SUMMARY

This thesis describes a study on the molecular mechanisms involved in the membrane fusion process of Semliki Forest virus (SFV). Extensive studies on the entry of SFV into cells have revealed that the virus enters target cells via receptor-mediated endocytosis (97, 98, 116, 144, 146-148). It has been shown that the fusion process in the endosomes is triggered by the low pH maintained in the endosomal lumen by an ATP-dependent proton pump. Several studies have demonstrated that SFV fuses rapidly and extensively with liposomes. This allows SFV membrane fusion to be investigated in detail in a cell-free model system. It has been demonstrated both with liposomes and cells that SFV fusion requires cholesterol in the target membrane (169, 227). Cholesterol is also required in the exit pathway of SFV (143). In **Chapter 2**, we demonstrate that besides its cholesterol dependence, SFV fusion also requires the presence of sphingolipids in the target membrane. Using a liposomal fusion assay, involving pyrene-labeled virus, it is shown that ceramide represents the minimally required sphingolipid. Sphingolipids exert their action at very low concentrations (at 2 mole% half-maximum fusion is obtained), suggesting a cofactor role for sphingolipids during the fusion process, rather than a structural role. Furthermore, we demonstrate that cholesterol is required for the initial step of fusion, the low-pH dependent binding of the virus to liposomes, and that sphingolipids, on the other hand, are required for the merging of the membranes. In **Chapter 3** it is shown that the ceramide analogues 3-Deoxy-ceramide and Dihydroceramide are unable to support fusion, indicating that the 3-OH group and the 4-5 *trans* double bond of the sphingosine backbone are essential for the sphingolipid-SFV interaction. In **Chapter 4**, the stereospecificity of the sphingolipid-SFV interaction is demonstrated by using the four possible stereoisomers of C_8-Ceramide. We show that only the naturally occurring D-*erythro*-C_8-Ceramide is able to support SFV fusion. The results presented in the third and fourth chapter confirm the notion that the SFV-sphingolipid interaction is very specific, further supporting our proposal of a cofactor role for sphingolipids during membrane fusion of SFV. In **Chapter 5**, Zn^{2+} ions are used to specifically inhibit SFV fusion. Inhibition of fusion is shown to be at the level of the spike conformational changes (E1 trimerization), while low-pH-dependent binding is not influenced by Zn^{2+} ions. This implies that the acid-induced conformational changes may occur after the initial binding of the virus to the target membrane. Hence, these conformational changes can be influenced by the lipids in the target membrane. Indeed, as shown in **Chapter 6**, cholesterol and sphingomyelin can have an independent stimulatory effect on the formation of E1 trimers. The effect of sphingolipids in cholesterol-depleted liposomes is quite remarkable, since the virus cannot bind to the liposomes, as shown in Chapter 2. We demonstrate that transient, weak interactions between the virus and the sphingolipid result in formation of the E1-trimer. The molecular specificity of the sphingolipid influence on E1 trimerization is demonstrated by the inert molecules 3-Deoxy-sphingomyelin and L-*erythro*-sphingomyelin. In addition, it is shown that the sphingolipid-dependence and cholesterol-dependence of SFV fusion can be circumvented in special cases, supporting the notion that sphingolipids act as a catalyst rather than a cofactor

during SFV fusion.

In **Chapter 7**, we investigate whether the SFV fusion process occurs via a hemifusion stalk intermediate. Inhibition of fusion by lysoPC and promotion of fusion by arachidonic acid suggests that SFV fusion occurs via a stalk hemifusion intermediate. Further support for a hemifusion intermediate comes from the data presented in **Chapter 8**. In this chapter, we show that fusion of SFV with liposomes is principally non-leaky, suggesting the existence of a fusion intermediate with well-defined boundaries, such as the stalk.

In **Chapter 9**, hydrophobic photolabeling with ^{125}I-TID-PC is used to identify the region of the SFV spike which penetrates into the target membrane to induce fusion. By using the soluble ectodomains of the SFV spikes we were able to label a region of the E1 subunit which contains the putative fusion peptide. Accordingly, additional experiments with viral particles identified the same region of the E1 subunit. Further analysis and fine-mapping of the labeled peptide should provide more precise data.

Finally, in **Chapter 10**, the fusion characteristics of Tick-Borne Encephalitis (TBE) virus and recombinant subviral particles (RSPs), are presented. TBE virus is a member of the family of the *Flaviviridae*, which, like the *Togaviridae*, belongs to the group of arboviruses. The influence of temperature, pH, and low pH preincubation are measured, using pyrene-labeled virus and RSPs in a liposomal assay. It is shown that TBE virus and RSPs fuse very rapidly and efficiently with liposomes. The fusion process of TBE virus has no specific lipid requirement. Compared to SFV, fusion of TBE virus occurs much more rapidly, as evidenced by the fact that low temperature hardly has any effect on the fusion rate of TBE virus. This suggests that formation of a fusion pore during TBE virus fusion is a highly efficient process, possibly explained by the parallel orientation of the TBE virus spikes to the viral membrane (172).

ROLE OF LIPIDS IN SFV FUSION

The lipid dependence of SFV fusion is a remarkable feature. First, the cholesterol-dependence has been quite well characterized. The 3β-OH group has been shown to be essential for fusion of SFV (118). Cholesterol has been demonstrated to be strictly required for the low-pH-dependent binding of SFV to liposomes, as demonstrated in Chapter 2. Also, cholesterol stimulates the formation of the E1 trimer, although the results in Chapter 6 show that these E1 trimers might be fusion-inactive. Cholesterol is not only required in the target membrane during entry of SFV in cells (169), but also in the exit pathway during maturation and budding of progeny virus (143).

Mosquitoes, the main vectors of SFV in nature, are dependent on their blood meals to acquire cholesterol. This implicates that SFV, taken up during a first blood-meal, cannot infect the mosquito, since at that time, the membranes of the mosquito do not contain cholesterol. The cholesterol-dependence of SFV fusion thus guarantees that SFV only infects mosquito cells with cholesterol-containing membranes, certifying that enough cholesterol is present for budding of newly synthesized virions.

The sphingolipid-dependence of SFV has been demonstrated more recently. It has been shown that the interaction between sphingolipids and SFV is highly specific

(see Chapters 2, 3 and 4 and (236)). It has also been demonstrated that sphingolipids stimulate E1 trimerization both in the virus (see Chapter 6) and in the soluble ectodomain, E1* (121).

It remains to be elucidated why the SFV fusion process has developed itself during evolution such that it requires sphingolipids. Preliminary experiments show that another alphavirus (Sindbis) also shows sphingolipid dependence, suggesting a general feature of alphavirus fusion (J. Smit, personal communication). Since sphingolipids are present in the outer layer of the plasma membrane of almost every cell, there seems no reason to develop a requirement for sphingolipids during fusion. Alphaviruses cause a wide range of diseases in animals and man, but have a preference to infect muscle, brain, reticuloendothelial system tissue and joints (79). There seems to be no correlation between the preference for these cell types and the sphingolipid dependence of membrane fusion developed by alphaviruses.

Over the last few years, it has become clear that the sphingolipid ceramide is a potent second messenger (for a review see (88)). Ceramide is a specific suppressor of cell growth and an inducer of apoptosis. Over-expression of the proto-oncogene Bcl-2 can inhibit ceramide-induced proteolysis and protect cells from ceramide-induced death (195). It is interesting to note that Bcl-2 also is able to inhibit SFV infection (177). Despite many papers about the role of ceramide as a second messenger, there has been no report about the ceramide-protein interaction at the molecular level. It has been demonstrated that stereospecific recognition of sphingolipids occurs in cells, further indicating that highly specific proteins must be involved (122). Recently, the interaction of the earthworm hemolysin with membranes was shown to be dependent on sphingolipids. This is an additional example of a sphingolipid-dependent protein-membrane interaction (125). Still, a sphingolipid-binding site on a protein has not yet been identified. Additional work with the relatively simple system of SFV and liposomes might result in the identification of a sphingolipid-binding site on a protein (E1) for the first time. Such a discovery may in turn stimulate also the work on other sphingolipid-protein interactions.

MODEL OF SFV FUSION

It is tempting to speculate on the molecular mechanism of SFV fusion and to try to propose a model. In such a model, the roles of cholesterol and sphingolipids need to be included, and also the data obtained using specific inhibitors such as Zn^{2+} ions and lysoPC. In the model we present here several steps can be distinguished upon acidification.

1. E2/E1 dissociation,
2. Cholesterol-dependent binding,
3. E1 trimerization, influenced by cholesterol and sphingolipids,
4. Cooperation of spikes to form a fusion site,
5. Formation of a hemifusion intermediate,
6. Formation of a fusion pore,
7. Completion of fusion.

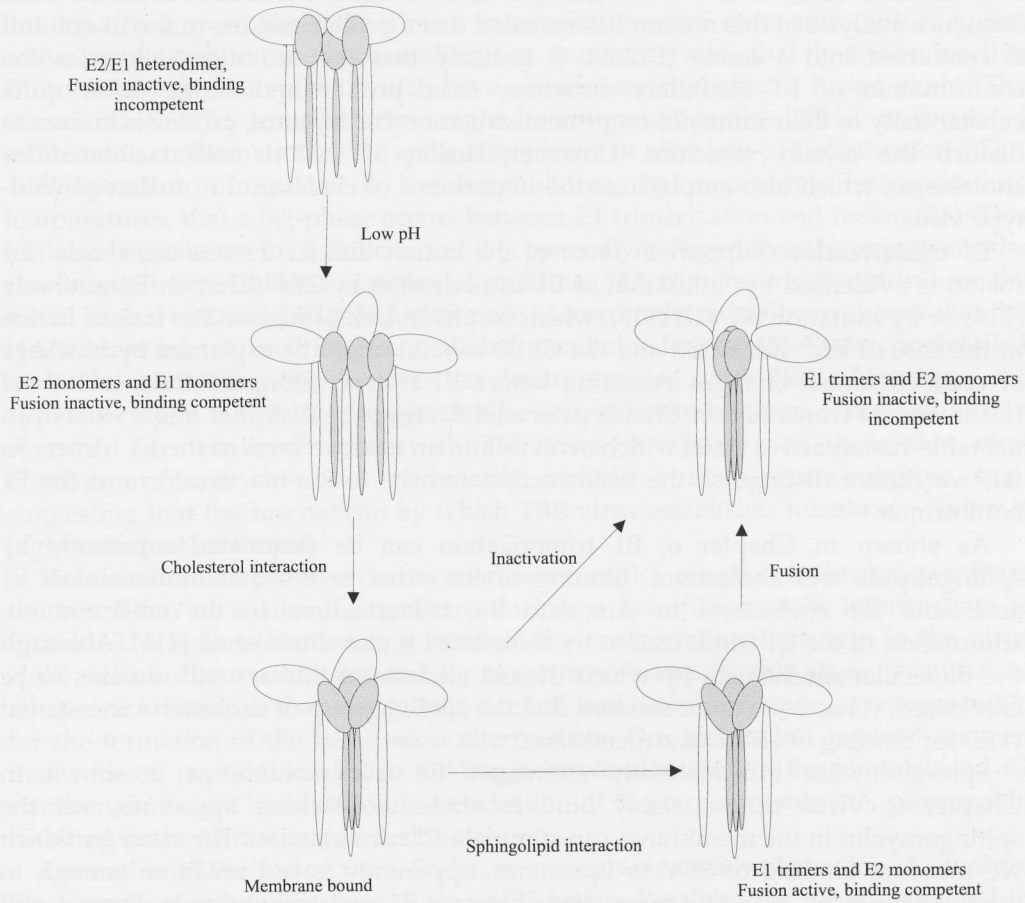

Figure 1: *Interactions of the SFV spike, resulting in formation of the E1 trimer, either fusion-active or fusion-inactive.*

E2/E1 dissociation. Upon low-pH induction, swiveling of the spikes occurs (68). These initial rearrangements ultimately result in E2/E1 dissociation. This dissociation is the only requirement for the next step, binding.

Cholesterol-dependent binding. As already mentioned above, the only requirement for binding to cholesterol-containing membranes is dissociation of the E2/E1 spike. In Chapter 5, we demonstrate that in the presence of Zn^{2+} ions low-pH-dependent binding and E2/E1 dissociation are not inhibited, but subsequent steps of fusion are. Additional evidence comes from the group of Kielian (117). In this study, SFV with a mutation in the putative fusion peptide (G91D), shows wild-type binding capacity and normal E2/E1 dissociation. However, E1 trimerization is inhibited, demonstrating that low-pH-dependent binding is independent of E1-trimerization.

It is clear from Chapters 2 and 6 that SFV cannot stably bind to cholesterol-lacking liposomes. However, very recently, a cholesterol-independent mutant of SFV has

been isolated (211). This mutant (srf-3) is able to infect cholesterol-depleted cells. Sequence analysis of this mutant has revealed that a proline residue in the E1 subunit is converted into a serine (P226S). It is likely that this mutation influences the conformation of E1 secondary structure, since proline and serine differ quite substantially in their influence on protein structure. For instance, proline is known to disturb the α-helix structure. However, fusion of srf-3 is still facilitated by cholesterol, which also emphasizes the importance of cholesterol in fusion of wild-type virus.

E1 trimerization. Support in favor of the notion that E1 trimers are crucial for fusion is evidenced by inhibition of E1 trimerization by Zn^{2+} (Chapter 5), antibody (216) or by mutation (G91D) (117), which results in lack of fusion. The lack of fusion in the case of Zn^{2+}-inhibition and the G91D mutant might be explained by blockage of conformational changes occurring before E1 trimerization, resulting in lack of fusion and E1 trimerization. Shortly after acidification, the E1 trimer might exist in an unstable fusion active state, which evolves into an inactive form of the E1 trimer. So far, we cannot distinguish the fusion-active and the fusion-inactive form of the E1 homotrimer.

As shown in Chapter 6, E1 trimerization can be stimulated separately by sphingolipids and cholesterol. Binding of the virus to the membrane makes E1 accessible for cholesterol to stimulate the trimerization. In the srf-3 mutant, stimulation of the E1 trimerization by cholesterol is also diminished (211). Although the molecular mechanism by which E1 and cholesterol interact still remains to be elucidated, it has been demonstrated that the 3β-OH group of cholesterol is essential (118) for binding of SFV to the membrane.

Sphingomyelin independently stimulates E1 trimerization, as is shown in Chapter 6. Virus cannot stably bind to cholesterol-lacking liposomes, yet the sphingomyelin in the membranes can stimulate E1 trimerization. The assay by which we measure binding of SFV to liposomes, apparently is not sensitive enough to measure the weak, transient interactions between E1 and sphingolipids. There is still no evidence for the direct interaction of E1 and sphingolipid. Only an indirect effect (E1 trimer stimulation) can be measured So far. Currently, we are investigating the possibility to extend and combine the results described in Chapters 6 and 8. It is conceivable that the sphingolipid-E1 interaction can be detected by hydrophobic photo labeling. Labeled sphingolipids should be used to identify the region of E1 which interacts with the sphingolipid.

In Chapter 6, we describe that PC/PE/Chol liposomes, to which SFV stably binds, can induce E1 trimerization. Yet, fusion does not occur with these liposomes, suggesting the formation of fusion inactive E1 homotrimers. On the other hand, PC/PE/SPM liposomes, to which SFV cannot stably bind, also stimulate E1 trimerization. Also in this case, no fusion occurs, but this can possibly be explained by the lack of stable binding. The fact that E1 trimerization can be stimulated by both cholesterol and sphingomyelin suggests that the E1 trimer can occur in at least two different forms. One, which is fusion-inactive, can be induced by cholesterol and one, which is fusion-active, might be enhanced by sphingolipids.

Evidently, a fusion inactive E1 trimer exists, since also low-pH inactivated SFV

contains E1 trimers. Also the extremely stable E1 trimers, existing long after fusion has occurred, most likely are trimers in a fusion inactive state, since SFV is fusion active for just a short time upon acidification. It is conceivable that the fusion inactive E1 trimers represent spikes, which have already accomplished a fusion event. The fusion inactive E1 trimeric form might be compared to the TBHA molecule of influenza: a conformation of the spike after fusion, or after low-pH inactivation (27).

Cooperation of spikes. It has been shown, by monitoring fusion at low temperatures, that a lag-phase occurs between E1 trimerization and fusion (23). This has also been shown for influenza and it is generally believed that this represents the time needed to establish a complex of multimer HA trimers, which can induce the initial stages of formation of a fusion pore. Accordingly, it has been shown that 3-4 HA trimers are required for HA-mediated membrane fusion (48). So far, no detailed research concerning this feature has been reported with SFV. However, the appearance of a lag-phase is suggestive for cooperativity between several spikes. In contrast, our results obtained with TBE virus in Chapter 10 show that viral membrane fusion does not necessarily include a temperature-sensitive step, suggesting that the mechanism by which TBE virus establishes membrane fusion is highly efficient and rapid.

Hemifusion. We propose that as soon as a complex of spikes is formed to initiate the formation of a site of membrane fusion, a hemifusion stalk intermediate is formed. The stalk is formed as a result of the conformational changes in the virus spike, which disrupt the viral and the target membrane. At this stage, it is unclear what conformation of the viral spike is needed, and how many spikes are responsible for the formation of the hemifusion intermediate. Our results in Chapter 9 suggest that a fusion peptide, located in the fragment we identified in the photolabeled E1 ectodomain, might be involved in penetration of the target membrane, presumably resulting in the formation of a fusion site.

Formation of the fusion pore. So far, no measurements have been done to analyze the formation of a fusion pore during SFV fusion. Possibly, the results obtained in Chapter 8 reflect the mechanism of fusion pore formation. The total lack of leakage during fusion suggests the existence of a specific, multimeric, protein/lipid complex, which is able to direct fusion in a highly organized form. It is unclear whether or not HA-mediated fusion is leaky. One report has been published which shows that influenza virus fusion is leaky (183). On the other hand, there are also reports of HA-mediated fusion of cells which suggest that this process is not leaky (21, 153, 248).

Merging of membranes. As a result of dilation of the fusion pore, most likely comparable with influenza HA-mediated fusion, full merging of the membranes occurs and fusion is completed, without leakage of contents.

Future prospects

The model of SFV fusion as proposed here is still far from complete. However, the research, described in this thesis, further contributes to conception of the SFV fusion process. Current investigations focus on further elucidation of the sphingolipid-dependence of SFV fusion. Also, the fusion characteristics of other alphaviruses, such as Sindbis virus and Ross River virus are currently under investigation. In this way,

general features of alphavirus fusion might be revealed. This may possibly lead to development of drugs or therapies against alphavirus infections, such as the human pathogens VEE, EEE and WEE.

As mentioned already, the postulated interaction between E1 and sphingolipids still needs to be demonstrated. Studies with photolabeled sphingolipids should provide enough information to demonstrate the direct interaction of E1 and sphingolipids. Furthermore, it might be possible to study the influence of SFV spikes, as particles or as soluble ectodomains, on lipid monolayers.

Determination of the 3-D structure of the SFV spike proteins also will provide a major contribution to the understanding of the membrane fusion process, by providing information about the location of amino acids, such as the G91, crucial for fusion, or the P226, involved in the cholesterol dependence of fusion. Specific structure-function studies can be performed as soon as the 3-D structure has been determined. In this way, the fusion process of SFV, and of alphaviruses in general, can be further unraveled.