Expression of Semliki Forest Virus E1 Protein in Escherichia coli
LOW pH-INDUCED PORE FORMATION

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Exposure of Semliki Forest virus 1 to mildly acidic conditions results in conformational changes of the viral spike proteins, which in turn leads to a pore formation across its membrane. The ability to form a pore has been ascribed to the ectodomain of the Semliki Forest virus (SFV) E1 spike protein. To elucidate whether the E1 protein per se is sufficient for low pH-dependent pore formation, we expressed E1 in Escherichia coli in an inducible manner using the pET11c expression system. The data obtained clearly showed that the E1 protein was expressed in the bacterial cell membrane and that exposure of E. coli expressing the SFV E1 protein to low pH (<6.2) resulted in a permeability change of the membrane. Thus, we conclude that the E1 protein of SFV per se is sufficient to promote pore formation under mildly acidic conditions.

The entry of a virus into a host cell is an essential step in the chain of events leading to infection. A multitude of viruses use the endocytic pathway to access host cells. As a model for the entry of enveloped animal viruses into cells, the α virus Semliki Forest virus has been extensively studied (1). Once attached, the virus is internalized via coated vesicles and transferred to the endosome. Due to the acidic conditions within this organelle, the lipid envelope of SFV fuses with the endosomal membrane of the target cell (2). This low pH-induced fusion is mediated by the so called virus spikes (3–5). Each spike is a heterotrimer, being composed of the type 1 integral membrane glycoproteins E1 (50.786 kDa) and E2 (51.855 kDa), plus the peripheral glycoprotein E3 (11.369 kDa), which is associated with E2 (6). Several functions have been ascribed to the spike proteins; e.g. the E2 and E3 precursor protein p62 forms a heterodimer with E1 in the endoplasmic reticulum and is responsible for the transport of the complex to the plasma membrane (6). The E1 protein is involved in the acid-induced fusion of the viral and endosomal membranes (7–9).

Under mildly acidic conditions (pH 5.8) the spike proteins undergo an irreversible conformational change that results in the dissociation of the E1/E2/E3 complex, the formation of an E1 homotrimer and the exposure of a fusion peptide on the E1 protein (7). This conformational change also leads to the formation of a pore, which causes an alteration in the permeability of the virion membrane or of a cell membrane expressing the spike proteins (10–14). It has been suggested that this acid-induced pore formation plays a crucial role in the penetration and uncoating process of SFV (15).

Several experiments have shown that pore formation is dependent on the ectodomain of the E1 spike protein (14, 16, 17). It has been speculated that the E1 protein per se would be sufficient for triggering acid-induced pore formation. So far, all attempts to express isolated E1 protein on the surface of eukaryotic cells have failed. The E1 protein was synthesized but retained in the endoplasmic reticulum, since efficient transport of the glycoproteins to the plasma membrane requires heterodimerization of E1 with E2/E3 (18). Therefore, to further investigate the role of E1 during pore formation, we decided to express E1 in the prokaryotic host Escherichia coli.

Our data clearly demonstrate that the E1 protein, although lacking a signal sequence, is transported to the plasma membrane of E. coli. Furthermore, we have shown that E1 is capable of modifying the membrane permeability of E. coli in a pH-dependent manner, leading to pore formation.

MATERIALS AND METHODS

Construction of the SFV E1 Expression Plasmid—DNA manipulations were performed by the use of standard cloning procedures (19). Polymerase chain reaction was used to amplify the SFV E1 gene from pSFv-E1. This plasmid contains the entire E1 gene and was derived by subcloning a 1951-bp SpeI-EcoRV fragment from pSP6-SFV4, which contains the full-length cDNA sequence of SFV (20, 21).

The primers were designed to introduce a start and an additional stop codon for translation. To facilitate cloning of the E1 gene into the expression vector pET11c (Stratagene AG, Amsterdam, Netherlands), the oligonucleotides encoded a unique NdeI and BamHI restriction site at their 5′ and 3′ end, respectively. Since the E1 gene contains an intragenic NdeI restriction site, the 3′ primer was designed to introduce a silent point mutation (A→G) that eliminated this NdeI cutting site.

The 1340-bp E1 polymerase chain reaction fragment was purified using a polymerase chain reaction purification kit (Qiagen AG, Basel, Switzerland), digested with the restriction enzymes BamHI and NdeI, and ligated into the pET11c vector. The ligation mixture was used to transform competent XL-1 blue E. coli cells and the resulting colonies screened by digestion of the isolated plasmid DNA with the appropriate restriction enzymes. The plasmid containing the E1 gene (pET11c-E1) was then used to transform BL21(DE3) E. coli cells.

Construction of the pET11c-E1/23_E2/423 Plasmid—Most of the E2 protein sequence was cut out from the plasmid pSP6-SFV4 (21) using the restriction enzymes BglII and SgrAI. The ends of the resulting fragment of 1262 bp were polished with Klenow enzyme and mung bean nuclease. The plasmid pMSEH1_E1/23_phoA (Nyfeler et al.) was digested with PsI and SalI, resulting in a 5371-bp fragment whose ends were blunted, too. This fragment was ligated with the 1262-bp-long

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Expression of the SVF E1 Protein in *E. coli*

Expression of the SVF E1 protein in *E. coli* was achieved by cloning the gene encoding the E1 protein into the pET11c cloning vector. The E1 protein was expressed as a fusion protein with a C-terminal tag of 432 amino acids. The expression was induced using 50 mM IPTG for 50 minutes. The expression level was monitored by Western blot analysis using antibodies against E1 and enzyme IICB, respectively. The expression level of E1 was determined by analyzing whole cell lysates using SDS-PAGE and subsequent Western blot analysis with a polyclonal rabbit anti-SV1 and rat anti-enzyme IICB antibody.

**RESULTS**

Expression of SVF E1 Protein in *E. coli* Cells—Comparison of the growth of noninduced bacteria harboring either the pET11c or pET11c-E1 at various pH (5.4–7.2) showed no significant difference. However, induction with 50 mM IPTG strongly hampered the growth of bacteria containing the pET11c plasmid, but not bacteria containing the pET11c-E1. Under mildly acidic conditions (pH 5) proliferation of induced cells containing the pET11c-E1 was strongly impeded, whereas at pH 6.4 and 7.4, respectively, growth appeared to be normal (data not shown). These results would be in agreement with a functional expression of the E1 protein in the *E. coli* membrane.

Expression of E1 was detected by analyzing whole cell lysates by SDS-PAGE and subsequent immunoblotting using a polyclonal rabbit anti-E1 antibody (data not shown). Separation of the bacterial plasma membrane from other cell components followed by Western blot analysis revealed that E1 was not solely localized in the membrane but also associated with cell wall fragments or other protein aggregates (Fig. 1A). To further determine whether the E1 protein found in the cell membrane fraction is indeed a membrane-bound protein and does not represent a contamination by inclusion bodies, we used 8 M urea (25, 26) to treat the fraction containing the bacterial membrane as well as the “cell debris” pellet. SDS-PAGE and subsequent Western blot analysis of the different fractions showed that the E1 protein remained associated with urea-treated membranes (Fig. 1B, lane M+U), whereas the corresponding supernatant fraction revealed no signal for E1 (lane M-U). Treatment of the cell debris pellet with 8 M urea demonstrated that a significant amount of E1 protein was most probably localized intracellularly in inclusion bodies or was associated with cell wall components (Fig. 1B, lane P/F). These results were further strengthened by demonstrating the co-localization of the E1 protein and the enzyme IICB in isolated membranes obtained from either a mixture of *E. coli* expressing E1 and enzyme IICB, respectively, or membranes isolated from *E. coli* that expressed both proteins simultaneously (Fig. 1, C and D).

Incorporation of the E1 protein into the plasma membrane of *E. coli* was further assessed by FACS analysis of spheroplasts generated from *E. coli* containing the pET11c-E1 plasmid that...
were labeled with rabbit anti-SFV and fluorescein isothiocyanate-anti-rabbit antibodies. FACS analysis in the presence of propidium iodide showed that the membranes of the spheroplasts were intact and non leaky (Fig. 2, A2 and B2). Hence, the positive signal depicted in Fig. 2, A1 proves that the E1 protein faces the extracellular space of the spheroplasts. To further analyze the orientation of the protein in the membrane, spheroplasts were exposed either in the presence or absence of Triton X-100 to proteinase K and the remaining membrane-associated protein fragments analyzed by SDS-PAGE and Western blot using anti-SFV antibodies. The results (Fig. 3) clearly show that a residual fragment of 3 kDa (lanes 2 and 3) was unaffected by the digestion. The size of the fragment and its reactivity with antibodies strongly suggest that it is the anchoring region (25 amino acids) of the E1 protein. Hence, the E1 protein was correctly oriented in the membrane.

Modification of the E. coli Membrane Permeability at Mildly Acidic pH—It has been shown previously that under acidic conditions, virus spike proteins can alter the host cell membrane permeability by pore formation (11). Several lines of evidence lead to the assumption that the SFV E1 protein is responsible for this process (14, 20). To test this hypothesis we have performed efflux experiments, as described under “Materials and Methods,” by using E. coli containing either the pET11c-E1, or as controls the pET11c_E1/23_E2/432 or pET11c François et al.
plasmids, respectively. At neutral pH, the [14C]choline efflux of E. coli cells expressing the E1 protein remained unchanged compared with controls. As depicted in Fig. 4, lowering the pH (pH = 5.85) resulted in an increase in choline release within the first 10 min in E. coli expressing E1. In contrast E. coli exposed to mildly acidic pH and harboring either the fusion protein E1/E2 or the pET11c plasmid only showed no or only a marginal increase in choline release compared with pH 7.5.

To test the pH influence on choline efflux, preloaded cells expressing the E1 protein were resuspended in medium ranging from pH 4 to 7. After 10 min of incubation, choline release was measured. As shown in Fig. 5, choline efflux in E1-expressing cells was strongly dependent on the pH of the extracellular medium, starting at a pH < 6.2 and reaching a maximum efflux rate at a pH of ~5.2.

**DISCUSSION**

The entry of many enveloped animal viruses into cells is mediated by conformational changes of the viral envelope proteins. These changes are triggered by binding of the virion to the receptor and/or by low pH, e.g., within the endosome, leading to fusion of the viral with the endosomal membrane. This membrane fusion is essential for a successful infection. In the case of the Semliki Forest virus this membrane fusion in the acidic milieu of the endosome is catalyzed by the envelope spike proteins (15). Previous findings indicate that these spike proteins may be responsible not only for membrane fusion but also for pore formation across the viral envelope (13, 14) and the membrane of infected insect cells (10, 11) under slightly acidic conditions.

It was postulated that this pore formation plays a crucial role in the penetration process of SFV (15), but the question which of the viral structural proteins plays the key role in this process remained open.

One possible candidate is the small structural membrane protein 6K, which is present only in small amounts in the viral membrane. Upon expression in E. coli 6K was capable of increasing the membrane permeability leading to cell lysis (22). Similar results have been demonstrated for poliovirus protein 3A (28). However, a deletion mutant of SFV lacking the 6K protein showed unaltered behavior with respect to low pH-induced pore formation in infected eukaryotic cells (20), although there was a reduction in virus release (21). This may indicate that the 6K protein plays a role in the budding process rather than in pore formation, as suggested by Loewy et al. (29).

It has been demonstrated previously that low pH-induced pore formation is dependent on the ectodomain of the viral spike (13). Furthermore, other findings using various mutant viruses strongly suggest that the E1 protein plays a crucial role in this process (20). Experiments showing that pore formation also takes place in the so-called E1 particles, where the E2 ectodomain has been removed by proteinase K (14) support this notion. However, since E1 particles still contain the transmembrane part of the E2 protein, an involvement of E2 could not be entirely excluded.

Independent expression of E1 protein on the cell surface of vertebrate cells has not yet been achieved (the protein is produced, but not transported to the plasma membrane) (18). Thus, it has not been possible to prove that the E1 protein presence is sufficient for the formation of pores across the membrane.

In this study we have therefore expressed the E1 protein in E. coli in an inducible manner using the pET11c expression system (30) and showed that these E1 proteins are indeed integrated into the plasma membrane of E. coli cells in an identical orientation as in SFV or SFV-infected cells. Fig. 3 clearly shows that in spheroplasts exposed to proteinase K the E1 protein is digested. An E1-derived peptide of ~3 kDa is protected from the proteinase digestion, i.e., is localized inside the periplasmic membrane. The size of this peptide is in agreement with the expected size of the anchoring region (2.9 kDa) and strongly supports our findings that the E1, in the E. coli cytoplasmic membrane, is correctly oriented.

To investigate whether pH-dependent pore formation occurs, [14C]choline release assays were performed. An enhanced choline efflux at pH 6–6.2 and below was found. It was maximal at a pH around 5.2, which is a pH similar to the one prevailing in endosomes. Lanrezin et al. (10) have reported corresponding results using SFV-infected insect cells: upon lowering the extracellular pH, efflux of a radiolabeled tracer molecule started at a pH of ~6.2 and reached a maximal level at a pH of ~5.5. Furthermore, these data are in accordance with what is known about the pH dependence of the SFV membrane fusion reaction (5) that in turn is dependent on the conformational change of the spike proteins.

The fact that exposure of E. coli expressing the E1 protein to mildly acidic pH results in a change of the membrane permeability, which shows the characteristics of previously described
acid-induced pore formation by the virus proteins, further strengthens the notion that the E1 protein is indeed incorporated into the cell membrane. The formation of pores at a pH below 6.2 also explains the observations that the growth of bacteria containing the pET11c-E1 plasmid was strongly hampered at pH 5, but not at pH 6.4 or 7.4, respectively.

In conclusion, the data presented demonstrate that the E1 protein gets inserted into the E. coli cell membrane. In a regular infection of eukaryotic cells the viral spike proteins are synthesized as a polyprotein, which is cleaved co-translationally into the single proteins, and the signal for the insertion of the E1 protein into the membrane is contained within the 6K protein that precedes E1 on the polyprotein (6). Within the membrane the E1 protein has a type I orientation (C terminus inside, N terminus outside). The membrane anchor sequence is located within the C-terminal 25 amino acids with just two arginines on the inside. With the selected strategy of cloning the 6K sequence was omitted and replaced by a start codon. Analysis of the E1 sequence (439 amino acids) for both pro- and eukaryotic signal sequences using the PSORT program (31, 32) predicted the protein to reside in the cytoplasm. Hence, the cloned DNA containing the sequence encoding the E1 protein lacks a known signal that would govern the insertion of the protein into the cell membrane. To identify the sequences within the E1 protein responsible for protein insertion into the membrane, further experiments are needed.

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