Mutagenesis of the Putative Fusion Domain of the Semliki Forest Virus Spike Protein

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Semliki Forest virus (SFV), an alphavirus, infects cells via a low pH-triggered membrane fusion reaction that takes place within the cellular endocytic pathway. Fusion is mediated by the heterotrimeric virus spike protein, which undergoes conformational changes upon exposure to low pH. The SFV E1 spike subunit contains a hydrophobic domain of 23 amino acids that is highly conserved among alphaviruses. This region is also homologous to a domain of the rotavirus outer capsid protein VP4. Mutagenesis of an SFV spike protein cDNA was used to evaluate the role of the E1 domain in membrane fusion. Mutant spike proteins were expressed in COS cells and assayed for cell-cell fusion activity. Four mutant phenotypes were identified: (i) substitution of Gln for Lys-79 or Leu for Met-88 had no effect on spike protein fusion activity; (ii) substitution of Ala for Asp-75, Ala for Gly-83, or Ala for Gly-91 shifted the pH threshold of fusion to a more acidic range; (iii) mutation of Pro-86 to Asp, Gly-91 to Pro, or deletion of amino acids 83 to 92 resulted in retention of the E1 subunit within the endoplasmic reticulum; and (iv) substitution of Asp for Gly-91 completely blocked cell-cell fusion activity without affecting spike protein assembly or transport. These results argue that the conserved hydrophobic domain of SFV E1 is closely involved in membrane fusion and suggest that the homologous region in rotavirus VP4 may be involved in the entry pathway of this nonenveloped virus.

Enveloped animal viruses use membrane fusion to transfer their genomes into the cytoplasm of the host cell. This critical membrane fusion reaction is mediated by the viral spike proteins and occurs either at neutral pH at the plasma membrane or in an endocytic compartment of acidic pH (reviewed in references 20, 32, 46, 49, and 55). Such viral membrane fusion reactions are a paradigm for the numerous fusion events which are an integral and ubiquitous part of cellular function. However, viral membrane fusion is presently more accessible to experimental dissection because of the clear identification of the virus spike protein as the fusogen. In addition, viruses that require a low pH for fusion can be very efficiently and synchronously triggered to undergo fusion *in vitro* upon exposure to acid.

Semliki Forest virus (SFV) is a well-characterized alphavirus that infects cells via endocytosis and an acid-triggered fusion step (reviewed in references 18 and 20). Membrane fusion is mediated by the viral spike protein, a heterotrimer of two transmembrane subunits, E1 and E2, and a peripheral protein, E3 (M_r , 50,786, 51,855, and 11,369, respectively). In addition to its low pH dependence, SFV fusion is characterized by a striking requirement for cholesterol in the target membrane (37, 50).

Upon exposure to a pH of 6.2 or below, both the SFV E1 and E2 subunits undergo irreversible conformational changes that result in altered protease sensitivity and reactivity with monoclonal antibodies (19, 21). The E2 subunit is synthesized as a precursor, p62, which must be proteolytically cleaved for efficient fusion in the physiological pH range (14, 28, 30). In spite of the acid-induced conformational change in E2 and its cleavage requirement for fusion, the available evidence points to E1 as being the fusion-active subunit of SFV. Thus, removal of E2 by proteolytic digestion results in E1 virus particles that can still fuse and infect cells (34). p62 expressed in the absence of E1 is fusion

negative (25). Significantly, the acid-induced conformational change in a truncated form of E1 requires both low pH and cholesterol, the critical target membrane component for fusion and infection (19, 21).

Sequence analysis of a number of alphaviruses demonstrated the presence of a highly conserved hydrophobic domain on the E1 subunit (see references in Fig. 1 legend). This region is 23 amino acids long and 75 residues from the N terminus of E1. Because of its apolar nature and strong conservation and by analogy with hydrophobic sequences in other virus spike proteins, it has been suggested to be the "fusion peptide" of SFV (8). Interestingly, the first 18 amino acids in this region are 45% identical and 27% similar to a sequence in the VP4 outer capsid protein from the nonenveloped rotaviruses (31).

We initiated experiments to test the role of the E1 hydrophobic domain in the membrane fusion activity of SFV. *In vitro* mutagenesis of a cDNA clone of the SFV spike protein was used to change specific amino acids within the conserved region. The spike protein was then expressed in COS cells and assayed for its cell-cell fusion activity at low pH. Here we present evidence that the internal hydrophobic domain of the SFV E1 protein plays a major role in membrane fusion. Mutation of specific amino acids in this domain had potent effects on the pH threshold and efficiency of fusion.

MATERIALS AND METHODS

SFV cDNA cloning and vector constructs. A plaque-purified isolate of SFV was grown in baby hamster kidney cells and purified as described previously (22). Viral RNA was isolated by extraction with phenol-chloroform and sodium dodecyl sulfate (SDS) (35). The RNA was annealed with oligo(dT) as a primer and used as a template to synthesize double-stranded cDNA (10, 39). The viral cDNA gave two prominent bands migrating at about 9 and 11 kb in agarose gel electrophoresis. To clone a 5-kb fragment containing the

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3' structural protein-coding sequences, the cDNA was digested with *Sall* and *HindIII* (Fig. 1). This fragment was ligated with the large fragment from a *Sall-HindIII* digest of pZ152, a pBR322-derived vector containing the M13 origin and intragenic region (57). A positive clone containing the 5-kb SFV insert was then digested with *HindIII* and partially with *EcoRI* to generate a 4-kb fragment containing the structural protein-coding sequence and about 30 nucleotides of 5' sequence (Fig. 1). After addition of *BamHI* linkers, the fragment was inserted into the *BamHI* site of pL2, a late simian virus 40-based vector (33), for expression in COS 1 cells. A positive clone, pL2-SFV-88, was analyzed by restriction enzyme digestion to verify the proper orientation and identity of the insert. pL2-SFV-88 was then used for further construction and as the wild-type control in transfections of COS 1 cells. All molecular biology procedures were performed by standard protocols (42).

Mutagenesis of the hydrophobic domain of E1. As a basis for mutagenesis studies, a 635-bp *ClaI* fragment containing the hydrophobic domain (Fig. 1) was subcloned from pL2-SFV-88 into pSP72 (Promega Corp., Madison, Wis.) and sequenced from the double-stranded plasmid with Sequenase as described by the manufacturer (United States Biochemical Corp., Cleveland, Ohio). The sequence was identical to the published SFV sequence (8) except for a silent C→T change at the third nucleotide of the codon for Cys-94 of E1.

Two strategies for mutagenesis of the hydrophobic domain were followed, with the mutagenic oligonucleotides listed below. The *ClaI* fragment was subcloned into pZ152 to allow production of single-stranded DNA in the presence of M13K07 helper phage (57). The mutations at positions 75 and 83 were then generated by in vitro mutagenesis with the Muta-gene kit manufactured by Bio-Rad (Richmond, Calif.). Mutants were screened by hybridization to a ³²P-labeled oligonucleotide and sequenced to verify the presence of the desired mutation. The pL2-SFV-88 wild-type construct was digested with *ClaI* and religated to generate plasmid pL2-SFV-32, which lacked the *ClaI* fragment. *ClaI* fragments carrying the desired mutation were subcloned into pL2-SFV-32 to regenerate the complete structural protein-coding sequence. All other single-amino-acid changes and the deletion mutant (Fig. 1) were generated by the overlap extension method by the polymerase chain reaction (PCR) (12). Ten nanograms of pSP72 containing the SFV *ClaI* fragment was used as the DNA template, 500 ng each of the T7 promoter and SP6 promoter primers (Promega Corp.) was used as the outside primers, and 250 ng of complementary mutagenic oligonucleotides was used as the internal primers. The resultant 750-bp PCR product was digested with *ClaI* and cloned into pL2-SFV-32 to regenerate the complete pL2-SFV construct. The entire *ClaI* fragment of each mutant was sequenced to confirm the absence of additional mutations. All mutagenizing oligonucleotides were synthesized in the Albert Einstein College of Medicine oligonucleotide synthesis facility. The sequences of the oligonucleotides and their orientation relative to the coding strand are listed below. Only one of each of the paired mutagenic oligonucleotides used for the PCR mutagenesis is shown. Mismatched bases are shown in lowercase letters: Asp-75→Ala, 3'TTCTCTTCg GACGGATGG5'; Lys-79→Gln, 5'GACTACCAATGCcAG GTT3'; Gly-83→Ala, 3'TCCAAATGTGTCgGCACA5'; Pro-86→Asp, 5'GTGTACgacTTCATGTGGGGAGGGGCA3'; Met-88→Leu, 5'GTGTACCCGTTcTGTGG3'; Gly-91→Pro, 5'TCATGTGGGGAccGGCAT3'; Gly-91→Ala, 5'ATG TGGGGAGcGGCATAT3'; Gly-91→Asp, 5'ATGTGGGGA

GacGCATATTGC3'; Δ83-92, 3'GTTACGTTCCAAATGT GT—ATAACGAAGACGCTG5'.

Transfection of COS1 cells. All constructs in the pL2 vector were expressed in COS1 cells to evaluate spike protein fusion activity, subcellular localization, and processing. Supercoiled plasmids prepared by CsCl gradient centrifugation were transfected into COS1 cells by using DEAE-dextran (3). Cells were washed twice with Dulbecco's modified Eagle's medium (DMEM) containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.0), and a mixture of 0.5 to 10 μg of DNA and 500 μg of DEAE-dextran (2,000,000 *M_w*) per ml in this medium was added to cells for 90 min at 37°C. The cells were then treated for 3 min with 10% dimethyl sulfoxide in DMEM with 10% serum, cultured for 5 to 8 h in DMEM with 10% serum and 100 μM chloroquine, and then cultured for a further 40 to 44 h in DMEM with 10% serum. Cells were assayed for spike protein expression or fusion activity as described below.

Localization of spike protein subunits by immunofluorescence. Expressing cells were fixed with either 3% paraformaldehyde for surface staining or methanol to permit internal staining. Cells were then treated with a polyclonal rabbit antiserum against the SFV spike protein or with mouse monoclonal antibodies against the E1 or E2 subunit (21), followed by fluorescein- or rhodamine-conjugated second antibodies (Organon Teknika-Cappel, Malvern, Pa.). Photographs were taken with a Zeiss Axioscop 20 with Kodak TMAX 400 film.

Cell-cell fusion assay. The ability of spike proteins to induce cell-cell fusion at low pH was examined. Wild-type and mutant SFV spike proteins were expressed in COS cells cultured on 22-mm square coverslips. Two days posttransfection, the cells were washed once with 1 ml of fusion medium (RPMI 1640 without bicarbonate, containing 0.2% bovine serum albumin, 10 mM HEPES, and 10 mM MES [morpholineethanesulfonic acid, pH 7.0]) per well and incubated for 1 min at 37°C with 3 ml of fusion medium adjusted to a pH of from 4.7 to 7.0. Cells were washed twice with DMEM and incubated at 37°C for 4 h to allow maximal SFV spike protein expression in the newly fused cells and morphological reorganization of cell nuclei. The cells were then fixed in paraformaldehyde and stained as above with a rabbit antibody against the SFV spike protein. After staining, the cells were permeabilized for 4 min in phosphate-buffered saline (PBS) containing 0.2% Triton X-100, and the cytoplasmic RNA was digested at 37°C for 1 h with 0.2 mg of RNase A per ml in 0.2 M phosphate buffer at pH 7.0. The nuclei were then stained for 10 min with propidium iodide (50 μg/ml) in 0.1% sodium citrate at pH 7.0. The number of nuclei per expressing cell was evaluated by fluorescence microscopy with fluorescein filters. Percent fusion was calculated as: % fusion = 100 × [1 - (cells/nuclei)] (53). From 25 to 50 fused cells or 100 to 200 single expressing cells were counted for each coverslip. A minimum of three cell-cell fusion assays was performed for each mutation, with the wild-type construct (pL2-SFV-88) used as a control.

Quantitation of cell surface expression of the SFV spike protein. Cell surface expression of the SFV spike protein was quantitated by using a polyclonal rabbit antiserum against the SFV spike protein and ¹²⁵I-labeled protein A (40). In brief, COS cells in six-well dishes were fixed in 3% paraformaldehyde at 2 days posttransfection. Duplicate wells were washed with PBS containing 0.2% gelatin and incubated with rabbit antispikes antiserum (1:100 dilution in PBS containing 0.2% gelatin) for 1 h on a rotary shaker at

FIG. 1. Mutations in the hydrophobic domain of the SFV E1 glycoprotein. A linear diagram of the 3' third of the SFV cDNA is shown, with the structural protein-coding sequence given as the large open box. The restriction enzyme sites used in the initial cDNA cloning, construction into the expression vector, and subcloning for mutagenesis are listed above. The order of the structural proteins is shown, where C is the capsid protein and p62 is the precursor to E3 and E2. Cross-hatches indicate the positions of the transmembrane domains. The E1 hydrophobic domain is depicted as the black box 75 amino acids from the N terminus of E1, and its amino acid and nucleotide sequences are shown below. Amino acids (aa) which are conserved among published alphavirus sequences are in uppercase letters. Beneath the arrows are listed the single-amino-acid changes and the deletion mutation, with the oligonucleotide-directed base changes given in lowercase letters. The sequences of E1 from the SFV, Sindbis, Ross River, Western equine encephalitis, Eastern equine encephalitis, Venezuelan equine encephalitis, and O'Nyong-nyong alphaviruses are given in references 8, 41, 4, 11, 2, 23, and 27, respectively. Residues 75, 83, 85, 86, 88, and 90 to 92 are conserved in SA11 rotavirus VP4 (31).

room temperature. The cells were then washed and incubated with 5×10^5 cpm of ^{125}I -protein A per well for 1 h. After additional washes, the bound radioactivity was eluted in 2% SDS at 60°C and quantitated by gamma counting. Untransfected cells were assayed to determine background binding. Protein A was iodinated by using Iodo beads as described by the manufacturer (Pierce, Rockford, Ill.).

Metabolic labeling, immunoprecipitation, and Endo H digestion. Transfected cells were labeled with [^{35}S]methionine in a pulse-chase protocol as described previously (21). The cells were then lysed on ice and reacted first with preimmune serum and fixed *Staphylococcus aureus* (Zysorbin; Zymed Labs, San Francisco, Calif.) and then with a rabbit antibody against the SFV spike protein. The immune complexes were precipitated with Zysorbin and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as described previously (21).

For some samples, immunoprecipitates were eluted from the Zysorbin by boiling in 1% SDS in 10 mM Tris (pH 6.8). The sample was adjusted to 40 mM citrate (pH 5.0) and digested overnight at 37°C with 0.05 U of endoglycosidase H (Endo H; ICN Biomedicals, Inc., Costa Mesa, Calif.); 2× SDS gel buffer (21) was then added, and the samples were boiled for 3 min and resolved on 12.5% SDS gels.

RESULTS

Mutagenesis of the SFV E1 hydrophobic domain. To ensure that the results of our analysis of the cloned protein would be completely comparable with those of our previous studies of the SFV fusion reaction, we cloned the structural proteins from our plaque-purified isolate of SFV (22). Cells transfected with the wild-type construct expressed spike proteins with subunit molecular weights comparable to those of purified virus. The expressed spike proteins showed efficient cleavage of the p62 precursor, cell surface localization of

both the E2 and E1 subunits, and membrane fusion activity with a pH dependence similar to that of wild-type virus. All of our analyses of both wild-type and mutant spike proteins were done with a simian virus 40-based vector transfected into COS cells and assayed 2 days after transfection (33). The COS cell expression system was especially useful for our cell-cell fusion studies, since the cells express large amounts of spike proteins and are well spread.

Nine mutations in the E1 hydrophobic domain were constructed and analyzed for their effects on the fusion activity of the spike protein (Fig. 1). Amino acids were selected for mutagenesis by several criteria: (i) amino acids conserved among alphaviruses and between alphavirus E1 and rotavirus VP4 (all of the selected amino acids except for Lys-79); (ii) charged amino acid residues within the conserved hydrophobic domain (Asp-75, Lys-79); (iii) amino acids with possible structural importance (Pro-86, Met-88); and (iv) conserved glycine residues in the central portion of the hydrophobic domain (Gly-83, Gly-91). In addition, we made a deletion of 10 amino acids from within the central hydrophobic core ($\Delta 83-92$).

Preliminary characterization of mutant spike proteins. Since our fusion assay was dependent on protein expression at the cell surface, our initial experiments assayed each mutant for expression and transport to the plasma membrane. Metabolic labeling and immunoprecipitation were used to evaluate the molecular weight of the expressed spike proteins and the cleavage of p62 (Fig. 2). After a 15-min pulse and a 30-min chase, the wild-type spike protein showed prominent bands migrating at the positions of authentic p62 and E1. A proportion of p62 was cleaved to E2 and E3, although E3 was not detectable at the levels produced in expressing cells. This cleavage occurs during or after the exit of the spike protein from the trans-Golgi network (5). The spike proteins produced by the Lys-79→Gln, Met-88→Leu, Asp-75→Ala, Gly-83→Ala, Gly-

FIG. 2. Metabolic labeling of SFV spike protein mutants expressed in COS cells. Two days posttransfection, cells expressing wild-type (wt) and mutant spike proteins were pulse-labeled for 15 min with [35 S]methionine and chased for 30 min with excess cold methionine. The cells were lysed, immunoprecipitated with a polyclonal antibody against the SFV spike protein, and analyzed by SDS-PAGE. The identity of each mutant is listed above the lane. UT, untransfected cells; pL2, cells which were transfected with the expression vector without any SFV sequences. The positions of the SFV proteins p62, E2, and E1 are indicated. The asterisk shows the higher-molecular-weight form of E2, and the arrowhead indicates the deleted form of E1.

91→Ala, and Gly-91→Asp mutants migrated identically to the wild-type proteins and showed similar amounts of p62 cleavage. In contrast, the Pro-86→Asp, Gly-91→Pro, and Δ83-92 mutants expressed p62 of predominantly wild-type molecular weight, but the E2 cleavage product migrated more slowly than authentic E2 (asterisk). The more slowly migrating E2 observed with these mutants is reminiscent of an SFV spike protein mutant with a deletion in the E1 subunit (25). This mutant did not transport E1 to the plasma membrane, and its E2 subunit was expressed on the surface in a higher-molecular-weight form.

Immunofluorescence experiments with monoclonal antibodies recognizing E1 or E2 were used to evaluate the surface localization of the spike protein subunits. This analysis revealed an E1 transport defect in the Pro-86→Asp, Gly-91→Pro, and Δ83-92 mutants, as discussed more fully below. The cell surface expression of the E1 and E2 subunits from all of the other mutants was indistinguishable from that of the wild type (data not shown). We also wished to quantitate the amount of spike protein and individual spike subunits at the cell surface for each mutant. For such quantitation, cells were fixed 2 days after transfection, polyclonal antispikes or anti-E1 or -E2 monoclonal antibodies were added, and antibody binding was detected by using 125 I-protein A (40) (Table 1). For mutants in which both subunits were transported to the plasma membrane, we found that equivalent levels of surface spike protein (as determined by polyclonal antibody binding) resulted in similar binding of monoclonal antibodies against the E1 and E2 subunits. Some mutants did show higher levels of binding of the anti-E2 antibody. The E1 transport mutants Pro-86→Asp, Gly-91→Pro, and Δ83-92 showed negligible binding of the anti-E1 antibody, a finding consistent with the transport defect described below.

We were concerned that cell-cell fusion might be greatly affected by the quantity of protein expressed at the cell surface. Since the mutants showed some variation in the levels of cell surface expression, we normalized the surface

TABLE 1. Cell surface expression of mutant and wild-type SFV spike proteins^a

Expt no.	Plasmid	DNA concn (μg/ml)	125 I-protein A binding (cpm) with antiserum:		
			Antispikes	Anti-E1	Anti-E2
I	None		5,388	1,249	1,076
	Wild type	2	51,019	3,728	9,436
	Lys-79→Gln	1	56,996	4,339	11,366
	Met-88→Leu	5	56,335	4,009	10,103
	Gly-83→Ala	3	44,628	3,097	10,327
	Gly-91→Ala	4	50,614	3,615	16,044
	Gly-91→Asp	3	57,364	4,099	14,208
	Pro-86→Asp	6	30,636	1,730	15,214
	Δ83-92	6	23,514	1,734	13,968
II	None		2,014	725	777
	Wild type	2	10,953	1,263	2,258
	Asp-75→Ala	6	11,163	1,207	3,119
	Gly-91→Pro	6	5,890	891	2,901

^a COS cells were transfected with wild-type and mutant plasmids and fixed 2 days after transfection. Duplicate samples were analyzed by the addition of a polyclonal antibody to the spike protein (antispikes) or a monoclonal antibody specific for the E1 subunit (antibody E1-2) or the p62-E2 subunit (antibody E2-1) (21). Antibody binding was detected by using 125 I-protein A as described in Materials and Methods.

amounts of mutant and wild-type spike proteins by using various amounts of DNA for transfection (Table 1) and used similar concentrations for subsequent fusion assays. However, when cell-cell fusion experiments were performed on cells transfected with from 0.5 to 3 μg of wild-type DNA per ml, the pH dependence and efficiency of cell-cell fusion were equivalent for each sample (data not shown). The cell surface expression in these samples varied over about a twofold range. This result suggests that within our working range of protein expression, the amount of spike protein at the surface is not limiting for fusion.

Mutants in which both subunits were transported to the cell surface were analyzed for pH-dependent fusion activity in a cell-cell fusion assay. Cells expressing wild-type or mutant spike proteins were treated with medium of the indicated pH to trigger fusion and returned to culture for an additional 4 h to permit expression of the spike protein on the surface of the polykaryons. The cells were then fixed and stained with an antibody to the spike protein to permit identification of expressing cells, and with a DNA stain to visualize the nuclei (Fig. 3). This assay permitted accurate visualization of fusion and reproducible quantitation of the fusion pH dependence. Since COS cells have a background level of polykaryon formation, it was also helpful to be able to visualize fusion specifically in the expressing cell population. Our mutants displayed four phenotypes, which are described in detail below.

Mutation of Lys-79 or Met-88 did not alter membrane fusion activity. Fusion assays were performed on COS cells transfected with the wild-type, Lys-79→Gln, and Met-88→Leu constructs (Fig. 4a). The expressed wild-type spike protein showed potent membrane fusion activity, with a threshold at pH 6.5. Fusion was maximal at about pH 6.2 and showed an efficiency of greater than 90%. These data agree with those from several types of fusion assays performed with purified wild-type virus (50, 52) and with those from previously published assays of expressed wild-type spike protein (25, 30). The Lys-79→Gln and Met-88→Leu mutants showed fusion activity which was indistinguishable

FIG. 3. Immunofluorescence assay of pH-dependent fusion in COS cells expressing the SFV spike protein. COS cells were transfected with the wild-type pL2 construct and assayed for membrane fusion 2 days later by treatment for 1 min at pH 7.0 (a) or pH 5.0 (b). After reculture for 4 h, cells were fixed and stained with a polyclonal antibody against the SFV spike protein and with propidium iodide to detect cell nuclei. Note large polykaryons in the expressing cells treated at pH 5.0. Bar, 10 μ m.

from that of wild-type spike protein. Lys-79 is conserved among SFV and the Ross River, Sindbis, and O'Nyong-nyong viruses but has been changed to an Arg, Ala, or Gln in Western, Eastern, and Venezuelan equine encephalitis viruses, respectively (see references in Fig. 1 legend). Although the positive charge is within the conserved hydrophobic domain, it appears not to be critical for the pH dependence of fusion. Met-88 is conserved among all known alphavirus E1 and rotavirus VP4 sequences (Fig. 1). Since Met has only one codon, conservation of this residue suggested a strong selective pressure and a possible role of the Met sulfur atom in the fusion mechanism. The efficient membrane fusion of the conservative Leu-88 replacement, however, excludes a specific requirement for Met in fusion. These results also agree with the observed sequence divergence within the hydrophobic domain, which suggests that while specific amino acids may be required for the spike protein fusion function, variation is permitted at other positions.

FIG. 4. Fusion activity of the Lys-79→Gln, Met-88→Leu, Asp-75→Ala, Gly-83→Ala, and Gly-91→Ala mutations. COS cells expressing wild-type (wt) and mutant SFV spike proteins were assayed for cell-cell fusion activity by treatment for 1 min at 37°C at the indicated pH. The cells were then processed as described in the legend to Fig. 3. Fusion was quantitated by counting the number of nuclei in expressing cells, with the formula given in the text (53). (a) These data are a representative example of three experiments: ●, wt; ◇, Lys-79→Gln; ▲, Met-88→Leu. (b) These data are the average of three separate experiments: ●, wt; □, Asp-75→Ala; △, Gly-83→Ala; ◆, Gly-91→Ala.

Mutations that shift the pH threshold of membrane fusion.

Three of the E1 mutations produced spike proteins with markedly more acidic fusion thresholds (Fig. 4b). The hydrophobic domain has strikingly conserved Gly at positions 83, 90, and 91. The Gly at position 83 was changed to Ala, a conservative replacement. This mutation resulted in spike proteins with a pH threshold of 6.1, maximal fusion at pH 5.3, and an unaltered final efficiency of fusion (>80%).

The putative E1 fusion domain is flanked by negatively charged Asp residues at positions 75 and 97 (Fig. 1). Replacement of the Asp at position 75 with Ala resulted in spike proteins with a fusion threshold of pH 5.8 and maximal fusion at pH 5.0 (Fig. 4b). In addition, the maximum efficiency of fusion was reduced to about 70% at pH 5.0 and did not change even after treatment at pH 4.7 (data not shown).

The amino acids 90 to 92 (Gly-Gly-Ala) are conserved among both alpha- and rotaviruses. Since Gly has no side chain hindrance, it increases the flexibility of the polypeptide backbone and can be an alpha helix breaker (45). We assessed the effect of a conservative mutation of Gly-91 to Ala. A dramatic pH shift of the fusion threshold to pH 5.4

FIG. 5. Gly-91→Asp mutation blocks membrane fusion. COS cells expressing wild-type and mutant SFV spike proteins were assayed for cell-cell fusion activity after pH treatment for 1 (○, ●), 3 (□), or 10 (△) min at 37°C. The 1-min graph shows the averages of three separate experiments. The 3- and 10-min graphs show the averages of two separate experiments.

was observed with this mutant (Fig. 4b). Fusion was maximal at pH 5.0, and the efficiency was reduced to ~70%, even after treatment at as low as pH 4.7 (data not shown).

One possible explanation for the shift in pH threshold of these three mutants is that the kinetics of the conformational change in E1 are slower. However, increasing the length of pH treatment from 1 to 3 or 10 min had no effect on the pH threshold or efficiency of fusion for either the wild type or the pH shift mutants (data not shown).

The single-amino-acid change Gly-91→Asp blocked membrane fusion. Since the most striking phenotype of the pH shift mutants was that of Gly-91→Ala, we explored the effects of other mutations at this position. Gly-91 was changed to Asp, inserting a negative charge within the conserved apolar region. Since Asp is a relatively small amino acid, this substitution is not uncommon in nature (see reference 45, p. 14 and 15), although the two amino acids differ substantially in their solvation free energies (7). As shown in Fig. 5, the Gly-91→Asp mutant was unable to mediate membrane fusion at any of the pH values tested. The complete block in membrane fusion was not reversed by treatment at low pH for 3 or 10 min (Fig. 5) or by incubation at pH 4.7 (data not shown). Antibody-binding data showed that both the E1 and E2 subunits were present at the cell surface within the population of cells expressing Asp-91 (Table 1). In addition, double immunofluorescence analysis of the Gly-91→Asp mutant was performed with a mouse monoclonal antibody against the E1 subunit and a rabbit polyclonal antibody which recognized both E1 and E2. Every cell which was surface positive with the rabbit antibody (indicating the presence of E2 and/or E1) was also positive with the E1 monoclonal antibody (data not shown). Thus, the block in fusion appears to be a specific consequence of the Gly-91→Asp mutation rather than an effect on overall spike protein assembly or transport. We have not yet evaluated the activity of this mutant in other assays for membrane fusion.

Pro-86→Asp, Gly-91→Pro, and Δ83-92 mutations cause an E1 transport defect. From our initial analysis, the Pro-86→Asp, Gly-91→Pro and Δ83-92 mutants appeared to have a defect in cell surface transport of the E1 subunit. Thus, a monoclonal antibody to the E1 subunit showed no cell surface binding (Table 1), and the E2 subunit migrated at a higher molecular weight (Fig. 2). To localize the E1 subunit within the cell, immunofluorescence was performed with an

FIG. 6. Localization of E1 from the Δ83-92 mutant. COS cells expressing the deletion plasmid construct were fixed with 3% paraformaldehyde (a) or with methanol to permeabilize the membrane (b). The cells were then stained with E1-1, a mouse monoclonal antibody against the E1 subunit (21), followed by a fluorescein-labeled goat antiserum against mouse immunoglobulins. Representative fields were photographed by fluorescence microscopy. The E1 staining pattern observed in panel b is indicative of endoplasmic reticulum localization. Bar, 10 μm.

E1-specific monoclonal antibody. Figure 6 shows the results for the Δ83-92 mutant. No cell surface staining of expressing cells was observed (Fig. 6a), and when the cells were permeabilized and stained, E1 was localized in a cytoplasmic network typical of the rough endoplasmic reticulum (RER) (Fig. 6b). Staining with an anti-E2 monoclonal antibody showed abundant E2 at the cell surface (data not shown). The results with the other two mutants were identical. Cell-cell fusion experiments were performed with all three mutants and showed a complete block in membrane fusion (data not shown). This result was expected, since the E1 subunit is required for fusion (25, 34).

Transport of E1 to the cell surface requires its association with E2, while E2 can be transported in the absence of E1 (29). To confirm the retention of the E1 subunit in the RER, cells expressing the mutant constructs were pulse-labeled for

FIG. 7. Endo H digestion of transport-defective mutants. COS cells expressing wild-type (wt) or mutant spike proteins were pulse-labeled for 20 min with [35 S]methionine, chased for 60 min, and immunoprecipitated with a polyclonal antibody against the SFV spike protein. Duplicate samples were untreated (-) or treated with Endo H (+) and analyzed by electrophoresis on 12.5% SDS gels. The higher-molecular-weight, Endo H-insensitive forms of p62 and E2 are indicated by p62' and E2', respectively.

20 min, chased for 60 min, immunoprecipitated, and digested with Endo H. Conversion of E1 to an Endo H-resistant form is indicative of its transport to the medial Golgi and acquisition of complex-type carbohydrate (26, 29), as shown for wild-type E1 in Fig. 7. In contrast, the E1 subunits from all three mutants were completely Endo H sensitive, indicative of retention within the RER (Fig. 7). E1 from the mutants was also turned over more rapidly, suggesting that it may be degraded within the RER degradative pathway (24). The wild-type E2 protein was Endo H sensitive, in agreement with the fact that E2 has one complex and one high-mannose oligosaccharide (13, 43). Surprisingly, the majority of the E2 subunits from the three mutants were Endo H resistant, suggesting that both glycosylation sites on mutant E2 were processed to complex carbohydrate. The aberrant molecular weight of E2 in these three mutants is consistent with correct p62 cleavage and such additional glycosylation.

DISCUSSION

Our working model of the SFV fusion mechanism is that low pH causes a conformational change in the E2 and E1 subunits. These conformational changes act to expose the previously hidden E1 hydrophobic domain, perhaps through altering the dimer interaction between the two subunits, as has been suggested by Lobigs et al. (30). The hydrophobic domain then interacts with cholesterol in the target membrane, allowing further conformational changes in E1 and ultimately leading to fusion of the viral and host membranes. As a first step in testing this model, mutagenesis of the conserved hydrophobic domain of the SFV E1 protein was used to evaluate its involvement in SFV's membrane fusion activity.

Our mutants had several phenotypes, defined by effects on fusion activity and spike assembly and transport. The Lys-79→Gln and Met-88→Leu mutations had no discernible effects on fusion, surface expression, monoclonal antibody binding, or glycosylation. We do not yet know the effects of these mutations in a virus particle, but if such spike proteins can assemble in a virion, they should be able to fuse with the host cell membrane.

The Pro-86→Asp, Gly-91→Pro, and Δ 83-92 mutations prevented exit of E1 from the RER without impairing the

transport of E2. Thus, these mutations appear to inhibit formation of the E1-p62 dimer required for E1 transport (reviewed in reference 29). The carbohydrate processing of E2 was altered in these mutants, perhaps from increased accessibility or longer exposure to processing enzymes in the Golgi complex. The effect of these mutations on E1-p62 dimerization and transport could imply a role of the E1 hydrophobic domain in directly interacting with p62. Given the nonconservative nature of these mutations, however, we believe that the transport defect is more likely due to an overall disruption in the conformation of E1, similar to that found in other E1 transport mutants that have been described (25). It is possible that the hydrophobic peptide must normally be masked in the endoplasmic reticulum by association of E1 and p62 in order for transport to proceed further. In the absence of dimerization, the hydrophobic peptide could cause E1 to self-aggregate or associate with molecules such as BiP that may bind to exposed hydrophobic moieties (36).

The Asp-75→Ala, Gly-83→Ala, and Gly-91→Ala mutations resulted in spike proteins that required a more acidic pH to trigger fusion. Early endosomes have a pH of ≤ 6.2 and gradually become more acidic with time, attaining a pH of ≤ 5.3 (44). Less acidic endosomes are found in the cell periphery, and more acidic endosomes and lysosomes are concentrated in the perinuclear region (47). Similar acid pH shift mutations of the spike proteins in a virus would probably alter the endosomal site of fusion and RNA release and thus could influence the infection pattern in cells and tissues. These three mutants indicate the importance of both the charges flanking the hydrophobic domain and the highly conserved glycine residues within the core of the hydrophobic region. The altered pH threshold may indicate that at each pH point, less mutant spike protein is converting to the active acid conformation, resulting in a decreased amount of fusion. Alternatively, equivalent amounts of wild-type and mutant E1 may convert at a given pH, but the mutant peptide itself may be less efficient in mediating the fusion reaction. It will be interesting to assay the pH shift mutants for the pH dependence of the E1 conformational change and E1-E2 dimer interaction (21, 48).

Substitution of an Asp for the conserved Gly at position 91 blocked fusion completely in our assay system, without noticeably affecting the transport of the spike protein or its epitopes for monoclonal antibody binding. We predict that this mutation would be lethal in a virus particle, resulting in the assembly of a nonfusogenic and hence noninfectious virion. This mutant again points out the importance of the Gly-Gly-Ala sequence within the fusion peptide, a region that is also strictly conserved in rotavirus VP4.

Rotaviruses are nonenveloped viruses with a double protein shell and a segmented double-stranded RNA genome (16). Unlike alphaviruses, rotaviruses have a very narrow host range, and entry into the host cell is independent of low endosomal pH (15, 17). In view of the many differences between alpha- and rotaviruses, the amino acid sequence identity between alphavirus E1 and the rotavirus outer capsid protein VP4 is very surprising. Structural analysis shows that VP4 projects in a spike from the surface of the virion (38, 56) and suggests that the hydrophobic domain is located at the distal tip of the spike protein (38). Given the sequence conservation, it was suggested that this domain on VP4 may be involved in the entry of rotavirus into the cell (31). Our data implicating the E1 hydrophobic domain in SFV fusion further strengthen the case for an important involvement of the VP4 sequence in the transfer of the

rotavirus genome across the host cell plasma membrane. Determination of the precise role of VP4 in virus entry awaits the development of assays for rotavirus-membrane interactions.

It is helpful to consider the results from the pH shift and Gly-91→Asp mutants in comparison with mutagenesis results from influenza virus, which also infects cells via endocytosis and acid-mediated fusion. To date, the influenza virus hemagglutinin (HA) is the best characterized virus fusion protein both structurally and mechanistically (see reference 54 for review). HA must be cleaved to HA1 and HA2 to become active, creating a new amino terminus on HA2 that contains the fusion peptide. This hydrophobic and highly conserved domain is normally buried in the interface between the trimeric HA spike subunits and becomes exposed during low-pH treatment. The exposed fusion peptide markedly increases the hydrophobicity of HA and interacts directly with membrane during fusion.

While the influenza virus HA and the SFV spike protein both mediate acid-induced membrane fusion, some interesting differences exist between the two proteins. The SFV spike protein is also proteolytically activated, but cleavage occurs on the p62 precursor of E2, not on the fusion-active E1 subunit (14, 28, 30). SFV fusion and infection are dependent on cholesterol in the target membrane, while fusion of influenza virus is independent of cholesterol (6, 51). The overall hydrophobicity of SFV E1 does not increase at acid pH (19; but see also reference 34), and the hydrophobic domains of SFV E1 and influenza virus HA2 have no sequence homology. Although these differences obviously mean that analogies must be made cautiously, some potential similarities are suggested by the results of mutagenesis of the two proteins.

A variety of mutations affecting the fusion activity of influenza virus HA have been described (reviewed in reference 54). Notably, mutations throughout the length of the trimer interface alter the fusion pH dependence, either by destabilizing the buried position of the fusion peptide or by modifying salt bridges and hydrogen bonding between trimer subunits. A complete block in cell-cell fusion was only observed with one mutation, a substitution of Glu for Gly at position 1 of HA2 (9). Thus, while mutations at many positions could affect the pH dependence of fusion, only a mutation within the fusion peptide itself was found to block fusion.

We report on mutations at three positions within the hydrophobic domain that shift the pH threshold of fusion to a more acidic range. The effects of several other alphavirus sequence alterations on fusion have been analyzed. Changes in two positions in the Sindbis virus E1 protein affect pH-dependent fusion (1). Either a Val-72→Ala or a Gly-313→Asp substitution causes an acid shift in the pH threshold of fusion and also appears to confer increased neurovirulence. While Ala-72 is adjacent to the conserved hydrophobic domain of E1 that we have analyzed here, Asp-313 is distant in the linear sequence. We have previously described an SFV mutant called *fus-1*, whose threshold for fusion is shifted to pH 5.3 (22). The molecular nature of the *fus-1* mutation is undefined at present, but sequence analysis of a *fus-1* cDNA clone showed that the sequence of the *Cla*I fragment including the E1 hydrophobic domain was identical to that of wild-type SFV (unpublished observations). Thus, sequence alterations outside of the E1 hydrophobic domain can affect the pH sensitivity of the spike protein. As in influenza virus HA, numerous residues may be responsible for the overall effect of pH on SFV spike

protein conformation, while key amino acids within the fusion peptide act to directly mediate fusion. The complete cell-cell fusion block in the Gly-91→Asp mutant thus is the most compelling evidence for the direct involvement of the E1 hydrophobic domain in fusion. It is possible that the Gly-Gly-Ala sequence in E1 is particularly important for interactions with cholesterol. Thus, one explanation for the complete fusion block in the Gly-91→Asp mutant is that the mutant E1 is no longer able to interact with cholesterol to bring about fusion.

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