in PE2 restore viral infection and membrane fusion, despite a sustained lack of PE2 cleavage.
In Chapter 9, the results and conclusions of this thesis are summarized and discussed.

References


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