Cell entry mechanisms of alphaviruses
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Chapter 7

Summarizing Discussion
7.1 Summary

One important aspect of the entry process of alphaviruses is the fusion of the viral membrane with the endosomal membrane in the host cell, resulting in the delivery of the viral genome into the cytosol. Without this fusion reaction the virus is destined for degradation (see Chapter 1). The fusion reaction can be monitored on-line in a model system using fluorescently labeled viruses and liposomes as model target membranes. The majority of the research described in this thesis has been performed using this model system. The advantage of liposomes is that the composition of the liposomal membrane can be varied in order to determine the effect of lipid species and membrane structure on membrane interaction and membrane fusion activity of the viruses. The incorporation of receptors in the liposomal membrane makes it possible to investigate virus-receptor interaction. In previous studies that used this model system, an interesting dependence of alphavirus fusion on the simultaneous presence of cholesterol and sphingolipids was revealed (Nieva et al., 1994). The sphingolipid dependence exhibited an exquisite molecular specificity (Corver et al., 1995; Moesby et al., 1995; Wilschut et al., 1995). The question as to the role sphingolipids play in alphavirus membrane fusion has been a central topic of the research project presented in this thesis.

There is extensive evidence for the presence of sphingolipid-cholesterol microdomains or lipid rafts in cellular membranes (see Chapter 1; Simons and Ikonen, 1997; Brown and London, 1998). The simultaneous requirement of sphingolipids and cholesterol in alphavirus fusion is suggestive of an involvement of lipid rafts in the entry of alphaviruses. Chapter 2 analyzes whether there is a requirement of lipid rafts in liposomes for SFV and SIN fusion. The presence of lipid rafts in the liposomal membrane was modulated by incorporating sphingolipid analogs and sterols varying in their ability to support raft formation. The results demonstrate that, in the absence of rafts, extensive SFV and SIN fusion occurs. SFV fusion was, in fact, lower with liposomes containing sphingolipid analogs or sterols that showed increased microdomain formation, suggesting an inhibitory effect of microdomain formation on fusion. The results indicate that sphingolipid-cholesterol microdomains or lipid rafts are not required for alphavirus fusion and that the role of sphingolipids in alphavirus fusion is not simply to form rafts with cholesterol, with these rafts then functioning as platforms for viral fusion in the target membrane.

In Chapter 3, we demonstrate that under non-physiological conditions, SFV has the capacity to fuse with liposomes in the absence of sphingolipids. However, the extent of this sphingolipid-independent fusion was limited compared with the extent of fusion in the presence of sphingolipids. Sphingolipid-independent fusion still required the presence of cholesterol and was dependent on the temperature and the fatty acyl chain composition of the glycerophospholipids in the membrane, suggesting a role of membrane lipid packing in the process. Although sphingolipids are not strictly required for SFV fusion, they always increase binding and fusion when present and are strictly required at physiological temperatures. A lack of SFV fusion with LUVs in the absence of sphingolipids was correlated with a lack of ability of the virus to bind to these LUVs, suggesting that sphingolipids are not only involved in the actual membrane fusion
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process, but also in the initial binding of the virus to target membranes. The results presented in Chapter 3 led us to reconsider the current idea about the role of sphingolipids in alphavirus fusion (see 7.2).

Low pH triggers the dissociation of the E2/E1 heterodimer allowing the formation of the E1 homotrimer, several copies of which are presumably involved in the fusion process (Bron et al., 1993; Ferlenghi et al., 1998; Justman et al., 1993; Wahlberg et al., 1992; Wahlberg and Garoff, 1992; Gibbons et al., 2003, 2004). The formation of a stable E1 homotrimer is irreversible. However, the results in Chapter 4 suggest that a reversible state of E1 precedes the formation of the homotrimer. We demonstrate that acidification of SFV alone for 1 min at 37°C renders the virus inactive in fusion. However, relatively independent of the length of the preceding acidification period, after neutralization the virus is able to infect cells and to fuse with liposomes upon reacidification. This shows that, contrary to common dogma, the virus is not inactivated by pre-exposure to low pH in the absence of target membranes. Fusion was optimal when the acid-treated virus was neutralized to pH 8.0. Density-gradient centrifugation analysis suggested that the E2/E1 heterodimer remains dissociated. When liposomes containing cholesterol and sphingomyelin are present during acidification, the virus binds to the liposomes, the extent of E1 trimerization increases, and the virus fuses with the liposomes. When the liposomes are added 2 min after acidification, the virus does not bind to the liposomes, there is no increase in E1 trimerization, and the virus does not fuse with the liposomes. The results presented in Chapter 4 indicate that a reversible state of E1 exists between heterodimer dissociation and trimerization. This reversible state is inactive in mediating virus-liposome binding and fusion. Furthermore, the results suggest that low pH triggers heterodimer dissociation and that the presence of membranes containing cholesterol and sphingomyelin triggers efficient E1 trimerization. A hypothesis with regard to the course of events in SFV fusion, from the low-pH trigger to membrane merging, is presented in 7.3.

Using liposomes supplemented with lipid-conjugated heparin, we investigated the role of the receptor in membrane fusion in a direct manner (Chapter 5). Heparin is an analog for cell-surface heparan sulfate (HS). Cell culture adapted SIN has been shown to efficiently bind BHK cells by interacting with HS, and it has been demonstrated that cell culture adapted SIN also binds to heparin (Klimstra et al., 1998). In Chapter 5, we show that HS-adapted SIN efficiently binds to heparin-containing liposomes at neutral pH. Non-adapted SIN does not bind to these liposomes at neutral pH. While adapted SIN efficiently interacts with heparin, no fusion of the viral membrane with the liposomal membrane at neutral pH occurred. Membrane fusion was only observed upon a low pH trigger with both HS-adapted and non-adapted SIN. The results demonstrate that virus-receptor interaction does not support membrane fusion and that low pH is the sole trigger for membrane fusion to occur. The results are in agreement with the idea that SIN enters its host cell by receptor-mediated endocytosis and fusion from within acidic endosomes, rather than by fusion with the plasma membrane at neutral pH.

The heparin-containing liposomes and the HS-adapted viruses were used as a tool to investigate the antiviral activity of human lactoferrin (hLF) (Chapter 6). hLF is a major component of breast milk protein and has antibacterial, antifungal, and antiviral
properties (Kanyshkova et al., 2001; Hanson and Korotkova, 2002). Most studies on the antiviral activity of hLF suggest that the protein inhibits virus entry into cells rather than later phases of viral replication. Lactoferrin, in principle, can prevent virus cell entry by binding to the virus particle or by binding to cell-surface molecules that viruses use as receptors or co-receptors. In either case, lactoferrin would prevent viral attachment to the cell surface (Meijer et al., 2001; van der Strate et al., 2001). It has been suggested that binding of hLF to cell-surface HS glycosaminoglycans (HSPGs) is involved in inhibition of viral infection (van der Strate et al., 2001). In order to determine whether hLF indeed exerts antiviral activity through interference with virus binding to HSPG receptors, we studied the effects of hLF on the cell entry and receptor binding of HS-adapted SIN and cell-culture adapted SFV. In Chapter 6, we demonstrate that hLF inhibits infection of cells by HS-adapted SIN and SFV and that hLF does not inhibit infection by non-adapted SIN. hLF inhibits binding of the HS-adapted viruses to cells and to heparin-containing liposomes. hLF does not inhibit membrane fusion of the adapted viruses with heparin-containing liposomes. Furthermore, we show that a positively charged derivative of human serum albumin has a similar antiviral effect against HS-adapted SIN and SFV, suggesting that the antiviral activity of human lactoferrin against alphaviruses is related to its positive charge. The results indicate that lactoferrin interferes with binding of viruses to cells rather than affecting the subsequent entry and membrane fusion process.

7.2 The Role of Receptor Interaction in Alphavirus Membrane Fusion

It is well established that SFV enters its host cell by receptor-mediated endocytosis and subsequent low-pH-induced fusion from within endosomes (Helenius et al., 1980 and 1982; Marsh and Helenius, 1980; Marsh et al., 1982, 1983; Izurun et al., 1997; DeTulleo and Kirchhausen, 1998; Glomb-Reinmund and Kielian, 1998 a and 1998b; see Chapter 1). It is likely that other alphaviruses follow the same route of viral entry into their host cells. However, there is controversy about this matter for SIN. Evidence is available suggesting that SIN undergoes fusion with the plasma membrane, at neutral pH, and that the receptor is involved in triggering the fusion process (Cassell et al., 1984; Coombs et al., 1981; Flynn et al., 1990; Edwards and Brown, 1991; Abell and Brown, 1993; Hernandez et al., 2001). On the other hand, other studies suggest that SIN, like SFV, does enter cells by receptor-mediated endocytosis and fusion from within acidic endosomes (DeTulleo and Kirchhausen, 1998; Glomb-Reinmund and Kielian, 1998b). Recent studies from our laboratory have shown that SIN fuses rapidly and efficiently with receptor-free liposomes (Smit et al., 1999), and that subtle shifts in the pH dependence of SIN membrane fusion directly correlate with the infectivity of the virus in cells (Smit et al., 2001). The results presented in Chapter 5, demonstrating that virus-receptor interaction does not support membrane fusion and that low pH is required for membrane fusion, and the results presented in Chapter 6, showing that blocking the viral attachment receptor by hLF did not inhibit membrane fusion, are in agreement with our previous studies and the studies of Glomb-Reinmund and Kielian.
Therefore, it appears that the sole trigger for SIN membrane fusion is low pH and that SIN infects its host by receptor-mediated endocytosis with the receptor required for cell attachment and endocytic uptake.

The reason for the discrepancy in the literature is not clear. Explanations regarding possible experimental side effects causing the occurrence of plasma membrane fusion at neutral pH have been proposed. Brown and coworkers would have used virus that had been frozen in phosphate-buffered medium. The pH of this medium decreases upon freezing and could have caused a rearrangement of the viral spike proteins, which would give the virus the ability to fuse with the plasma membrane at neutral pH (DeTulleo and Kirchhausen, 1998; Ferlenghi et al., 1998). However, Brown and coworkers have denied using virus prior frozen in phosphate-buffered medium (Hernandez et al., 2001). Moreover, this explanation of the cause of the discrepancy is not very likely because we demonstrate for SFV that acidified virus which is subsequently neutralized (by thawing in this case) must be reacidified in order to fuse with liposomes. A more likely explanation is that the cause of the discrepancy lies with the types of fusion assays used (Glomb-Reinmund and Kielian, 1998b). The cell-cell fusion assay used by Brown and coworkers relies on the occurrence of morphological changes of the cells after fusion. These morphological changes could also be caused by secondary effects.

Based on earlier research of our laboratory (Smit et al., 1999; Smit et al., 2001) and on research presented in this thesis (Chapters 5 and 6), we have concluded that SIN and other alphaviruses, like SFV, enter the host cell by receptor-mediated endocytosis and subsequent, low-pH-dependent, fusion from within endosomes.

7.3 The Role of Sphingolipids and Cholesterol in Alphavirus Membrane Fusion

Reconsideration of the Role of Sphingolipids in Alphavirus Membrane Fusion

Previous research from our laboratory showed that SFV membrane fusion is dependent on the presence of both cholesterol and sphingolipids in the target membrane (Nieva et al., 1994). Only a very low concentration of sphingolipid is needed to mediate optimal fusion. In the absence of sphingolipids, SFV was found to be able to bind to liposomes provided that cholesterol is present (Nieva et al., 1994). Based on these results, it was suggested that cholesterol and sphingolipids play divergent roles in the fusion process, the presence of cholesterol in the target membrane being necessary and sufficient for binding of the virus to the liposomes, and the sphingolipids being essential for induction of membrane merging. Sphingolipids would play the role of a cofactor activating the fusion function of E1, possibly by inducing a specific conformational change in the protein. In agreement with the cofactor hypothesis, it was shown that the sphingolipid dependence exhibits molecular specificity, including stereospecificity (Corver et al., 1995; Moesby et al., 1995).

In Chapter 3 it is demonstrated that SFV has the capacity to fuse in the absence of sphingolipids at 20°C, although to a limited extent. Furthermore, it is demonstrated
that the virus is not able to bind to cholesterol-containing liposomes lacking sphingolipids at 37°C. When the virus is able to bind to liposomes, it also fuses at least in part with the liposome. These results are inconsistent with the idea of completely divergent roles of cholesterol and sphingolipids proposed in our previous work mentioned above. The results of this previous work, in fact, are in agreement with the results presented in Chapter 3. However, we did not realize the importance of low temperature in fusion without sphingolipids. Low temperature decreased the rate and extent of fusion in sphingolipid-dependent fusion, and thus we did not anticipate an increase of fusion at lower temperatures with liposomes lacking sphingolipids. For convenience, the binding experiments were done at 20°C but fusion was measured at 37°C and the results were compared. Our new results presented in Chapter 3 show that at physiological conditions sphingolipids, together with cholesterol, are required for efficient SFV binding to the target membrane, which indicates that the roles of cholesterol and sphingolipids in SFV fusion cannot be readily separated. However, it is important to note that extensive fusion occurred only in the presence of sphingolipids. Based on the results presented in Chapter 3, we now propose that sphingolipids are primarily required to interact with cholesterol in order to create the membrane binding site for SFV E1. Furthermore, sphingolipids are able to elevate fusion to higher extents, possibly by interacting with E1.

**Where and How Do Sphingolipids and Cholesterol Create the SFV Binding Site?**

While the simultaneous requirement of sphingolipids and cholesterol suggests that rafts could be the binding sites for SFV, the results presented in Chapter 2 suggest that SFV fusion with LUVs is independent of lipid raft formation and therefore does not necessarily involve lipid rafts. Small complexes of both lipids could be acting as binding sites for the virus. Since SFV also fuses extensively with raft-containing liposomes, these small complexes could reside in between rafts or at raft boundaries. The results in Chapter 2 also demonstrate that extensive detergent-insoluble complex formation, mediated by sphingolipid analogs or sterols other than cholesterol, partially inhibit SFV fusion. The bilayer of the liposomes containing these sphingolipid analogs or sterols could have a larger part of the surface covered by rafts and have less space in between the rafts, resulting in fusion inhibition. The rafts themselves would then be portions of the membrane unfavorable for fusion because (i) the stable lamellar organization of rafts (Brown and London, 2000; Rietveld and Simons, 1998; Harder and Simons, 1997) might be difficult to disrupt, while disruption of the lamellar organization is involved in membrane fusion (Wilschut, 1991); (ii) the stable organization of rafts might shield the 3\(\beta\)-hydroxyl group of cholesterol, as evidenced by studies showing that the 3\(\beta\)-hydroxyl group is shielded from cholesterol oxidase in sphingolipid-cholesterol monolayers (Grönberg and Slotte, 1990; Slotte, 1992), such that virus binding to cholesterol would be inhibited. Another result in favor of the idea of fusion in between rafts is the low concentration of sphingolipid required for fusion. A non-raft pool of cholesterol has been suggested to exist in between rafts (Simons and Ikonen, 1997; Harder and Simons, 1997) and it is likely that a non-raft pool of sphingolipids also exists. However, because of the preference of sphingolipids for rafts (Brown and London, 2000; Rietveld and Simons, 1998; Harder and Simons, 1997), the
non-raft pool of sphingolipid may be small. Yet, the low concentration of sphingolipids required for optimal fusion of SFV in liposomal model systems suggests that even low concentrations of sphingolipid in non-raft pools might suffice for supporting efficient fusion in cells. Binding of SFV to liposomes has been shown to require the 3β-hydroxyl group of cholesterol (Kielian and Helenius, 1984). Epi-cholesterol, having a 3α-hydroxyl group which lies parallel to the plane of the membrane, is not able to mediate SFV fusion, indicating the importance of the positioning of the hydroxyl group. Recently, it was shown by Brzustowicz and colleagues (2002) that the tilt angle of the axis of cholesterol with the membrane normal in membranes containing PC and PE species with one saturated and one polyunsaturated acyl chain was influenced by the temperature. Raising the temperature from 20°C to 40°C resulted in an increase of the tilt of cholesterol through an increased disorder of the membrane structure. Thus at 20°C cholesterol has a more upright positioning in the membrane. No change in the tilt of cholesterol was seen with membranes containing dipolyunsaturated PC and PE species, indicating a role for saturated acyl chains. The importance of the positioning of the 3-hydroxyl group, demonstrated by Kielian and Helenius (1984) and the results in Chapter 3, have interesting similarities with the observations of Brzustowicz et al. We demonstrate binding and fusion of SFV with liposomes at 20°C but not at 37°C, and optimal fusion at 20°C occurring with POPC/POPE/cholesterol membranes, i.e. with PC and PE species containing one saturated acyl chain. On the basis of the similarities we propose that lowering the temperature from 37°C to 20°C decreases the tilt of cholesterol and thereby positions the 3-hydroxyl group of cholesterol in a more upright fashion making it available for SFV binding at this temperature. However, a very tight membrane organization, while bringing cholesterol in an upright position, is not beneficial for SFV binding and fusion (Chapter 3). This suggests that, for SFV binding, the positioning of the 3-hydroxyl group has a very subtle optimum. When this importance for the positioning of cholesterol is extended to the role of sphingolipids in the creation of the virus binding site, it can be hypothesized that sphingolipids, by interacting with cholesterol, position cholesterol optimally for SFV binding. It is interesting to note that androstenol in the absence of sphingolipids was unable to mediate SFV binding and fusion at 20°C (Chapter 3). In contrast to cholesterol, androstenol lacks the isooctyl side chain. This structural difference may result in a greater tilt of androstenol in the membrane, and lowering the temperature to 20°C would not decrease the tilt enough to make the 3-hydroxyl group available for SFV binding. Sphingomyelin, on the other hand, by interacting with the sterol nucleus, would be able to optimally position androstenol, resulting in extensive binding and fusion of the virus with androstenol-containing membranes in the presence of sphingomyelin (Chapter 3).

The Involvement of the Sphingolipid Molecular Specificity

Specific molecular characteristics of sphingolipids have been found to have an influence on SFV fusion. The 3-hydroxyl group cannot be altered or deleted without affecting SFV fusion, while the 4,5-trans double bond is required for fusion (Corver et al., 1995). Furthermore, it has been demonstrated that the 4,5-trans double bond in the sphingoid backbone cannot be replaced by a 5,6-trans double bond (He et al., 1999). SFV fusion also exhibits a remarkably strict dependence on the presence of the D-
erythro isomer of the sphingolipid. The other three diastereoisomers have been shown to be unable to support SFV fusion (Moesby et al., 1995).

The molecular specificity of SFV fusion could be a result of the specific molecular features of sphingolipids on their interaction with cholesterol. Changes in the interaction between sphingolipids and cholesterol could influence the formation of the binding site for SFV E1 (Chapter 3). The removal of the 4,5-trans double bond, resulting in the formation of dihydrosphingomyelin, has been shown to cause a stronger interaction between this sphingolipid and cholesterol (Kuikka et al., 2001). The stronger interaction with cholesterol increases the formation of condensed domains, which are more stable than in the case of sphingomyelin. This could lead to a decrease in the concentration of “free” sphingolipids in between the domains or rafts, which in turn could inhibit SFV binding and fusion at physiological conditions. Another possibility is that a stronger interaction with cholesterol abrogates the formation of an optimal binding site for E1, because the tight interaction limits optimal positioning of the 3-hydroxyl group of cholesterol or decreases cholesterol’s degree of flexibility that may be needed to create the binding site.

The finding that only the D-erythro isomer of sphingolipids promoted fusion can also be reconciled with altered interactions of the sphingolipid with cholesterol. In a recent study comparing the biophysical properties of D-erythro-sphingomyelin with racemic sphingomyelin, a mixture of D-erythro and L-threo sphingomyelin, Ramstedt and Slotte (1999) demonstrated that cholesterol extraction from lipid monolayers by β-cyclodextrin was significantly slower from D-erythro-sphingomyelin monolayers than from racemic sphingomyelin monolayers. Furthermore, D-erythro-sphingomyelin monolayers contain significantly more condensed domains than racemic sphingomyelin monolayers. These results indicate a significant difference between D-erythro-sphingomyelin and racemic sphingomyelin in intermolecular interaction with cholesterol, with D-erythro-sphingomyelin having a stronger interaction with cholesterol. This difference may have an effect on the creation of the binding site for SFV, resulting in sphingolipid stereospecificity in SFV fusion.

The removal of the 3-hydroxyl group of sphingolipids results in a loss of a hydrogen donor and acceptor group (Ohvo-Rekilä, 2002), which could weaken the interaction if this group is important for the interaction. However, the 3-hydroxyl group of sphingomyelin has been suggested not to be critically important for sphingomyelin-cholesterol interactions (Kan et al., 1991; Grönberg et al., 1991).

Thus, many aspects of the molecular specificity of sphingolipids promoting SFV fusion can be reconciled on the basis of sphingolipid-cholesterol interactions, the sphingolipid being indirectly involved in virus-membrane interaction. Yet, it is likely that sphingolipids in addition play a crucial role in the actual fusion process. The presence of sphingolipids markedly increases the extent of fusion compared to fusion in the absence of sphingolipids, indicating such a direct fusion-promoting function (Chapter 3). More research is required to reveal which aspects of the molecular specificity of sphingolipid's role in SFV fusion are related to this direct fusion-promoting function of sphingolipids.
**Chapter 7**

E1 Trimerization and the Role of Sphingolipids and Cholesterol

Based on research performed in our laboratory and by Kielian and coworkers it is currently believed that SFV E1 homotrimer formation occurs after membrane binding of the virus. For example, Zn$^{2+}$ inhibits SFV fusion and homotrimer formation, but permits liposome binding and heterodimer dissociation (Corver *et al.*, 1997). Furthermore, Kielian *et al.* (1996) demonstrated that a SFV mutant containing a mutation in the fusion peptide, E1:Gly91Asp, is inactive in homotrimer formation and membrane fusion, while this mutant is able to bind to liposomes to similar extents as wild-type SFV. These results not only indicate that the homotrimer is important for fusion but also show that binding is possible without homotrimer formation, suggesting that binding precedes E1 trimerization. Our recent results show that SFV does not bind to PC/PE/cholesterol liposomes at 37°C and also does not bind to PC/PE/androstenol liposomes at 20°C and 37°C (Chapter 3). In an earlier report, Klimjack *et al.* (1994) show that trimerization of SFV E1 occurs in the presence cholesterol-containing liposomes with or without sphingomyelin at 37°C (Klimjack *et al.*, 1994). We have reproduced these results with SFV E1 at 37°C and also found similar results at 20°C (J. M. Smit, B. -L. Waarts, J. Corver, unpublished results). We have also found extensive E1 trimerization with androstenol-containing liposomes at both 20°C and 37°C with or without sphingomyelin (B. -L. Waarts, unpublished results). These results show that extensive E1 trimerization occurred irrespective of the occurrence of SFV-liposome binding and that E1 trimerization requires the presence of cholesterol- or androstenol-containing liposomes irrespective of the presence of sphingolipids.

Taken together, it appears that membrane binding and E1 trimerization are processes that occur independently of each other, but both processes require cholesterol. One possibility is that induction of E1 trimerization occurs via a different kind of interaction with cholesterol than the interaction resulting in E1 binding to cholesterol. This is supported by the finding that epi-cholesterol also induces trimerization, suggesting that the β-hydroxyl group, which is required for binding, may not be involved or that its positioning (α or β) is not important for trimerization (Klimjack *et al.*, 1994; unpublished results). However, it is also possible that in both cases the interaction between cholesterol and E1 is the similar, always inducing E1 trimerization, but the simultaneous presence of sphingolipids will result in the binding of the virus to the membrane via the sphingolipid-cholesterol binding site. Clearly, both E1 trimerization and membrane binding are required for the subsequent fusion event.

**7.4 Alphavirus Fusion: Time Course of Events**

Upon exposure of SFV to low pH, the E1/E2 heterodimer dissociates (Walhberg *et al.*, 1992; Wahlberg and Garoff, 1992). It has been suggested from cryo-EM data that the E2 subunits move away from the center of the spike while the E1 subunits move toward the center, closer to each other (Haag *et al.*, 2002; see also Fig. 7.1). We propose that E1/E2 heterodimer dissociation brings E1 in a “reactive” state, which scans the target membrane. Sphingolipid- and cholesterol-independent membrane interactions, which have been detected in black lipid membranes as conductance
increases (Samsonov et al., 2002), could represent this membrane scanning. The fusion peptide loop (Lescar et al., 2001) would probably be responsible for this scanning function. The fusion peptide may insert into the target membrane by an acid-induced conformational change of E1 moving the protein from its position parallel to the membrane (Lescar et al., 2001) to a more perpendicular position, as proposed for dengue virus (Modis et al., 2004; see also Fig. 7.1). In the absence of target membrane, E1 rapidly switches into a non-reactive state in which it is no longer capable of interaction with membranes. This hypothesis is supported by the results presented in Chapter 4, showing that adding liposomes 2 min after acidification did not lead to virus-liposome binding and increased trimerization. Neutralization switches E1 into an intermediate state and subsequent reacidification brings E1 back in its reactive state able to interact with membranes again. This is supported by results showing that neutralized and reacidified SFV was bound to liposomes, had increased trimerization, and fused with liposomes (Chapter 4). We hypothesize that E1 in its reactive state interacts with cholesterol. The presence of cholesterol then triggers trimerization of E1, as shown by Klimjack et al. (1994) and our unpublished results (see 7.3). When cholesterol is not present, E1 probably switches to its non-reactive state, as is the case when no target membrane is present. The trimerization reaction is inhibited by Zn$^{2+}$ (Corver et al., 1997) or the mutation E1:Gly91Asp (Kielian et al., 1996). The simultaneous presence of sphingolipids results in an optimal positioning of cholesterol in the target membrane, creating the E1 binding site, proposed above (7.2), resulting in stable E1 binding to cholesterol involving the $3\beta$-hydroxyl group of cholesterol. The importance of the positioning of the 3-hydroxyl group has been reported earlier (Kielian and Helenius, 1984). Recent structural data on trimers of the E1 ectodomain formed by acidification in the presence of sphingomyelin and cholesterol show the presence of rings of several complexed trimers (Gibbons et al., 2003, 2004). We propose that stable association of E1 homotrimers with the binding site created by sphingolipids and cholesterol subsequently brings the homotrimers together, resulting in the formation of a ring of trimers which eventually drive fusion of the viral membrane with the target membrane (Fig. 7.1; Gibbons et al., 2003, 2004). Sphingolipids may exert their additional function in the fusion process itself (Chapter 3) by promoting the ring formation.

### 7.5 Perspectives

The last three years have witnessed major progress in the field of alphavirus research. In 2001 the crystal structure of SFV E1 was resolved. The protein turned out to be surprisingly similar to the fusion protein E of tick-borne encephalitis (TBE) (Lescar et al., 2001). This result led to the grouping of alphaviruses together with flaviviruses in the category of viruses with “class II” fusion proteins in order to distinguish them from viruses having structurally different “class I” fusion proteins. The class I fusion proteins are represented by the influenza virus glycoprotein hemagglutinin (HA), which was the first viral envelope glycoprotein for which the 3-D structure was resolved at the atomic level (Skehel and Wiley, 2000). TBE is a member of the family Flaviviridae, which also includes yellow fever virus, hepatitis C virus, and
Figure 1. Proposed model of the sequence of conformational changes occurring in the viral spike upon a low-pH trigger. See text for details. Chol=cholesterol.
dengue virus, and the similarities among the fusion proteins suggested that alphaviruses may have the same fusion mechanism as flaviviruses. Because of major structural differences between class I and class II fusion proteins, the class II fusion mechanism was thought to be fundamentally different from class I membrane fusion. However, as described in Chapter 1, very recent structural data appear to suggest that, despite the structural differences between the fusion proteins, the fusion mechanisms could well be surprisingly similar (Gibbons et al., 2003; Gibbons et al., 2004; Modis et al., 2004, Bressanelli et al., 2004).

The research presented in this thesis contributes to the understanding of the precise fusion mechanism of alphaviruses and possibly other viruses having class II fusion proteins. Although the results presented in this thesis provide novel clues with regard to the involvement of cholesterol and sphingolipids in the fusion process of alphaviruses, and the role of the viral fusion protein in the process, the model presented in Fig. 7.1 remains hypothetical and lacks specific details. For example, it is not clear what the difference is between the reactive state and the non-reactive state of E1. It is also not clear how cholesterol mediates E1 trimerization and whether this function of cholesterol is different from that in mediating virus binding to membranes. Another major question is whether lipids are indeed involved in the formation of the ring of homotrimerers that appears to form the basis of the final fusion machine. Last but not least, it is not clear whether the specific molecular features of sphingolipids, which have been shown to be involved in SFV fusion, have a function in the interaction of sphingolipids with cholesterol or in a hypothetical direct interaction of sphingolipids with the viral E1. An intriguing question is whether sphingolipids are involved in the E1 homotrimer ring formation. Thus, the riddle of the role of sphingolipids in alphavirus fusion has not yet been fully solved. In fact, the riddle is being extended, giving continued credit to the intriguing name of these lipids.

The sphingolipid requirement of SFV fusion has only been demonstrated in liposomal model systems and has not yet been verified in cells. This is mainly because of experimental difficulties. Cells do not tolerate extensive sphingolipid depletion. Yet, since fusion of alphaviruses requires only very low levels of sphingolipid in the target membrane, such extensive depletion appears to be necessary to unequivocally demonstrate that sphingolipids are crucial for alphavirus cell entry. On the other hand, the cholesterol requirement of SFV fusion has been demonstrated in liposomal model systems and in cells, indicating that the results acquired from model systems can be translated to the situation in vivo. Therefore, we believe that sphingolipids are indeed required in the cell entry mechanism of alphaviruses. Sphingolipids appear to be critically involved in the creation of a membrane binding site for the viral E1 fusion protein probably in between rafts in the endosomal membrane. Interaction with this binding site results in E1 trimerization and stable binding of the virus to the endosomal membrane. This is followed by membrane fusion and delivery of the viral genome to the cytosol, initiating the infection process and the production of progeny virus proceeds.

The primary aim of the work presented in this thesis was a further elucidation of the molecular mechanisms underlying cell entry and membrane fusion of alphaviruses, SFV and SIN in particular. Although SFV and SIN are minor human pathogens, we hope that the results that were obtained with these viruses will contribute to a further
understanding of the cell entry mechanisms of viruses with class II viral fusion proteins in general. These include major human pathogens like dengue virus, yellow-fever virus, hepatitis C virus, and tick-borne encephalitis virus. A thorough understanding of the cell entry mechanisms of class II viruses is likely to facilitate the development of antiviral drugs interfering with infection at the level of virus entry into the host cell.

7.6 References

Summarizing discussion


