Low pH-induced pore formation by spike proteins of enveloped viruses

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Exposure of Aedes albopictus cells infected with Semliki Forest virus (SFV; Togaviridae) to mildly acidic pH (5-6) results in a dramatic increase in the host cell membrane permeability due to pore formation by the virus spike proteins. Identical results were obtained when the cells were infected with two other viruses, Sindbis virus (SIN, Togaviridae) and vesicular stomatitis virus (VSV, Rhabdoviridae). This permeability change could also be observed on isolated virions of SFV, SIN and VSV by measuring the influx of propidium iodide, a nucleic acid-specific fluorescent marker, into the virions. This influx was dependent on the presence of the ectodomains of the viral spikes and could be hampered by zinc ions. Furthermore, haemagglutinin, a membrane protein of influenza A virus (Orthomyxoviridae), expressed in Aedes cells induced a change in membrane permeability identical to that induced by the spike proteins of SFV, SIN and VSV when exposed to low pH. Thus acid-induced membrane permeability changes produced by spike proteins of three different virus families could be demonstrated in infected cells as well as in virions. Therefore, the low pH-induced pore formation by viral spike proteins seems to be more than an event specific for togaviruses and might well be an inherent property of enveloped viruses that use the endocytotic pathway to infect a cell.

Introduction

The entry of enveloped viruses into cells using the endocytotic pathway can be divided into the following steps: (i) virus attachment; (ii) virus uptake via coated vesicles into the endosome; (iii) low pH-triggered fusion of the viral with the endosomal membrane; and (iv) virus uncoating, resulting in release of the genome (reviewed in Marsh & Helenius, 1989; Lanzrein et al., 1994a).

One of the best-studied viruses with respect to the entry pathway is Semliki Forest virus (SFV; alphavirus, Togaviridae). SFV is composed of five structural proteins. The capsid (C) protein (240 copies), together with the positive-sense RNA, forms the nucleocapsid, which is surrounded by a lipid envelope. The envelope contains 80 spikes composed of the two major transmembrane proteins, E1 and E2, and the peripheral E3 protein (240 copies of each). Additionally, minor amounts of the small 6K protein are found in the virus membrane (approximately 3% compared to the spike proteins; reviewed in Strauss & Strauss, 1994).

Several functions have been attributed to the virus membrane proteins. The precursor of the E2 and E3 proteins, the p62 protein, may be responsible for transport of the E1 protein to the plasma membrane. The E1 protein is involved in the acid-induced fusion process (Kondor-Koch et al., 1983). At mildly acidic pH the spike proteins undergo a conformational change that results in the dissociation of the E1–E2–E3 complex, the formation of a E1 homotrimer and the exposure of a fusion peptide on the E1 protein (Justman et al., 1993). Furthermore, the conformational change of E1 leads to a permeability change in the membrane due to the formation of a pore (reviewed in Lanzrein et al., 1994a). This pore formation is dependent on the ectodomain of the spike and was detected in infected cells expressing the virus proteins (Lanzrein et al., 1992) as well as in isolated virions (Schlegel et al., 1991). Several lines of evidence have shown that the E1 protein plays a crucial role in the pore-forming process (Lanzrein et al., 1994b; Spyrl et al., 1995; Dick et al., 1996). It was also shown that the 6K protein modifies membrane permeability after its expression in Escherichia coli (Sanz et al., 1994).

Another well-studied virus is influenza A virus (Orthomyxoviridae). The membrane of influenza A virus contains three proteins: haemagglutinin (HA), neuraminidase and M2. It is
known that HA is responsible for virus fusion (Sarkar et al., 1989). The M2 protein was shown to form a cation-selective, pH-regulated ion channel (Pinto et al., 1992) and to modulate the intracellular pH in infected cells (Ciampor et al., 1992). It is believed that pH regulation by M2 in the trans-Golgi network plays an important role in virus assembly by hindering the acid-dependent conformational changes in HA during its export via the trans-Golgi network, thus preventing the fusion-mediated low pH form (Sugrue et al., 1990; Steinhauser et al., 1991; Grambas & Hay, 1992).

It was postulated that pH regulation and the pore-forming activity of M2 initiates the uncoating of the virus inside endosomes (Helenius, 1992). On the other hand, there are indications that influenza virus HA can also function as a channel or pore. It has been reported that HA expressed by Gpbind4 cells induces a proton influx into the cell when exposed to the low pH conditions that are required for fusion (Kempf et al., 1987). In addition, alterations in membrane permeability by influenza virus have been reported (Patel & Pasternak, 1983; Sato et al., 1983; Kobrinskij et al., 1992) and an unspecific permeability change was observed in HA-expressing 3T3 cells (Sarkar et al., 1989). These results suggest that the HA protein changes the permeability of a cell membrane when exposed to low pH. Thus, HA might form pores by itself, in a way similar to the E1 protein of SFV. Hence, it could be speculated that pore formation by spike proteins might be an inherent property of enveloped viruses that enter cells via endocytosis.

### Methods

#### Cells and media

*Aedes albopictus* cells, clone C6/36 (Igarashi, 1978), were grown at 28 °C in Mitsuhashi–Maramorosch (MM) medium (400 mosm; Amimed) supplemented with 16% fetal calf serum (FCS). 100 µg/ml streptomycin and 100 U/ml penicillin. Cells were passaged weekly at a split ratio of 1:15 dilutions. BHK-21 cells were grown at 37 °C in Glasgow medium (Gibco) supplemented with 5% FCS, 10% tryptose phosphate broth. 20 mM-NaCl. 100 µg/ml streptomycin and 100 U/ml penicillin.

#### Virus propagation

SFV, Sindbis virus (SIV) and vesicular stomatitis virus (VSV) were propagated in Aedes cells. Briefly, cells were infected with approximately 5 p.f.u. per cell. At 24 h post-infection (p.i.) the medium was harvested and cellular debris removed by centrifugation (600 g, 10 min). The virus-containing supernatant was aliquoted and stored at −80 °C. Virus titres were determined on Vero cells by endpoint titration according to established methods. Titres were calculated according to the method of Spearman (1908) and Kaerber (1931).

#### Preparation of purified virus

Aedes cells were infected with virus at a m.o.i. of 10 p.f.u. per cell. At 6 h p.i. the medium was replaced with MM medium diluted 1:10 with PBS (adjusted to 400 mosm) containing 0.2% (w/v) BSA. At 24 h p.i. the medium was harvested and cellular debris removed by centrifugation (3000 g, 10 min). The virus was purified and concentrated by centrifugation through a cushion of 12% sucrose in PBS at 130000 g for 2 h 40 min. The virus pellet was resuspended in PBS or MES-buffered saline (MBS; 20 mM-MES, 190 mM-NaCl) and analysed by SDS–PAGE. Protein concentrations were determined by the method of Lowry et al. (1951).

#### Efflux of radiolabelled marker

Aedes cells grown to confluency on 6-well tissue culture plates (TPP) were infected with virus (m.o.i. approximately 10 p.f.u. per cell) and used for experiments at 16 h p.i. The cells were washed twice with PBS pH 7.3 (400 mosm) containing 10% glucose and subsequently loaded with n-[1-3H]carnosine (AIB; Du Pont NEN) by adding 0.75 ml PBS containing 0.25 µCi AIB per well. The cells were incubated for 30 min at 28 °C resulting in an uptake of approximately 200 pmol per 106 cells. The cells were then washed three times with PBS pH 7.3 to remove excess marker. At the start of each experiment PBS pH 7.3 was replaced with 1 ml PBS of pH 7.3 or 5.6, and at different times 100 µl aliquots were taken. Totals were obtained by lysing the cells at the end of the experiment in 0.1% Triton X-100. The aliquots were mixed with 3 ml of phenolxylxylene-based scintillation fluid (Beckman) and the radioactivity measured in a Kontron MR 300 β-counter.

#### Influx of fluorescent marker into infected Aedes cells

Confluent monolayers of Aedes cells grown in MM medium in cell culture flasks were infected with virus (m.o.i. approximately 10 p.f.u. per cell) and used for experiments 16 h p.i. The cells were harvested by flushing them off the culture flask with MBS pH 7.3, washed and resuspended in MBS. After addition of 20 µl propidium iodide (PI; 1 mg/ml; Fluka) to a 2 ml cell suspension (approximately 5 × 106 cells/ml), the suspension was allowed to equilibrate in a 1 cm fluorescence cuvette under constant stirring. The pH was lowered to 5.6 by adding 1 M-HCl (time zero). The fluorescence was monitored in time drive mode on a Perkin Elmer LS-5b Luminescence Spectrometer (excitation wavelength 528 nm; emission wavelength 600 nm; slit ex 5 nm; slit em 10 nm).

#### Influx of fluorescent marker into isolated virions

To freshly purified virus (50 µg protein; approximately 1011 particles) in 500 µl MBS pH 7.3, 8 µl of PI (1 mg/ml) was added. The virus suspension was allowed to equilibrate in a 0.5 cm fluorescence cuvette. After a constant baseline of fluorescence was established, the pH was lowered to 5.6 by adding 1 M-HCl (time zero) and the fluorescence was monitored in time drive mode on a Perkin Elmer LS-5b Luminescence Spectrometer (excitation wavelength 528 nm; emission wavelength > 570 nm; slit ex 10 nm; slit em 20 nm).

For control experiments, 'shaved' particles were produced by proteolytic digestion of the ectodomains of virus envelope proteins with either bromelain (Schlegel et al., 1991) or trypsin (Spyr et al., 1995).

#### Expression of influenza virus HA in Aedes cells

HA was expressed in Aedes cells using the pSP6-SFV4 replicon system described by Liljestrom & Garoff (1991). The cDNAs used encoded either the structural proteins of SFV (helper) or the HA protein of influenza A virus (Puerto Rico strain; the corresponding cDNA was kindly provided by P. Palese (Mount Sinai School of Medicine, New York, N.Y., USA) and H. Garoff (Karolinska Institute, Huddinge, Sweden)). The amplification of the DNA was performed in *E. coli* strain UT 580. Plasmid DNA was isolated with the Qiagen Plasmid Mega kit according to the manufacturer’s protocol. The redissolved DNA was transcribed to RNA using SP6 RNA polymerase and products were visualized on an agarose gel. The transcription was aliquoted and stored frozen. Approximately 107 BHK-21 cells in 0.8 ml PBS containing HA and helper1 transcription mixture were electroporated, applying two pulses, in a 4 mm electroporation cell (Bio-Rad) with 850 V, 25 µF, 0.2 (resulting in τ of 0.4 to 0.5 ms). The cells were then seeded on 10 cm Petri dishes.
After incubation of the cells in BHK-21 medium for 24 h at 37 °C, the supernatant containing recombinant RNA packaged in infectious particles was harvested. Aedes cells were infected with these viral constructs. Expression of HA was tested by SDS-PAGE and subsequent detection of the HA by Western blot. Briefly, 16 h p.i. cells were treated with trypsin (10 μg/ml; 10 min) and lysed with 0.05% Triton X-100 in the presence of proteinase inhibitors (0.1 μM-TPCK, 0.1 μM-PMSF and 10 μg/ml soybean trypsin inhibitor). The cell lysates of recombinant virus-, mock- and SFV-infected Aedes cells were separated by SDS-PAGE (10–15%) under reducing conditions. Immunoblotting was performed with anti-HA goat polyclonal antibody (Anawa) and alkaline phosphatase-conjugated rabbit anti-goat IgG (Sigma) as the secondary antibody.

Results

It has previously been demonstrated that SFV spike proteins expressed on the cell membrane of Aedes cells form pores when exposed to mildly acidic conditions. This was shown by efflux experiments (Lanzrein et al., 1992) and confirmed by patch-clamp studies (Lanzrein et al., 1993). Experiments using isolated purified virions and so-called E1 particles, generated by proteolytic digestion of the E2 protein, demonstrated that the pores were also formed on the virus particle (Spyr et al., 1995). The latter experiments were based on measurements of influx of a fluorescent dye, PI (molecular mass 668 Da). Both approaches, efflux of AIB from and influx of PI into virus-infected cells and influx into isolated virions, were used to investigate the capability of two other enveloped viruses, SIN (Togaviridae) and VSV (Rhabdoviridae), to form low pH-induced pores. In addition the behaviour of influenza A virus (Orthomyxoviridae) HA expressed in Aedes cells was tested.

Efflux of AIB from virus-infected Aedes cells at mildly acidic pH

To detect a change in membrane permeability, cells were infected with SIN or VSV and loaded 16 h p.i. with [14C]AIB. After exchanging the extracellular medium for a buffer of mildly acidic pH (5.6), aliquots were taken at different times. The radioactivity was measured and the efflux calculated for each time point. As controls, either non-infected Aedes cells (mock) were used or infected cells were kept at neutral pH (7.3). As depicted in Fig. 1, cells infected with either VSV (Fig. 1a) or SIN (Fig. 1b) clearly showed an acid-induced increased loss of [14C]AIB. In contrast, with mock-infected cells at pH 7.3 or 5.6 (Fig. 1c) and infected cells at neutral pH (Fig. 1a, b) a maximum AIB efflux of approximately 20% within 30 min could be observed. When the experiments were performed in the presence of millimolar amounts of Zn2+, [14C]AIB efflux was inhibited, as previously reported for SFV-induced pore formation (Lanzrein et al., 1992).

Acid-induced pore formation measured by influx of PI

The membrane-impermeant dye PI, normally utilized as a vital cell marker in flow cytometry (Bhakdi & Martin, 1991; Ni et al., 1993), has been used to detect pore formation (Spyr et al., 1995). This dye shows an increased fluorescence upon DNA or RNA binding.

When PI was incubated with virus-infected Aedes cells, the cells showed an increase in the monitored emission signal at 600 nm after acidification (pH 5.6), indicating a pH-dependent influx of PI into the cells. In control experiments, either mock-infected Aedes cells or infected cells kept at neutral pH (7.3) were tested. As depicted in Fig. 2 (a–c), cells infected with either SFV, VSV or SIN clearly showed an influx of the fluorescent dye upon acidification of the extracellular medium, whereas no
effect could be observed with mock-infected Aedes cells (Fig. 2 d), indicating that a low pH-induced permeability change had taken place in infected cells. The acid-induced permeability increase was strongly hampered by addition of ZnCl₂ (2.5 mM), as previously described for SFV (see above). Chelating the zinc ions with EGTA reversed this inhibition (Fig. 2 b, c).

To measure pore formation on isolated purified virions, PI was added to virus suspensions. Freshly prepared virus was always used, since freeze–thawing may damage the virus membrane (Young et al., 1983). As shown in Fig. 3, acidification of the medium resulted in a marked increase in the fluorescence signal for all viruses tested. Thus, VSV (Fig. 3 b) and SIN (Fig. 3 c) showed pore formation identical to SFV (Fig. 3 a). For control purposes the ectodomains of the virus spike proteins were removed by proteolytic digestion with either bromelain or trypsin. These shaved particles showed no increase in fluorescence after acidification (Fig. 3 a–c). As a control for the presence of an intact lipid envelope during the experiment, virions were lysed finally by addition of Triton X-100 (0.1% final concentration), which further augments the accessibility of the RNA and therefore resulted in an additional increase in fluorescence (data not shown). As described for the flux experiments mentioned above, addition of Zn²⁺ resulted in inhibition of the flux across the membrane.

**pH-dependent permeability changes in HA-expressing cells**

It had previously been postulated that HA might function as a proton 'channel' in a similar way to the spikes of SFV (Kempf et al., 1987). In order to demonstrate a similarity between the HA protein of influenza A virus and the spike proteins of SFV, VSV and SIN, the HA protein was expressed in Aedes cells using the SFV replicon system (Liljestrom & Garoff, 1991) and flux experiments were performed with these cells.

Aedes cells were infected with viral constructs consisting of the SFV structural proteins packaged with RNA coding for influenza virus HA. Expression of HA on the cell surface was confirmed by immunofluorescence labelling, haemadsorption tests (data not shown) and by SDS–PAGE and subsequent Western blot analysis using a polyclonal antibody against HA.
As depicted in Fig. 4, mainly HA₀ (the precursor form of HA) into HA₁ and HA₂ generates the fusion-active form of HA. The amount of partially cleaved HA₀ in Aedes cells was dependent on the recombinant virus batch used to infect cells, and could be increased by exposing the cells to trypsin (Fig. 4). Functionality of the HA was tested by a fusion assay. Cell–cell fusion requires the cleaved form of the HA (HA₁). Infected cells that tested positive for fusion were used for the flux experiments. As depicted in Fig. 5(a), such cells showed a clear increase in their permeability when exposed to mildly acidic pH. The rate of AIB efflux was found to be pH-dependent. In Fig. 5(b) the initial velocity of AIB efflux (expressed as percentage of total efflux per minute) is plotted against pH values. A pH maximum for the permeability change of 4.8 was obtained. As depicted in Fig. 6, the low pH-induced permeability change could also be observed when the test was performed with the previously described PI assay. Further experiments revealed a correlation between the amount of cleaved HA₀ and the observed AIB efflux at pH 5.6; when no cleaved HA could be detected by Western blot analysis or by a fusion assay, no pore formation was observed (data not shown). Thus, low pH-induced change in membrane permeability, monitored either by influx of PI or efflux of AIB, was dependent on a functional HA.

Discussion

Many viruses including a number of non-enveloped viruses have been shown to be internalized by endocytosis. In the endosome they reach the first acidic compartment in the endocytotic pathway. This low pH is generated by membrane-bound H⁺-ATPases (Mellman et al., 1986). For enveloped viruses the low pH leads to a conformational change of the spike proteins that culminates in the fusion of the viral with the endosomal membrane. Additionally, it was previously described that the conformational change of the spike proteins of SFV also results in the formation of a pore across the viral membrane (Schlegel et al., 1991; Spyr et al., 1995) or the membrane of infected cells (Lanzrein et al., 1992). This pore formation was demonstrated by flux measurements and confirmed by patch–clamp experiments. A pore size of 1–2 nm in diameter was calculated (Lanzrein et al., 1993). However, so far it has not been determined whether this low pH-induced pore formation is a specific property of SFV or inherent to various enveloped viruses.

Thus, in this study the behaviour of three additional enveloped viruses was tested under fusogenic conditions. The pore-forming potential of the spike proteins of two viruses, SIN (belonging to the same family and genus as SFV) and VSV (a member of the family Rhabdoviridae) was tested on isolated virions as well as with infected cells that expressed the spike proteins in the plasma membrane. Pore formation on infected cells was tested with two previously described assays: the measurement of AIB efflux (Lanzrein et al., 1992) and PI influx into the cells. The latter assay had been described before by Spyr et al. (1995) and was used to demonstrate pore formation by SFV spikes on isolated virions. The membrane-impermeant dye PI was used to measure permeability changes. The fluorescence of PI is enhanced when bound to RNA or DNA. As virus genomic RNA is the only nucleic acid present in the assay, the detected increase in fluorescence when lowering the pH must be due to the binding of PI to the virus RNA. Liposomes were used to exclude an effect of lipids; they showed no effect on the fluorescence of PI (data not shown).

As demonstrated in the present studies, both SIN and VSV showed identical behaviour to SFV, namely low pH-inducible pore formation. Control experiments with isolated virions clearly revealed that, as described for SFV, the ectodomains participate in pore formation. When the ectodomain was removed proteolytically to produce shaved particles, low pH-induced pore formation was abolished. The virus envelope was not damaged during the treatment with proteases, as shown by subsequent addition of a detergent to solubilize the membrane.
Thus the virus genome was made accessible to PI, which in turn led to an increase in its fluorescence. The observed low pH-induced permeability change in cells infected with VSV or SIN or in either of the isolated viruses could be hampered, in a reversible manner, by millimolar concentrations of zinc ions, a characteristic described for the pores formed by SFV. This provides further evidence that well-defined, proteinaceous pores are mediating the effect (Lanzrein et al., 1992). The sensitivity towards Ca$^{2+}$ and Zn$^{2+}$ is a common effect observed in many pore-forming agents like mellitin, bacterial toxins and virus proteins (Bashford et al., 1986, 1988). It has been proposed that the action of Zn$^{2+}$ is due to specific binding rather than non-specific screening of surface charge (Bashford et al., 1988). In conclusion, it can be stated that the spike proteins of both VSV and SIN form low pH-induced pores when expressed in the plasma membrane of infected cells and within the virus envelope in the same way as previously described for SFV. For SFV, strong evidence exists that it is the E1 protein out of the five structural proteins (C, E1, E2, E3 and 6K) that is responsible for pore formation. It has been demonstrated that (i) the E1 protein is mandatory for pore formation (Dick et al., 1996) and (ii) pore formation also takes place in so-called E1 particles (Spyr et al., 1995). Thus, it can be assumed that the E1 protein also represents the pore-forming element in SIN. In the case of VSV, the spikes consist of only one protein, the G protein. This transmembrane glycoprotein is responsible for virus attachment to the host cell membrane and for membrane fusion (reviewed in Gaudin et al., 1995).

Finally, the widely reported permeability changes of influenza A virus when exposed to low pH were compared with the findings in SFV, SIN and VSV. In influenza virus it is known that the M2 protein acts as an ion channel (Pinto et al., 1992). Apart from the pH regulatory function in the trans-Golgi network (Sugrue et al., 1990), it has been postulated that the M2 protein plays a role in the virus uncoating process (Helenius, 1992). Briefly, within the endosome where the virion encounters a mildly acidic pH, M2 allows protons to cross the virus membrane. This in turn leads to structural changes of the capsid, priming it for the uncoating process. However, some observations make it doubtful whether M2 is solely responsible for the proton translocation in the endosome. There are two major facts that could imply the involvement of supplementary 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 (Zebbedee & Lamb, 1988). Second, the role of the anti-influenza virus drug amantadine (acting on the M2 protein) early in infection is so far not fully understood. Some strains such as the Rostock strain are particularly sensitive to amantadine late during the infectious cycle but remain relatively insensitive at early stages (Hay & Zambon, 1984). One possible explanation for these findings could be that other virus 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 described that HA constitutively expressed in Gpbind4 cells can induce a proton influx into the cell below the pH required for fusion (Kempf et al., 1987). Other groups have reported that influenza virus (Patel & Pasternak, 1983; Sato et
in this report fully support these observations. As shown in Fig. 5, HA forms pores at mildly acidic pH. The pore formation is dependent on the presence of the cleaved HA, and the described pH optimum correlates with the one observed for fusion. Thus, taken together these results suggest that the low pH form of influenza virus HA represents a pore-forming element. Consequently, one would have to revise the hypothesis on influenza virus uncoating and postulate that HA might play a key role. This would also mean that the acid-induced conformation change of viral envelope proteins which leads to the formation of pores for low molecular mass compounds can be seen as a common step in the entry pathway of enveloped viruses using low pH for triggering fusion with the host cell membrane. The proteins involved in the observed formation of such a pore would be E1 (SFV and SIN), G (VSV) and HA (influenza A virus). Hence the present report suggests that pore formation by virus spike proteins might be an inherent property of viruses using the endocytic pathway to infect cells.

In conclusion one can postulate that the opening of such a pore and the implied fluxes may be a common step in the entry pathway of enveloped viruses. The pore may be involved in transmitting an initial signal over the viral membrane for uncoating the capsid and the release of infectious RNA into the host cell cytoplasm (Lanzrein et al., 1994a).

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References


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