REVIEW ARTICLE

Entry and uncoating of enveloped viruses

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INTRODUCTION

Understanding of the entry pathway of enveloped viruses has increased due to extensive research efforts during the last few years. Detailed information is now available especially for orthomyxoviruses (e.g. influenza A) and alphaviruses [e.g. Semliki Forest virus (SFV)]. The pathway that has emerged is probably not only used by these two virus families but also by others, including rhabdo- and retro-viruses. The pathway can be divided into four stages: (i) virus attachment; (ii) internalization via coated vesicle and transfer to endosomes; (iii) low-pH-triggered fusion of the virion membrane with the endosome membrane; and (iv) virus uncoating, resulting in release of the genome, which in this way becomes susceptible to the cellular replication machineries.

In this review, we will discuss stages (i) and (ii) briefly and then focus on membrane fusion and especially on virus uncoating. Uncoating is the process that opens the rigid structure of the nucleocapsid which contains the viral genome. The mechanism of virus uncoating has long been of interest, as it could be a possible target for antiviral therapy. The therapeutical prospect becomes even more intriguing assuming the possibility that uncoating could be a step solely mediated by viral proteins and not involving host-cell proteins.

The best-studied viruses, with respect to entry and uncoating, are influenza A and SFV. This review will therefore mainly compile data from these two prototypes.

VIRUS ATTACHMENT AND INTERNALIZATION

An overview of the life cycle of an enveloped virus is given in Figure 1. To begin a successful infection, the virus binds to the surface of a susceptible cell (reviewed by Marsh and Helenius, 1989) by means of surface proteins of the virion interacting with structures on the target cell. The binding properties determine the viral tropism. Some viruses bind with high affinity and specificity [e.g. human immunodeficiency virus (HIV) binding to CD4 on T-cells] and therefore have a narrow host range. Others bind with considerably higher specificity to molecules that are abundant on various cell types (e.g. influenza virus, which binds to cell-surface sialic acid residues). A number of viruses have a broad host range and can bind to several molecules on the cell surface. It has been shown that viruses could infect cells devoid of their receptor and that viruses could undergo fusion with liposomes (White and Helenius, 1980). Nevertheless, the binding of the virion to its receptor probably facilitates entry into the cell by providing an initial physical association between the surface and the virus particle.

Many viruses, including a number of non-enveloped viruses, have been shown to be internalized via endocytosis. There are mainly three lines of evidence that support the view that virions are taken up by endocytosis (reviewed by Kielian and Helenius, 1986; Marsh and Helenius, 1989; Koblet, 1990): (i) they require low pH to trigger membrane fusion; (ii) lysosomotropic weak bases increasing the pH of endosomal organelles inhibit virus entry; and (iii) morphological studies have shown virion particles trapped in endosomes and fusing with endosomal membranes. However, some enveloped viruses are able to penetrate directly at the plasma membrane (paramyxo-, herpes-, retro-, and corona-viruses). Accordingly, they do not need low pH values to trigger membrane fusion.

MEMBRANE FUSION

The acidic pH (5–6) within the endosome triggers fusion of the virion membrane with the endosomal membrane. Due to the fact that the virus-induced fusion reaction is the only membrane fusion so far known to be catalysed by identified viral proteins, i.e. the envelope spike proteins, it has been extensively studied (for reviews see White et al., 1983; Stegmann et al., 1989; White, 1990, 1992; Bentz, 1991). Despite this, the molecular mechanism of virus-induced fusion is not yet fully understood. For viruses that enter the cell by the endocytotic route, an acid-induced conformational change in the fusogenic spike proteins starts the fusion procedure (Skehel et al., 1982; Kielian and Helenius, 1985).

In the case of influenza virus, the fusion protein is haemagglutinin (HA). HA is one of the best-characterized proteins. The three-dimensional structure of the HA ectodomain (Wilson et al., 1981) has contributed much to the understanding of the fusion mechanism. The trimeric form consists of a fibrous stem domain protruding from the membrane and holding a globular head domain. The length of the trimer, from the junction with the membrane to the distal tip of the globular head is approx. 135 Å. Each subunit contains a fusion peptide, an N-terminal sequence containing many hydrophobic amino acids. The fusion peptide is thought to interact with the target bilayer (Harter et al., 1988, 1989; Stegmann et al., 1991; Tsurudome et al., 1992). Mutations within the fusion peptide can impair fusion (Daniels et al., 1985; Gething et al., 1986). In the native conformation, the fusion peptides are buried within the stem region, approx. 100 Å away from the top of the trimer. It is believed that at low pH, the globular head domains partially dissociate (Kemble et al., 1992), thereby exposing the previously buried N-terminal fusion peptides. In the native HA, a three-stranded coiled-coil domain of the stem is preceded by a loop region. Carr and Kim (1993) recently proposed that at low pH, the coiled coil is extended to

Abbreviations used: SFV, Semliki Forest virus; HIV, human immunodeficiency virus; HA, haemagglutinin; SIN, Sindbis virus; vRNPs, viral ribonucleoproteins.

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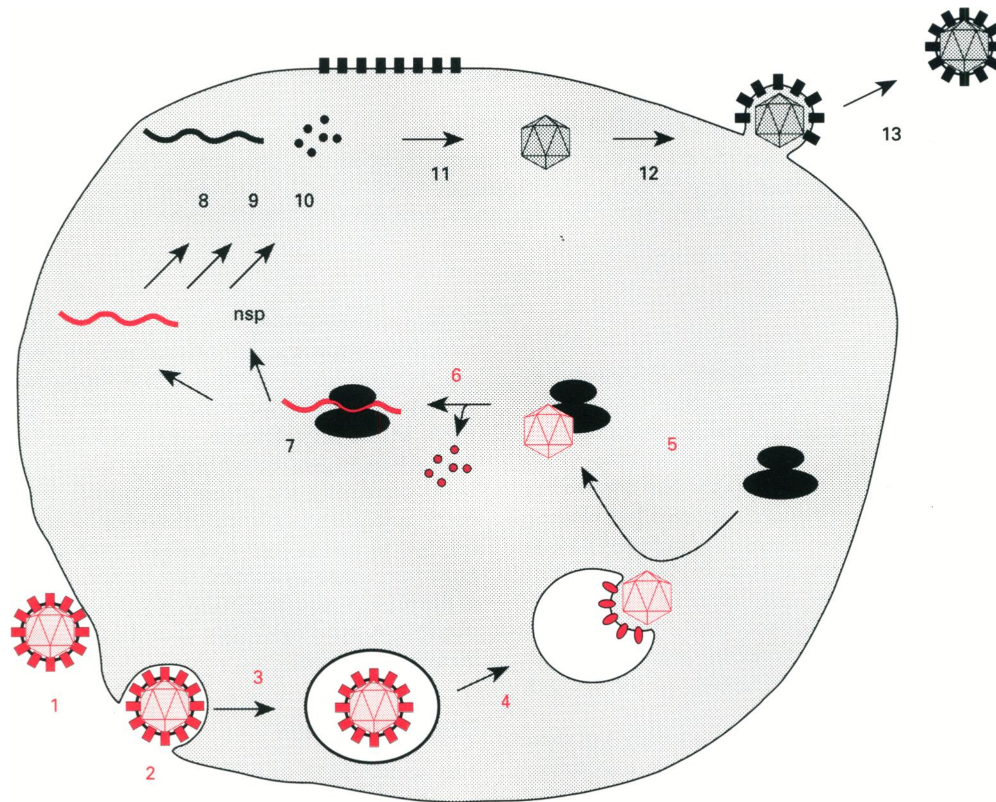

Figure 1 The life cycle of alphaviruses: penetration, uncoating and replication

Red numbers indicate penetration and uncoating stages: 1, binding; 2, uptake via coated pits, endocytosis; 3, acidification of endosome; 4, conformational change of spike, membrane fusion and release of capsid; 5, association of capsid with ribosome; 6, uncoating. Black numbers indicate replication stages: 7, synthesis of non-structural proteins (nsp); 8–10, replication of 49S RNA, synthesis of 26S RNA and synthesis of structural proteins; 11, assembly of capsids; 12 and 13, assembly and budding of progeny virus.

include the whole loop region and an adjacent helix. This would relocate the fusion peptides by 100 Å toward the target membrane. The hypothesis has been substantiated by measurements of the conformations of peptides corresponding to the domains of HA involved. It provides an interesting working model to the question of how the conformational change of HA pilots the fusion peptides to interact with the target bilayer. The SFV E_1 protein also contains a fusion peptide (Garoff et al., 1980a), and its involvement in fusion is supported by site-directed mutagenesis (Levy-Mintz and Kielian, 1991).

Biophysically, the initiation of the fusion reaction mediated by viral spike proteins encompasses: (i) recognition of the appropriate target membrane; (ii) approach of the two membranes into molecular contact; and (iii) breakdown of the hydration barrier, which represents the major repulsive physical force between bilayers (Rand, 1981). The approach of the membranes into molecular contact is presumably realized by the insertion of the fusion peptide into the target membrane, providing a molecular bridge between two bilayers. What drives the breakdown of the hydration barrier, however, remains elusive. In this context, it is apparently energetically most favourable if the fusion is initiated locally at a focal site in a controlled manner. Such a situation is created by the viral spike proteins. It has been proposed that several HA trimers assemble to form a collar or ring (Bentz, 1991; White, 1992; Tse et al., 1993). The mixing of lipids from the outer leaflets is then initiated in the interior of this ring. It is thought that the fusion peptides align along the interior of the fusion pore creating a hydrophobic channel, thereby promoting lipid mixture from viral and target membrane. Alternatively, the fusion peptides might bind to the outer leaflets of both membranes, inducing a non-bilayer structure in the interior of the aggregate. The protein-ring model is based mainly on electrophysiological data (Spruce et al., 1989, 1991; Lanzrein et al., 1993a; Tse et al., 1993). These studies describe the existence and properties of a so-called fusion pore, the molecular structure that transiently connects the lumens of two compartments during their fusion. It has been shown that cell-membrane fusion induced by influenza virus HA began with the abrupt opening of a fusion pore, 1–1.5 nm in size (Spruce et al., 1989, 1991). The pore exhibited flickering, i.e. reversible openings and closings, a phenomenon common to ion channels. In an elegant study, combining capacitance measurements and video imaging, it was shown that lipid flux between fusing membranes started after establishment of intercellular conductance, i.e. when the pore size had reached a certain threshold (Tse et al., 1993). If the fusion pore was purely lipidic, lipid flux would have to start before, or concomitant with, the occurrence of intercellular conductance. Hence, the early HA fusion pore is presumably made of a ring of proteins (possibly containing immobile lipids as well). The pore expands later by lipid flux into its circumference, which disjoins the protein ring. The early stages of SFV-induced cell–cell fusion have been studied by using the double-patch-clamp technique (Lanzrein et al., 1993a). The development of intercellular conductance after initiation of fusion could be divided into two stages. The first stage was characterized by abrupt transitions to several stable levels of intercellular

conductance, consistent with the opening of several fusion pores. In contrast with influenza virus no flickering was observed, the pores remained stable for up to several seconds. The second stage consisted of a more gradual increase in conductance, implying a gradual dilation of the previously formed fusion pores. These observations might be interpreted in agreement with the model for influenza put forward by Almers and colleagues (Tse et al., 1993). Hence, the first stage represents openings of proteinaceous fusion pores, whereas the second stage represents pore dilation due to lipid flux into its circumference. Fusion pores with similar properties were also described for the fusion involved in secretion (reviewed in Almers, 1990; Monck and Fernandez, 1992).

An alternative model for virus-induced membrane fusion predicts that the function of HA or other viral fusion proteins is only to bring two bilayers so close together that they start to fuse on their own. Fusion begins with the formation of a lipidic intermediate, possibly an inverted micellar structure (Rand and Parsegian, 1986; Bentz, 1991). In this case, lipid mixing commences before the opening of an electrolytic connection between the fusing compartments (fusion pore). Interestingly, it was recently reported that glycosylphosphatidylinositol (GPI)-anchored HA mediated lipid mixing (hemifusion) but not membrane fusion (Kemble et al., 1994). This finding however, does not necessarily support the hypothesis of lipid mixing starting on its own after membrane approach, because lipid motion in the fusion pore might be constrained by the HA transmembrane domain (Tse et al., 1993). More work will definitely be needed to assess the validity of the models describing the early events in fusion.

The membrane-fusion reaction in the endosome liberates the virus from its lipid envelope and provides access for the nucleocapsid to the cytosol. In order to make the genome accessible for subsequent translation and replication, the capsid has to be opened, or fall apart. Conceivably, this must be a triggered process, because according to current knowledge, the nucleocapsid might not be disassembled in the cytosolic environment as such. This can be concluded from the fact that only a few hours after entry in the same cell, newly formed progeny nucleocapsids remain intact and will perform successful budding.

UNCOATING OF INFLUENZA A VIRUS

The proposed structure of influenza A virus is shown in Figure 2. The virus genome consists of eight separate, negative-stranded RNA molecules. These are individually packed into viral ribonucleoproteins (vRNPs). vRNPs and the matrix protein M1 assemble together to form the capsid structure (reviewed by Lamb and Choppin, 1983; Lamb, 1989). The uncoating of the influenza capsid consists of dissociation of M1 from vRNPs. The vRNPs then enter the nucleus through the nuclear pores by an active mechanism (Martin and Helenius, 1991b).

In the virus, the capsid is surrounded by a membrane that contains three proteins: HA, neuraminidase and the minor coat protein M2. Recent work has suggested that M2 plays a crucial role in the uncoating of influenza virus (Helenius, 1992).

M2 protein

M2 protein is a small (97 amino acids) transmembrane protein which has been shown to be the main target of the anti-influenza virus drug amantadine (Hay et al., 1985). The site of action of the drug is located within the transmembrane domain of the protein, as could be deduced from the sequences of drug-resistant mutants. Drug resistance is confined to single amino acid changes in this domain (Hay et al., 1985). Cross-linking experiments have shown that M2 formed tetramers stabilized by disulphide bonds, resembling the structure of a membrane channel (Sugrue and Hay, 1991). These findings allowed the suggestion that M2 forms a transmembrane channel capable of translocating ions across the membrane. This suggestion was supported by the finding that M2 modulated intracellular pH in virus-infected cells (Ciampor et al., 1992). Pinto and collaborators have provided direct evidence that M2 expressed in *Xenopus* oocytes acted as a cation-selective, pH-dependent ion channel, and moreover that channel activity was blocked by amantadine (Pinto et al., 1992).

Amantadine, the only anti-influenza virus drug known so far, specifically blocks the release of virus particles from infected cells. Susceptibility to the drug is retained if the drug is added shortly after infection (Hay and Zambon, 1984). Hence, in addition to its early effect in virus uncoating (see below), M2 is thought to have an important function in the assembly of influenza virus. The assembly of the virus at the plasma membrane is preceded by transport of HA through trans-Golgi vesicles. The HA molecules are thereby exposed to the acidic environment present in these vesicles. Consequently, a conversion of HA into its low-pH form can take place, resulting in failure of virus assembly. The M2 channel, as a cation channel, can provide the means for the regulation of pH of the trans-Golgi vesicles in order to prevent the conversion of HA into the low-pH form (Sugrue et al., 1990; Ruigrok et al., 1991; Steinhauer et al., 1991; Grambas and Hay, 1992; Grambas et al., 1992).

More important in our context was the discovery that the dissociation of M1 from vRNPs was inhibited by amantadine (Bukrinskaya et al., 1982; Martin and Helenius, 1991a). Therefore, M2, which is the target of amantadine, most probably has a crucial role in influenza virus uncoating. The work of Pinto et al. (1992) showed that the M2 channel opened at low pH. Hence, when the virion stays in the endosome during entry, the channel should be opened due to the low intracompartmental pH and protons should enter the virion at this stage. Accordingly, the following model for the mechanism of influenza virus uncoating could be postulated: incoming virus particles encounter mildly acidic pH conditions in the endosome which trigger membrane fusion and activation of the M2 channel. M2 mediates an influx of protons into the virion, which in turn triggers nucleocapsid disassembly. Support for this model was provided by an *in vitro* study with detergent-solubilized virions, demonstrating that the interaction of M1 and vRNPs was disrupted upon exposure to mildly acidic pH (Zhirnov, 1990).

Since M2 is a cation channel activated at low pH values, it is probably contributing to uncoating. However, some observations question whether M2 is solely responsible for the proton translocation in the endosome. There are two major facts that could imply involvement of complementary factors: first, although M2 is found abundantly in the plasma membrane of infected cells, it is greatly under-represented in virions, as only 4–16 channels are incorporated into the envelope (Zebedee and Lamb, 1988). Secondly, the role of amantadine early in infection is so far not fully understood. Some strains, e.g. the Rostock strain, are particularly sensitive to amantadine late during the infectious cycle but remain relatively insensitive at the early stage (Hay and Zambon, 1984). One possible explanation for these findings could be that other viral proteins, e.g. HA, might serve complementary roles in the modification of the pH within the virion upon its entry. Indeed, there are indications that influenza virus HA can function as a channel or pore. It has been reported that influenza HA, constitutively expressed in GPbind4-cells, induced a proton influx into the cell if the extracellular pH was set below the threshold pH required for fusion (Kempf et al., 1987). Other groups have reported that influenza virus (Patel and Pasternak,

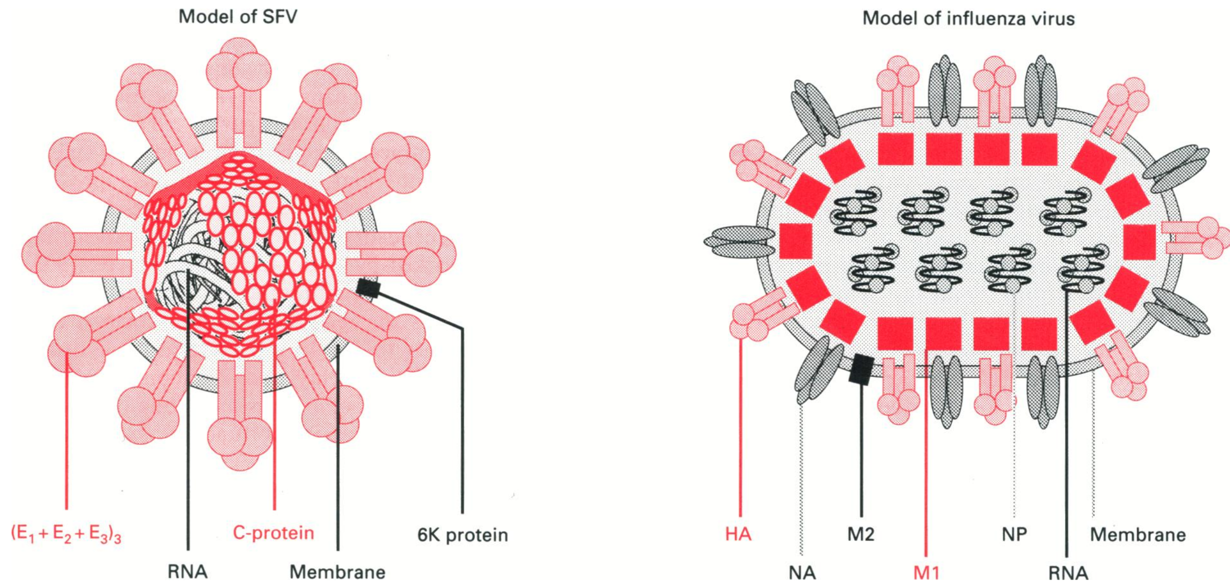

Figure 2 Schematic drawing of the structural models for SFV and influenza A virus

1983; Kobrinskij et al., 1992) or HA, expressed in 3T3-cells (Sarkar et al., 1989), caused unspecific alterations in membrane permeability upon exposure to low pH conditions (e.g. pH 5). Thus these results suggest that the low-pH form of influenza HA increases the permeability of a cell membrane for protons and could therefore be involved in uncoating.

UNCOATING OF ALPHAVIRUSES

Structure of alphaviruses

The two most prominent and best-studied members of the alphavirus genus (family: togaviridae) are Semliki Forest virus (SFV) and Sindbis virus (SIN). The structure of SFV and SIN is well-documented (Figure 2). The particle has a radius of 23 nm and carries 80 surface projections (spikes) 6–10 nm in length, anchored in the lipid bilayer. The spikes consist of trimers of a protein composed of three subunits E_1, E_2 and E_3 (50.786, 51.855 and 11.369 kDa respectively). The polypeptide composition of one spike is therefore $(E_1E_2E_3)_3$. In SIN, E_3 is lacking. The amino acid sequences of the spike proteins are known (Garoff et al., 1980a). The spikes are arranged on a T = 4 lattice (von Bonsdorff and Harrison, 1978; Adrian et al., 1984; Vogel et al., 1986; Choi et al., 1991). The envelope additionally contains low amounts (3% compared with the spike proteins in SFV) of a small transmembrane protein, the 6K protein (Lusa et al., 1991) which is thought to be involved in the regulation of virus budding (Liljeström et al., 1991; Gaedigk-Nitschko and Schlesinger, 1991). The nucleocapsid is composed of 240 copies of the C-protein (30 kDa) (Coombs and Brown, 1987) that are most likely also arranged in a T = 4 quasisymmetry (Choi et al., 1991). The equivalent symmetries of the spikes and the nucleocapsid suggest an interaction between the cytoplasmic tails of the spike proteins and the C-protein, which in turn would drive virus budding. Most plausibly, the cytoplasmic domain of E_2 interacts with C (Suolamainen et al., 1992), as the E_1-tail was shown to have no role in budding (Barth et al., 1992). However, direct proof is lacking. The nucleocapsid contains the single-stranded 11.5 kb (42S) RNA genome of positive polarity. The entry pathways of SFV and SIN are currently being investigated by several groups. Detailed information is now available about the fate of core proteins during the infectious cycle, allowing the development of concepts about the uncoating mechanism.

Fate of the incoming nucleocapsid and the core proteins

Recently, Singh and Helenius (1992) have analysed the fate of incoming SFV C-protein in BHK-21 cells. It was found that incoming virus capsids disassembled very rapidly, i.e. within 1–2 min of the nucleocapsids entering the cytoplasm. The C-protein appeared to bind to 60S ribosomal subunits and uncoating was dependent on ribosomes. If Triton X-100-solubilized virions were added to isolated ribosomes, the capsids disassembled. These results are in agreement with previous work on SIN, where incoming cores were also found to bind to the large ribosomal subunit (Wengler et al., 1984, 1992). The specific ribosome-binding site on the C-protein has been localized between amino acids 94 and 105, which is a highly conserved sequence among alphaviruses (Wengler et al., 1992). Newly formed SFV C-protein has also been reported to bind to ribosomes (Ulmanen et al., 1979).

Wengler and Wengler (1984) have proposed a model for the mechanism of alphavirus nucleocapsid uncoating. According to this model, incoming capsids are disassembled by an interaction with ribosomes. The stability of progeny capsids later in infection is maintained by saturation of ribosomes by the capsids themselves. Thus incoming and exiting capsids may have the same conformation. The model assumes that the specific ribosome-binding site on the C-protein is exposed on the surface of the nucleocapsid. However, for structural reasons, this supposition is controversial. The core protein of alphaviruses is composed mainly of two domains (Figure 3). There is a globular domain containing a serine proteinase active site at the C-terminus. The crystal structure of this region, beginning with residue 114 in the SIN capsid protein, could be determined (Choi et al., 1991; Tang et al., 1993). The structure of the N-terminal domain has not been resolved. It contains numerous positively charged residues and is thus thought to interact with the viral RNA (Garoff et al.,

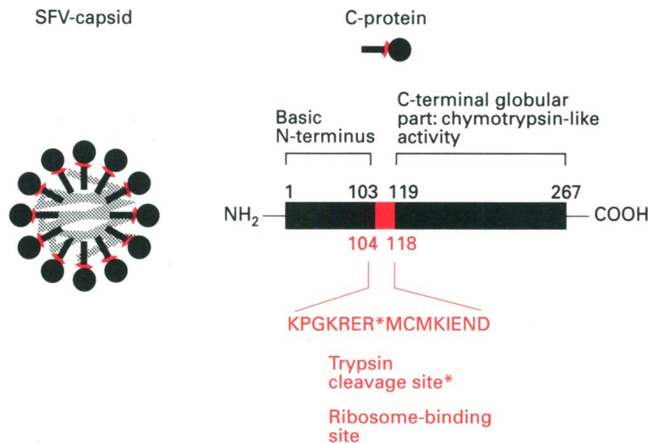

Figure 3 Schematic drawing of the SFV-nucleocapsid structural model derived from cryoelectromicrographic and crystallographic studies (Vogel et al., 1986; Choi et al., 1991)

The C-protein is composed of a C-terminal globular domain and a basic N-terminal domain. The two domains are separated by a short, highly conserved stretch of 15 amino acids that is shown in red. This stretch contains the ribosome-binding site, and a trypsin cleavage site inaccessible in the native capsid.

1980b). Consequently, this region is more likely to be buried inside the core. The ribosome-binding site is located between these two domains and it is thus uncertain whether it is exposed on the surface of an intact nucleocapsid (see Figure 3). Recent work suggested the binding site for viral RNA is most probably located in exactly the same region, namely between amino acids 97 and 106 in the SIN capsid protein (Geigenmüller-Gnirke et al., 1993). Furthermore, within the ribosome-binding/RNA-binding site there is a trypsin cleavage site (Wengler et al., 1992). Strong and Harrison (1990) have shown that this cleavage site was not accessible to trypsin in intact nucleocapsids. In order to get trypsin cleavage, separating the globular C-terminal part from the basic N-terminal stretch, the nucleocapsids had to be unravelled by addition of high concentrations of NaCl.

Altogether, these structural and biochemical data suggest that the ribosome-binding site is not directly accessible in the intact capsid. Hence, it might be that the incoming capsid changes its conformation and thereby exposes the ribosome-binding site. However, it was also shown that isolated capsids uncoated when added to cell lysates containing ribosomes (Wengler et al., 1992; Singh and Helenius, 1992). Therefore, several options have to be considered. For example, it is possible that the C-protein has two distinct ribosome-binding sites, one of which is exposed to the outside. Or there could be other unknown proteins that bind to the capsid and alter its conformation before it binds to the ribosome. But it is also possible that the capsid isolation procedure, which involves detergent solubilization of the virions, has changed the capsid conformation in a way that means the previously hidden ribosome-binding site now becomes accessible.

An important issue is the maintenance of the stability of newly formed capsids later in infection. If incoming nucleocapsids would be disassembled only by interaction with ribosomes, how could freshly assembled progeny capsids be prevented from binding to ribosomes? The Wengler model (Wengler and Wengler, 1984) suggests that ribosomes would be saturated with newly synthesized C-protein. In contrast with that, it was shown that in SFV, only 20% of the total cellular ribosomes were associated with C-protein 8 h after infection (Ulmanen et al., 1979). Moreover, cell lysates of infected or uninfected cells had similar efficiencies in uncoating Triton X-100-solubilized virus *in vitro* (Singh and Helenius, 1992). As pointed out above, it is conceivable that the capsid has to undergo a structural change during entry. Such a structural change would provide a regulatory element for disassembly/assembly.

Effect of acid exposure on the capsid conformation

During entry, the virion is exposed to acidic conditions. Several groups have investigated the effects of acidic conditions on alphavirus nucleocapsids or related nucleocapsids. Exposure of isolated SFV nucleocapsids to mildly acidic pH conditions (pH < 6) lead to a marked shrinking and altered sedimentation behaviour (Soederlund et al., 1972). Furthermore, it was shown that acid exposure of isolated capsids led to a partial auto-proteolytic cleavage of the C-protein, resulting in the formation of a 17.5 kDa fragment which could be identified as the globular C-terminal part (Schlegel et al., 1993). The cleavage indicates a conformational change in the C-protein, because the chymotrypsin-like active site is sterically inaccessible in the native conformation (Choi et al., 1991). Mauracher and co-workers (1991) have shown that nucleocapsids isolated from rubella virus, another member of the family togaviridae, released their RNA at mildly acidic pH values in the presence of Triton X-114. It is not clear to what extent the findings on isolated capsids represent the situation in the intact virus. Stubbs et al. (1991) used purified SIN for X-ray solution scattering studies. It was found that exposure to low-pH conditions did not alter the scattering density in the capsid shell, indicating that the capsid's diameter was not changed. This does not however rule out a change in fine structure not resolvable with this method. In contrast, intact SFV particles exhibited marked capsid shrinking at low pH (Schlegel et al., 1991). Hence, SFV and SIN capsids might differ in their behaviour at low pH, despite the high sequence identity of their C-proteins.

In the endosome, the virus capsid is probably exposed to acidic conditions, since alphaviral proteins have channel activities (see next section). An acid-induced conformational change in the alphavirus capsid during entry could have several biological functions. First, it might lead to exposure of the previously hidden ribosome-binding site, enabling uncoating to occur and providing a regulatory element for assembly/disassembly. Secondly, it is also conceivable that the acid-induced change in conformation is not strictly necessary but that it would destabilize the rigid structure and thereby prime the capsid for more efficient uncoating induced by ribosomes. In fact, both mechanisms could operate in a redundant or complementary fashion. An interesting possibility is that shrinking observed in SFV might help to disrupt the C-protein–E_2 interaction. This might be an important step, ensuring the capsid's disconnection from the virion membrane. It is not known whether the lateral forces imposed on the virion membrane during fusion alone are sufficient to achieve this separation.

Do alphavirus proteins have channel activities?

This section summarizes recent findings that describe channel- or pore-forming activities of alphavirus envelope proteins, apparently indicating certain parallels to what has been described for the influenza virus M2 protein. By analogy to influenza and with respect to the effects of acid exposure on alphavirus capsids, alphaviral channels might play an important role in uncoating. It was shown by electron microscopy and sedimentation analysis that exposure of purified SFV to pH 5.8 leads to a shrinking of the nucleocapsid (Schlegel et al., 1991). The shrinking of the

capsid indicates exposure to low-pH conditions (Soederlund et al., 1972), i.e. the interior of the virus particle must have been acidified. The shrinking effect was dependent on the presence of intact spike proteins, as protease digestion of the spike proteins abolished the shrinking. These experiments were in fact the first to provide evidence for an ion flux through a virus envelope membrane and they indicated that the spike protein ectodomains might mediate this ion flux. Additional studies, using SFV-infected insect cells, revealed that the putative channel formed by SFV envelope proteins was an unspecific pore, allowing passage of ions and molecules up to 900 Da (Lanzrein et al., 1992). The ability of SFV-envelope proteins to form pores in membranes at low pH was confirmed using the patch-clamp technique (Lanzrein et al., 1993b). SIN exhibited similar pore-forming activities (F. Kaesermann and C. Kempf, unpublished work). The SFV-induced change in membrane permeability was found to be sensitive to millimolar concentrations of Zn^{2+} and Ca^{2+}. These ions are known to block permeability changes induced by pore-forming agents, such as bacterial toxins and viruses (Bashford et al., 1986). Thus under physiological conditions, which means in the presence of 2 mM Ca^{2+}, the pores are blocked.

Young et al. (1983) have analysed the effects of Sendai, influenza, SFV and vesicular stomatitis virus on planar bilayer membranes. They found that the viruses only elicited changes in conductance when freeze–thawed and concluded that the changes resulted from incorporation of damaged viral membranes into the planar bilayer by fusion. The data from planar bilayers contrast with recent findings from both alphaviruses and influenza virus. As for the latter, it is now generally believed that its M2 protein has a channel-forming activity. The reason why no additional currents were detected after fusion of intact alphavirus might be that the measurements were carried out in buffer containing 3 mM Ca^{2+}, where alterations in conductance caused by viral proteins could have been blocked (Lanzrein et al., 1992, 1993b). In another publication (White and Helenius, 1980), the fusion of SFV and liposomes was found to be non-leaky with respect to high-molecular-mass compounds (> 10 kDa), confirming the notion that the alphavirus pores pass only low-molecular-mass compounds (Lanzrein et al., 1992).

Which of the envelope proteins could be involved in pore formation? SFV and SIN envelopes contain a small integral membrane protein, the 6K protein. This protein is, like the M2 protein, abundant in the plasma membrane of infected cells, but under-represented in virions (Lusa et al., 1991). It has been proposed as a candidate for a putative ion channel. However, there are several lines of evidence that exclude a function of 6K in alphavirus uncoating. (i) Proteolytic digestion of SFV particles abolished the proton influx, indicating that the ectodomains of the SFV spike proteins are involved in the formation of the channel (Schlegel et al., 1991). (ii) A 6K deletion mutant was as infectious as wild-type virus, and therefore 6K does not seem to be required for virus penetration (Liljeström et al., 1991). (iii) The same mutant did not elicit any changes in membrane permeability of infected cells (M. Dick and C. Kempf, unpublished work). Hence, it appears that the 6K protein does not possess channel activity and is not involved in uncoating. Consequently, it may be the SFV spike protein, composed of the three subunits E_1, E_2, and E_3, that could be responsible for the pore-forming activity. This proposal is corroborated by the finding that the low-pH-induced increase in permeability of infected cells could be strongly impeded by preincubation with a monoclonal antibody directed against E_1, but not by antibodies against E_2, suggesting that E_1 is involved in the low-pH-induced pore formation (Lanzrein et al., 1994). Interestingly, it was reported that under low-pH conditions, E_1 dissociated from the

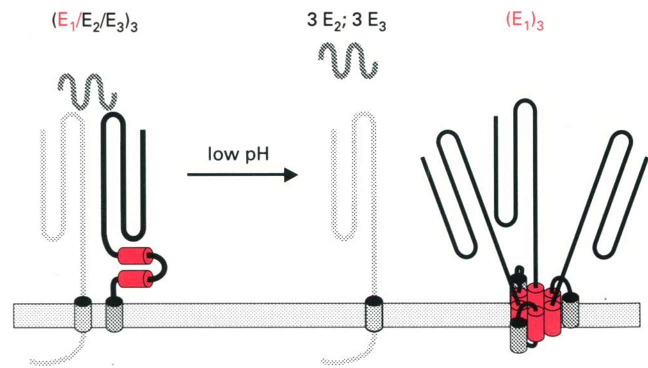

Figure 4 Hypothetical model of a pore formation by the SFV-E_1 protein

The E_1 protein contains a putative membrane-associated amphipathic helix (V352–A372) with a channel motif (Lear et al., 1988; Degrado and Lear, 1990). It has been speculated that this helical domain might insert into the membrane at low pH (Kempf et al., 1990; Schlegel and Kempf, 1992). The model also predicts that E_1 subunits oligomerize into trimeric or possibly higher oligomeric states.

E_1–E_2–E_3 complex to form homotrimers or even higher homo-oligomers (Wahlberg et al., 1992; Wahlberg and Garoff, 1992; Bron et al., 1993). E_1 dissociation was blocked in a mutant deficient in cleavage of p62, the precursor of E_2–E_3 (Salminen et al., 1992). The same mutant failed to elicit permeability changes in cell membranes (M. Dick and C. Kempf, unpublished work).

Although it is pure speculation to date, one might assume that the E_1 homo-oligomers could form a pore in a barrel-stave-like structure (Ojcius and Young, 1991). It is also conceivable that E_1 oligomers might constitute the SFV fusion pore (Lanzrein et al., 1993a), as it was shown that E_1 is the subunit that catalyses fusion (Omar and Koblet, 1988). It can further be speculated that the permeability pore represents a fusion pore that has not contacted a target membrane and therefore forms a pore in the residing membrane. A putative model for pore formation in SFV is shown in Figure 4. As outlined above, there is strong evidence that alphavirus envelope proteins, most likely the spike proteins, might function as channels or pores in the absence of Ca^{2+}. The extracellular fluid usually contains about 2 mM Ca^{2+}. The concentration of Ca^{2+} in the endosome is not known. However, it has been demonstrated that the endosome membrane is permeable to Ca^{2+} (Diaz et al., 1989). The cytoplasmic Ca^{2+} concentration is usually approx. 100 nM. Hence, one can expect a low Ca^{2+} concentration in the secondary endosome due to leakage of Ca^{2+} from endosomes to the cytosol down the large concentration gradient. For the incoming viruses, this would give rise to an increased permeability of the envelope membrane to ions and small molecules. However, it should be mentioned that there is one older report that apparently conflicts with this concept: White et al. (1980) found that low-pH-induced fusion of SFV with the plasma membrane of susceptible cells led to successful infection in the presence of millimolar Ca^{2+}.

Model for alphavirus uncoating

A hypothetical model for alphavirus uncoating is presented in Figure 5. It relies on the findings discussed in the previous sections, namely the pore-forming activities of alphavirus spike proteins, the effects of acid exposure on capsids and the evidence that capsids have a specific ribosome-binding site. Thus low-pH conditions in the endosome trigger a conformational change in the spike proteins, leading to pore formation. Inflowing protons

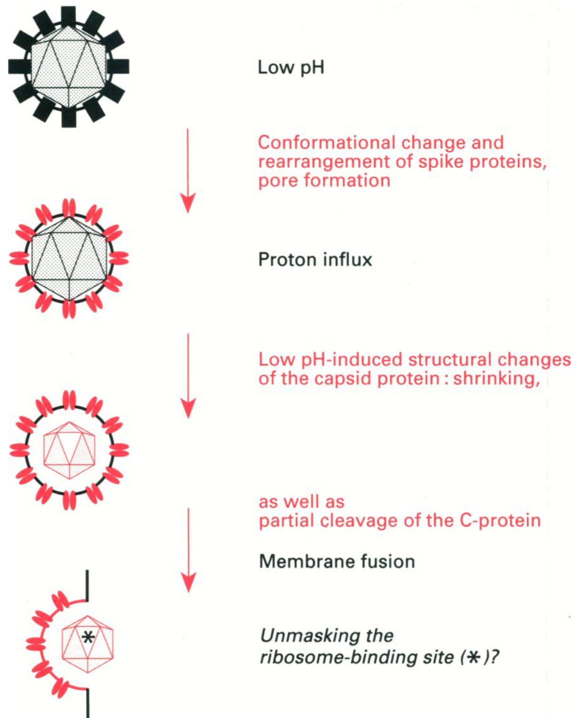

Figure 5 Model of how proton influx triggers capsid disassembly

Uncoating occurs in endosomes, where low pH (5–6) activates the spike proteins to form pores in the viral membrane, as well as to catalyse membrane fusion. Pore formation changes the pH inside the virion, thereby inducing structural changes such as autoproteolytic cleavage. This could lead to either disassembly of the capsid or unmasking of the ribosome-binding site. In the latter case, subsequent binding to ribosomes would accomplish the uncoating process.

induce structural changes in the capsid. Such changes might expose the ribosome-binding site and capsid shrinking might help to loosen the interaction with the E_2 tail. Finally uncoating is accomplished after binding to the ribosome large subunit. It should be emphasized that the model presented here is speculative, although there are plenty of data supporting it. Nevertheless, it is also conceivable that alphavirus uncoating is exclusively based on the interaction with ribosomes (Figure 1; for this alternative view see Wengler and Wengler, 1984; Singh and Helenius, 1992).

Conclusions

The entry pathways of influenza and alphaviruses are now remarkably well documented. Both pathways involve a pH-dependent membrane fusion within the endosome. Recent electrophysiological studies have revealed that one of the earliest events in fusion is the opening of a proteinaceous pore connecting the two fusing bilayers. The pore can have a lifetime of several seconds before it widens due to lipid flux. It is believed that fusion proteins oligomerize to form a ring attached to both fusing membranes. This structure will facilitate lipid contact and initiate controlled lipid mixing at a local point.

The fusion is followed by the disassembly of the viral nucleocapsid. For influenza virus, it is now generally believed that this step is triggered by an influx of protons into the virion mediated by the M2 protein. The protons stimulate the dissociation of M1 and the vRNPs. Recent data suggest that alphavirus uncoating could include a similar mechanism as influenza virus.

It may well be that other viruses that enter the cell by endocytosis also use analogous mechanisms for uncoating. For example vesicular stomatitis virus, a rhabdovirus, appeared to exhibit pore-forming activities similar to SFV (F. Kaesermann. and C. Kempf, unpublished work).

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