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Mutations at Two Conserved Acidic Amino Acids in the Glycoprotein of Vesicular Stomatitis Virus Affect pH-Dependent Conformational Changes and Reduce the pH Threshold for Membrane Fusion

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Recently, we had shown that amino acid substitutions at residues 124, 127, and 133 either abolished or drastically altered the fusion activity of the glycoprotein (G protein) of vesicular stomatitis virus (VSV), indicating that this region is important for membrane fusion and may constitute an internal fusion domain. In this report we show that amino acid substitutions at two conserved acidic residues located at the C-terminal end of the putative fusion domain also affect the fusion activity of G protein. Two substitutions, D137-L and E139-L, slightly reduced the pH threshold at which G protein mediates membrane fusion. However, both D137-L and E139-L had fusion activities equivalent to that of wild-type G protein after exposure to a pH of 5.7 or below. To determine if the fusion activity of G protein required an acidic residue in this region both D137 and E139 were replaced with serine. This double substitution also reduced the pH threshold for fusion activity, but the effect appeared to be less severe than that for either of the single substitutions. The mutated G protein cDNAs were also introduced into a VSV minigenome to examine the effect of the substitutions on virus assembly and infectivity. All three mutant G proteins assembled into particles and these virions were infectious despite the altered fusion activities of the mutant G proteins. Although particles containing the mutant proteins were infectious they appeared to be attenuated, suggesting that these two acidic residues, which are conserved in several distantly related rhabdoviruses, play an important role in maintaining virus fitness.

INTRODUCTION

Vesicular stomatitis virus (VSV) is an enveloped, negative-strand RNA virus which enters a host cell through receptor-mediated endocytosis (Matlin *et al.,* 1982). VSV encodes a single envelope glycoprotein (G protein) which mediates both binding of the virus to the cell surface and fusion between the viral envelope and the endosomal membrane after endocytosis. Although the mechanism of G protein-mediated membrane fusion remains to be elucidated, the low pH of the endosome is thought to induce conformational changes in G protein that result in the exposure of a domain which interacts with the target membrane and initiates the fusion event (Blumenthal *et al.,* 1987; Crimmins *et al.,* 1983; Doms *et al.,* 1987; Puri *et al.,* 1988).

We and others have previously demonstrated that mutations between amino acids 117 and 136 either greatly reduce or abolish the fusion activity of G protein (Fredericksen and Whitt, 1995; Li *et al.,* 1993; Whitt *et al.,* 1990; Zhang and Ghosh, 1994), and it has been proposed that this region, which consists of primarily uncharged amino acids, may constitute an internal fusion domain (Fredericksen and Whitt, 1995; Ohnishi, 1988; Whitt *et al.,* 1990; Zhang and Ghosh, 1994). It has recently been reported that a large segment of G protein (amino acids 59 to 221), which contains the putative fusion domain, becomes preferentially labeled by a hydrophobic photoaffinity reagent during the initial stages of membrane fusion with large unilamellar vesicles (Durrer *et al.,* 1995). This result is the first direct demonstration that a specific region of the G protein ectodomain interacts with target membranes and provides support for the hypothesis that amino acids 117–136 may constitute an internal fusion domain.

Internal fusion domains are thought to consist of 16 to 20 primarily hydrophobic and uncharged, polar amino acids, with positively or negatively charged residues at the C-terminal end of the domain (White, 1990). Immediately following the putative fusion domain of VSV G protein there are two acidic amino acids, D137 and E139, which are conserved between all vesiculoviruses, and the equivalent position for E139 is present in several of the more distantly related lyssaviruses (Durrer *et al.,* 1995). This degree of conservation suggests that these acidic amino acids play an important role in maintaining the structure and/or function of rhabdovirus G proteins.

We had previously reported that the substitution of a leucine for either D137 or E139 had little to no effect on the fusion activity of G protein when examined at pH 5.7

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using a cell–cell fusion assay (Fredericksen and Whitt, 1995). However, we consistently observed a reduction in fusion activity compared to that seen with wild-type G protein at pH 6.3, which is near the threshold for wild-type G protein fusion activity. In a separate study, Zhang and Ghosh found that substituting asparagine for D137 also shifted the pH optimum for cell–cell fusion and they suggested that this mutation may increase the stability of G protein such that more acidic conditions are required to expose the fusion domain (Zhang and Ghosh, 1994). To gain a better understanding of the importance of amino acids 137 and 139 in both G protein fusion activity and pH-dependent conformation changes we have made several new mutations at both of these residues and examined the effects of these mutations on G protein structure and function.

The majority of substitutions made at positions 137 and 139 resulted in G proteins with fusion activities similar to that of wild-type G protein at all pHs examined using a cell–cell fusion assay. However, three of the substitutions shifted the pH threshold for membrane fusion to more acidic conditions. Substituting leucine for either amino acid 137 or amino acid 139 (D137-L and E139-L), or replacing both residues with serine (DE-SS) resulted in G proteins with only low levels of fusion activity when exposed to pHs above 5.9, but each of these mutants was capable of inducing syncytia formation at levels equivalent to that of wild-type G protein at pH 5.7. These results indicated that neither D137 nor E139 is essential for the fusion activity of G protein, but the presence of at least one of the acidic residues appears to be important for maintaining the relatively high pH threshold for fusion activity characteristic of rhabdovirus G proteins. Using replication-competent VSV minigenomes we found that the mutations which altered the fusion activity of G protein also reduced the number of cells that became infected during subsequent passages. This effect on virus spread could not be fully explained by reduced accumulation of the mutant G proteins on the cell surface; instead, these mutations appear to slightly reduce virus infectivity. The effects of amino acid substitutions at D137 and E139 on both membrane fusion activity and virus infectivity may explain why these residues are conserved in rhabdovirus G proteins.

MATERIALS AND METHODS

Cell culture, viruses, transfections, and rescue assay

HeLa cells and baby hamster kidney (BHK-21) cells were grown in high-glucose Dulbecco's modified Eagle medium (DMEM) containing 5% fetal bovine serum, 100 units penicillin per milliliter, and 100 mg streptomycin per milliliter. Cell transfections were performed as described previously (Whitt *et al.,* 1991b) using a suspension of liposomes composed of dimethyldioctadecyl ammonium bromide (DDAB) and L-α-dioleoylphosphatidylethanolamine (DOPE) at a weight ratio of 1:2.5, respectively (Rose *et al.,* 1991).

Plasmids and oligonucleotide-directed mutagenesis

The construction of mutants D137-L, E139-I, E139-L, E139-S, and E139-T has been described previously (Fredericksen and Whitt, 1995). All other mutations were introduced into pBS-G (Fredericksen and Whitt, 1995) by overlap PCR. Using pBS-G as a template, DNA fragments encoding the desired mutations were generated by PCR amplification using sense-strand mutagenic primers which contained the appropriate nucleotide changes for the indicated amino acid substitutions and an antisense, nonmutagenic oligonucleotide overlapping the *Nco*I site. The mutagenic primers removed the *Sfa*NI site from this segment of the G protein gene. A second fragment was generated by PCR amplification of the 5′ end of the G protein gene using pBS-G as a template and an oligonucleotide complementary to the T7 promoter and a downstream antisense, nonmutagenic primer complementary to nucleotides 494 to 527 in the G protein cDNA. The second PCR fragment contains the wild-type *Sfa*NI site and overlaps approximately 200 bp of the 5′ end of the first PCR fragment, which contains the desired mutations. The desired full-length fragment was generated by using the two PCR fragments as templates for another round of PCR amplification in which the T7 promoter oligonucleotide and the nonmutagenic oligonucleotide overlapping the *Nco*I site were used as primers. The products of this amplification were extracted with phenol–chloroform, precipitated with ethanol, resuspended in TE buffer [10 m*M* Tris, 1 m*M* EDTA (pH 8.0)], digested with *Sfa*NI to remove wild-type products, and the undigested fragment was gel purified. The recovered fragment, which should contain the desired mutations, was subsequently digested with *Apa*I and *Nco*I and then used to replace the corresponding wild-type fragment in pBS-G. Clones were screened for the presence of a new *Rsa*I restriction site which was introduced by the mutagenic primer and that did not alter the amino acid sequence of the protein. PCR-amplified regions were sequenced to verify that the appropriate mutations were present and that no additional nucleotide substitutions were introduced during PCR amplification. Sequences of the oligonucleotides and the conditions used for PCR amplification for the individual clones are available upon request.

To generate minigenome constructs containing the desired mutations the mutated G protein cDNAs were digested with *Dra*I and *Nco*I and a 459-bp fragment was purified by gel electrophoresis and recovered following electroelution. These fragments were then subcloned into pBS-GMMG (Stillman *et al.,* 1995) using a three-component reaction in which a *Mlu*I to *Dra*I fragment from pBS-GMMG and the *Dra*I to *Nco*I fragment recovered from the mutated G genes were ligated to a vector

consisting of pBS-GMMG that had been digested with *Mlu*I and *Nco*I.

Surface expression and syncytium formation assays

Duplicate plates of HeLa cells were rinsed two times with serum-free DMEM (SF-DMEM) and then infected with the recombinant vaccinia virus vTF7-3 (Fuerst *et al.,* 1986) which expresses bacteriophage T7 RNA polymerase at a multiplicity of infection (m.o.i.) of 10 for 30 min at 37°. The inoculum was removed and the cells were transfected with plasmids encoding either wild-type or mutant G proteins as described previously (Whitt *et al.,* 1991b) or were incubated in SF-DMEM without DDAB:DOPE liposomes (mock-transfected). At 7 hr posttransfection, cells from one set of duplicate plates were processed for flow cytometry as described previously (Whitt *et al.,* 1990). To examine low-pH-induced membrane fusion activity, cells from the other set of duplicate plates were rinsed once with fusion medium [10 m*M* Na_2HPO_4, 10 m*M* NaH_2PO_4, 150 m*M* NaCl, 10 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES), and 10 m*M* *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid, titrated to the indicated pH with HCl] and then bathed for 1 min in fresh fusion medium at room temperature. After 1 min incubation, the fusion medium was replaced with DMEM containing 5% FBS and the plates were returned to a 37°, 5% CO_2 incubator for 20 min. Following the 20-min incubation, the medium was removed and ice-cold phosphate-buffered saline containing 0.9 m*M* $CaCl_2$ and 0.5 m*M* $MgCl_2$ (PBS^+) was added. The cultures were maintained at 4° to prevent further cell–cell fusion until individual cultures could be examined for syncytium formation using an inverted light microscope and phase contrast optics. After the addition of ice-cold PBS^+, the number of syncytia and the number of single (unfused) cells were counted in three to five separate fields containing approximately 100 cells per field for each of the mutants and for parallel cultures of cells that were either mock-transfected or expressing wild-type G protein. Fusion activities were quantitated by determining the percentage of fused cells per field for each of the mutants. The percentage of fused cells was determined by first subtracting the number of syncytia containing three or more nuclei present in the mock-transfected cultures from the number of syncytia seen in the cells expressing either wild-type G or the G protein mutants. This value represents the corrected number of fused cells ($fused_{corr}$) and excludes any background fusion resulting from spontaneously fused cells or from cells that may have fused due to the activity of the vaccinia virus fusion protein (Doms *et al.,* 1990; Rodriguez *et al.,* 1987). The percentage of fused to unfused cells was then determined by calculating the ratio of $fused_{corr}$ to unfused cells in each field. The percentage cells fused represents the average value obtained from each of the fields.

Metabolic labeling, transport kinetics, trimer stability assays, and trypsin sensitivity assays

HeLa cells expressing either wild-type or mutant G proteins were rinsed once with methionine-free SF-DMEM (Met-free DMEM) at 2 to 4 hr posttransfection, incubated for 10 min in Met-free DMEM, and then radioactively labeled for various amounts of time in 1 ml of Met-free DMEM containing sufficient Express Protein Labeling mix (DuPont/New England Nuclear) to give 50 μCi [^{35}S]methionine per milliliter. Following the pulse, the radioactive labeling medium was removed and either the cells were lysed immediately by adding 1 ml of detergent lysis buffer (10 m*M* Tris, pH 7.4, 66 m*M* EDTA, 1% Triton X-100, 0.4% deoxycholic acid, 0.02% sodium azide) or the labeling medium was replaced with DMEM containing 5% FBS and 2 m*M* additional methionine (chase medium). Immunoprecipitation was performed essentially as described previously (Rose and Bergmann, 1982) except that the postnuclear cell lysates were made to 0.3% sodium dodecyl sulfate (SDS) and the antigen–antibody complexes were formed for 30 min at 37°. Digestion of immunoprecipitates with endoglycosidase H was performed according to the manufacturer's instructions (New England Biolabs).

Trimer stability was examined by lysing radioactively labeled cells with 1% Triton X-100 in 2× MNT buffer (40 m*M* MES, 60 m*M* Tris, 200 m*M* NaCl, 2.5 m*M* EDTA) at the pH indicated as described previously (Whitt *et al.,* 1991a). Cell lysates were centrifuged on 5–20% sucrose gradients as described (Whitt *et al.,* 1991a). The fractions were immunoprecipitated with polyclonal anti-VSV serum, electrophoresed through 10% polyacrylamide gels containing SDS (Laemmli, 1970), and the resolved proteins were visualized following fluorography (Bonner and Laskey, 1974).

To examine trypsin sensitivity of G protein 1×10^6 cells were radioactively labeled and then lysed with 1 ml 2× MNT containing 1% Triton X-100 at pHs indicated. The lysates were centrifuged at 14,000 *g* for 5 min to remove nuclei and cell debris. Aliquots containing 170 μl of the postnuclear supernatants were then incubated with or without 10 μg TPCK–trypsin for 30 min at 37°. Aprotinin (0.1 trypsin inhibitor unit) was added to all samples to inhibit further digestion and the G proteins were immunoprecipitated using a polyclonal anti-VSV serum. Proteins were resolved on a 10% polyacrylamide gel containing SDS and visualized by fluorography.

Expression and passage of minigenomes

Passages of minigenomes were carried out as previously described (Stillman *et al.,* 1995) with minor changes. BHK-21 cells grown in 35-mm-diameter dishes were infected with vTF7-3 at a m.o.i. of 10 for 1 to 1.5 hr. Cells were subsequently transfected with plasmids encoding GMMG and the nucleocapsid (N), phosphopro-

TABLE 1

Surface Expression of G Protein Mutants

Protein	% of cells positive	Fluorescence intensity	Relative surface expression[a]
WT-G	29.5	24.5	+++
D137-A	59.5	32.1	+++
D137-E	25.3	30.4	+++
D137-L	31.2	25.7	+++
D137-S	60.7	34.3	+++
DE-LL (137/139)	6.2	6.5	+
DE-SS (137-139)	68.4	27.2	+++
E139-D	42.3	26.4	+++
E139-I	59.1	29.9	+++
E139-L	35.0	24.6	+++
E139-Q	0.1	10.6	−
E139-S	40.0	29.2	+++
E139-T	48.1	31.4	+++

[a] Relative surface expression = (% positive cells in mutant population) (mean fluorescence intensity)/(% positive cells in wild-type population) (mean fluorescence intensity). Values of 1.0 or higher = (+++); 0.1 to 1.0 = (++); 0.01 to 0.1 = (+); 0.001 to 0.01 = (−).

tein (P), and large catalytic subunit (L) proteins at a ratio of 10:5:4:1 μg plasmid DNA, respectively, per dish. At 24 hr posttransfection the medium was removed and the cells were fixed with 3% paraformaldehyde in PBS. Indirect immunofluorescence was used to visualize G protein expression on the cell surface and was performed as described previously (Stillman *et al.,* 1995).

To passage particles 0.5 to 2 ml of medium from the primary transfection was added to cells expressing the N, P, and L proteins only at 5 hr posttransfection. After incubating for 60 min 0.5 to 1.5 ml of fresh medium was added directly to the plate containing the inoculum. The cells were then incubated for 24 hr, fixed, and probed for G protein expression using immunofluorescence microscopy.

RESULTS

The region between amino acids 117 and 136 has been suggested to constitute an internal fusion domain of G protein (Fredericksen and Whitt, 1995; Ohnishi, 1988; Whitt *et al.,* 1990; Zhang and Ghosh, 1994). Two charged residues, aspartic acid 137 and glutamic acid 139, are located at the C-terminal end of this region. To determine if these two charged residues are important for the membrane fusion activity of G protein we have made several substitutions at both positions and examined the effect of these mutations on the transport, surface expression, and pH-dependent fusion activity of G protein.

Cell surface expression and membrane fusion activity of mutant G proteins

To examine the fusion activities of the mutant G proteins we assayed the extent to which cells expressing these proteins form syncytia when exposed to acidic pH. Because syncytia formation requires expression of G protein on the cell surface we first examined the accumulation of the mutant G proteins on the plasma membrane using flow cytometry. Most of the mutant G proteins were expressed on the plasma membrane at slightly lower levels than wild-type G protein when equal amounts of plasmid DNA were used to transfect cells (data not shown), and two of the mutants, DE-LL and E139-Q, were either not expressed or expressed at levels significantly less than that of wild-type G protein. These results indicated that all of the substitutions affected either the transport or the stability of G protein. However, when the amount of plasmid DNA encoding wild-type G protein was reduced to 1 μg most of the mutants were expressed at levels similar to that of wild-type G protein (Table 1). Both the number of cells expressing the mutant G proteins and the mean fluorescence intensity, which is a measure of the relative surface density of G protein per cell, were similar to those of wild-type G protein. Therefore, the levels of mutant G proteins present on the cell surface were equivalent to that of wild-type G protein, allowing us to directly compare the fusion activities of the mutants with wild-type G protein using a syncytium formation assay.

The fusion activities of the mutant G proteins were examined by treating cells with fusion media buffered from pH 6.5 to 5.2 for 1 min at 20° and then quantitating the number of syncytia that had formed after incubating at neutral pH for 30 min at 37°. Cells expressing wild-type G protein began to form syncytia at pH 6.3, and when exposed to pH 6.1 or below most of the cells had fused to form large polykaryons. The majority of the substitutions at amino acids 137 and 139 resulted in G proteins with fusion profiles similar to that of wild-type G; however, three substitutions, D137-L, E139-L, and DE-SS, resulted in G proteins with altered fusion thresholds (Fig. 1). Both D137-L and E139-L were significantly reduced in their ability

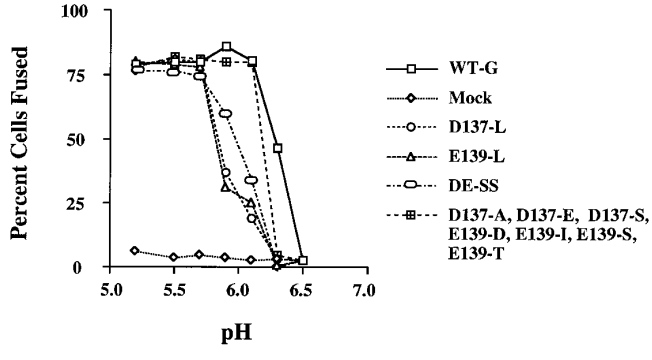

FIG. 1. pH dependence of G protein membrane fusion activity. HeLa cells expressing equivalent amounts of either wild-type G protein (WT-G) or mutant G proteins on the cell surface, or that were infected with vTF7-3 only (mock) were treated with fusion medium at the pH indicated. The percentage of fused cells in the culture was determined as described under Materials and Methods.

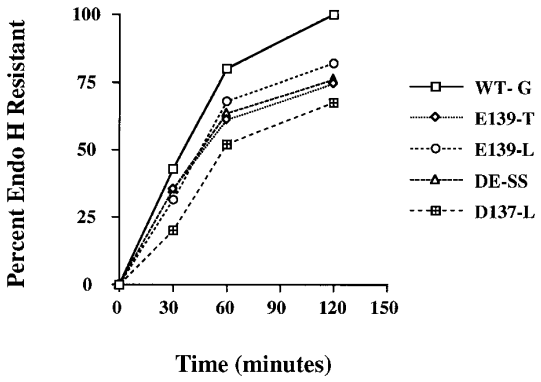

FIG. 2. Transport kinetics of wild-type and mutant G proteins. HeLa cells expressing wild-type G protein (WT-G), D137-L, E139-L, E139-T, or DE-SS were labeled with [^{35}S]methionine for 15 min at 3 hr post-transfection and then chased for the times indicated in medium containing excess methionine. The G proteins were then immunoprecipitated from cell lysates, and one half of the immunoprecipitate was digested with Endo H. The Endo H-sensitive and resistant forms of G protein were resolved by SDS–PAGE and visualized following fluorography. The amount of Endo H-resistant G protein was determined using NIH Image after the fluorogram was digitized using a flatbed scanner.

to induce syncytia formation at pHs of 5.9 and above. Nevertheless, at pH 5.7 these mutants induced syncytia formation at levels equivalent to that seen with cells expressing wild-type G protein. The third mutant, DE-SS, also had an altered pH threshold for fusion activity. The effect of this double substitution on cell–cell fusion appeared to be less dramatic than that observed for either D137-L or E139-L between pH 6.3 and 5.9, but like the single amino acid substitutions, fusion activity equivalent to wild-type G protein did not occur until exposure to pH 5.7. To further examine the fusion activities of D137-L, E139-L, and DE-SS cells expressing the mutant G proteins were exposed to pH 6.1 for 5 min at 37° and then incubated for 30 min at 37° at neutral pH to allow syncytia to form. Under these conditions all three mutant G proteins were capable of inducing syncytia at levels equivalent to that of wild-type G protein (data not shown), suggesting that these mutations may slow the rate at which the mutant G proteins are able to undergo fusion-activating conformational changes.

Transport of G protein

Although most of the mutant G proteins were transported to the cell surface, all of the mutations resulted in some reduction in surface expression compared to wild-type G protein. To determine if this effect was due to reduced transport out of the endoplasmic reticulum (ER) we examined the rate at which the two N-linked oligosaccharides on G protein became resistant to endoglycosidase H (Endo H). As found previously (Rose and Bergmann, 1983; Whitt *et al.,* 1989), the majority of wild-type G protein was resistant to Endo H by 60 min. In contrast, all of the mutants that were examined were transported slower than wild-type G protein (Fig. 2). The reduced rate of transport was most apparent after 120 min of chase where 20–35% of the labeled mutant G population remained sensitive to Endo H. These results indicated that a fraction of the mutant proteins were retained in the ER, possibly because they were misfolded. Although all of the mutants exhibited transport defects, the reduced levels of expression on the cell surface did not appear to be due to reduced protein stability since none of the mutants were degraded faster than the wild-type protein (data not shown). Importantly, the reduced transport rates did not appear to correlate with an altered fusion activity, as shown by the mutant E139-T.

Stability of mutant G protein trimers

Upon exposure to acidic pH, G protein is thought to undergo a series of conformational transitions that are essential for initiating membrane fusion (Puri *et al.,* 1988). To examine pH-dependent conformational changes in the mutant G proteins we monitored the ratio of G protein trimer to monomer after centrifugation through acidic sucrose gradients. No significant differences were observed in the trimer stability profiles of D137-L, E139-L, DE-SS, or E139-T compared to wild-type G protein (Fig. 3). The trimer stability of E139-T was examined to ascertain whether a substitution that had no effect on fusion activity would alter the trimer stability of G protein. These results indicated that mutations at either position 137 or position 139 did not significantly affect the low-pH-induced conformational changes required for trimer stability; however, it should be noted that the structural transitions associated with trimer stability may not correspond to those required for membrane fusion (Durrer *et al.,* 1995).

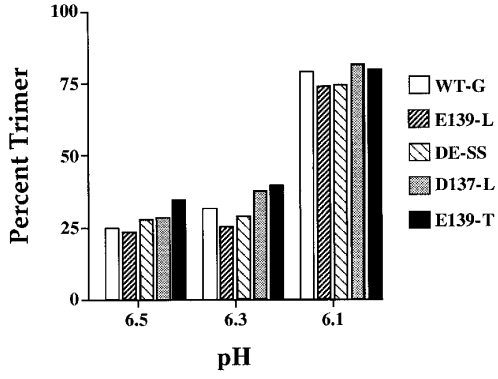

FIG. 3. Trimer stability of G protein. HeLa cells expressing wild-type G protein (WT-G), D137-L, E139-L, E139-T, or DE-SS were labeled for 15 min with [^{35}S]methionine and then chased for 2 hr in the presence of excess methionine. Cell were lysed at the pH indicated, and lysates were centrifuged through 5–20% sucrose gradients buffered at the same pH as the lysis solution. Fractions of the gradient were collected, and the G protein in each fraction was immunoprecipitated and analyzed by SDS–PAGE and fluorography. The amount of G protein found in the trimer and monomer fractions was determined as described in the legend to Fig. 2. The percentages of G protein which formed stable trimers at the various pHs are shown.

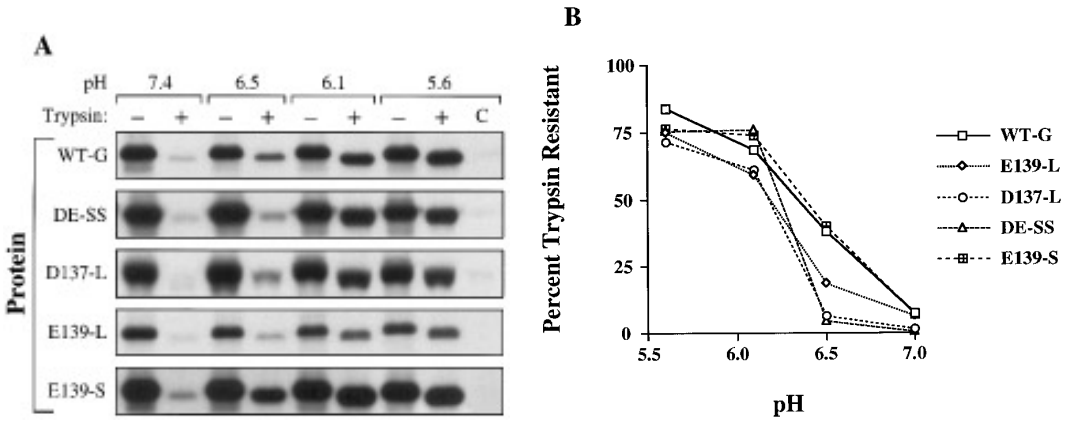

FIG. 4. pH-dependent sensitivity of G protein to digestion by trypsin. HeLa cells expressing wild-type G (WT-G), D137-L, E139-L, or DE-SS were radioactively labeled for 30 min and than chased for 1 hr in the presence of excess methionine. Cells were lysed at the pH indicated and incubated with or without 10 μg trypsin for 30 min at 37°. Samples in lane C were incubated in 0.3% SDS to denature proteins before the addition of 10 μg of trypsin. (A) G proteins were immunoprecipitated from all samples, subjected to SDS–PAGE, and visualized by fluorography. (B) The amount of G protein remaining after trypsin digestion was determined as described in the legend to Fig. 2 and the percentage trypsin resistant was calculated.

pH-dependent trypsin sensitivity of G proteins

To determine if we could detect any differences in conformation between the wild-type and mutant G proteins which were not detected by the trimer stability assays we next examined the sensitivity of the mutant G proteins to digestion by trypsin at different pHs. In an initial screen of several different proteases we found that detergent-solubilized wild-type G protein was digested by trypsin at neutral pH, but became increasingly resistant to proteolysis as the pH was lowered (Fig. 4). Resistance to digestion by trypsin at the lower pHs was not due to reduced proteolytic activity of trypsin since denatured G protein was completely degraded by trypsin at pH 5.6 (Fig. 4A, lane C). Therefore, the loss of trypsin sensitivity at low pHs most likely reflects acid-induced conformational changes in G protein. When the mutants D137-L, E139-L, and DE-SS were examined with this assay we found that they were more sensitive to proteolysis than wild-type G protein at pH 6.5 (Fig. 4B). In contrast, the mutant E139-S had a trypsin sensitivity profile identical to that of wild-type G protein (Fig. 4B). These results indicated that the mutant G proteins which exhibited an acid-shift in the threshold for fusion activity also required a slightly lower pH to achieve a trypsin-resistant conformation similar to that of wild-type G protein. Although these differences were observed at pH values above the threshold for membrane fusion activity, the results suggest that the altered fusion thresholds of the mutant proteins may be due to an increased requirement for protonation to induce the necessary conformational changes required to initiate membrane fusion.

Incorporation of mutant G proteins into minigenome particles

To determine if the mutant G proteins with altered fusion thresholds could assemble and function in virus particles we introduced these mutations into the minigenome construct pBS-GMMG (Stillman *et al.,* 1995). This plasmid encodes a negative-sense RNA, expressed from the T7 promoter of pBluescript, that contains the trailer and leader regions of the VSV genomic RNA and both the G and the M (matrix) protein genes. Infectious particles are formed when cells expressing T7 RNA polymerase from the recombinant vaccinia virus vTF7-3 (Fuerst *et al.,* 1986) are transfected with pBS-GMMG as well as plasmids encoding the N protein and the two polymerase proteins (P and L) of VSV. When these particles are used to infect cells that are expressing the N, P, and L proteins only, an increase in the number of cells expressing G protein is observed and more infectious particles are produced (Stillman *et al.,* 1995).

After constructing minigenomes containing substitutions at positions 137 and 139 we examined whether these mutant G proteins could function in particle assembly and subsequent entry into cells. To determine if infectious particles containing the mutant G proteins were produced we recovered the media from individual primary transfections and added these media to cells expressing N, P, and L proteins of VSV. The cells were then examined for G protein expression using immunofluorescence microscopy. All three mutants could be passaged, but the infectivity of particles containing D137-L, DE-SS, and E139-L appeared to be reduced compared to particles containing either wild-type G protein or E139-T. For example, after three passages there was only a 100-fold increase in the number of cells that expressed D137-L, DE-SS, or E139-L whereas there was at least a 500- to 1000-fold increase in the number of cells replicating the wild-type G or E139-T minigenomes. Consistent with this observation, significantly more particles containing wild-type G protein or E139-T were isolated after three passages compared to the other three mutants (Fig. 5A). This reduced particle pro-

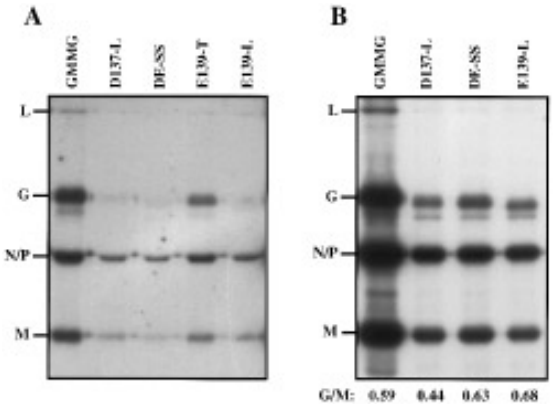

FIG. 5. Replication and infectivity of VSV minigenomes encoding mutant G proteins. BHK-21 cells were infected with vTF7-3 and then transfected with pBS-GMMG or plasmids expressing minigenomes which encode the mutant G proteins, together with plasmids encoding the N, P, and L proteins of VSV. After incubating for 24 hr the medium was recovered and added to fresh cells expressing the N, P, and L proteins only. Cells from the primary transfection, and all subsequent passages, were examined for G protein expression by immunofluorescence microscopy. (A) After two passages, minigenome particles were radioactivity labeled by adding [^{35}S]methionine directly to medium during the third passage. After 24 hr the medium was removed, passed through a 0.2-μm filter, and virions were recovered by centrifugation through a sucrose cushion. Viral proteins were immunoprecipitated with polyclonal anti-VSV serum, resolved on a 10% polyacrylamide gel containing SDS, and then visualized following fluorography. (B) Supernatants containing enough infectious particles to give between 50 (GMMG) and 10–30% (D137-L, E139-L, DE-SS) G-positive cells by immunofluorescence microscopy were added to cells expressing the N, P, and L proteins. Minigenome particles were labeled, isolated, and viral proteins were resolved on 10% SDS–PAGE as previously stated. The ratio of G to M protein (G/M) was determined using NIH Image as described in Fig. 2.

duction did not correlate with reduced accumulation of the mutant proteins on the cell surface since particles containing E139-T, which was transported with kinetics similar to the other mutant G proteins, were amplified at rate comparable to wild-type particles. To determine whether this difference was due to reduced amounts of G protein incorporated into virions we recovered radioactively labeled particles by centrifugation and quantitated the amount of G protein present in the virions. Each of the mutant G proteins were incorporated into particles at levels similar to that of wild-type G protein when the amount of G protein was normalized to the amount of M protein present (Fig. 5B). Together with the passaging data, these results indicated that the mutant G proteins were assembled into virions and could function in virus entry; however, fewer particles were produced after multiple passages possibly because the particles enter cells less efficiently than virus containing wild-type G protein.

DISCUSSION

The G proteins of the two major serotypes of VSV, Indiana and New Jersey, are approximately 50% identical at the amino acid level (Gallione and Rose, 1983). However, aspartic acid at position 137 and glutamic acid at position 139 are conserved not only between these two VSV serotypes but also between more distantly related vesiculoviruses such as Piry and Chandipura, which share only 40% amino acid identity with VSV–Indiana. This suggests that these two amino acids may have an important role in maintaining virus fitness by providing essential interactions required either for the proper folding of G protein or for some aspect of its biological function. G protein mediates both binding and entry of virus into host cells as well as virus assembly at the plasma membrane. Aspartic acid 137 and glutamic acid 139 could possibly be involved in any or all of G protein's functions. Because these two charged residues are located at the C-terminal end of the putative G protein fusion domain, we examined the role of these residues in membrane fusion activity. Site-directed mutagenesis was used to make specific amino acid substitutions that were predicted either to increase the hydrophobicity of the domain or to disrupt the local charge distribution in this region of G protein.

Many pH-dependent fusion proteins have acidic amino acids either in or near the fusion domain (Durrer *et al.,* 1995; Fredericksen and Whitt, 1995; Levy-Mintz and Kielian, 1991; White, 1990; Wiley and Skehel, 1987; Zhang and Ghosh, 1994). It has been suggested that these residues must become protonated so that the fusion domain can interact with the lipid bilayer of the target membrane during the initial stages of membrane fusion (Ohnishi, 1988). Alternatively, the acidic residues may provide a destabilizing effect that facilitates the transition from a fusion-inactive state at neutral pH to the fusion-active conformation at low pH, culminating in the exposure of the fusion domain such that it can interact with the target membrane (Zhang and Ghosh, 1994). Mutations that reduce this destabilizing effect may cause a shift in the pH threshold to more acidic conditions by increasing the activation energy required to achieve the fusion-active conformation.

Previously, we had shown that substitution of either D137 or E139 with leucine resulted in mutant G proteins that had fusion activities equivalent to that of wild-type G protein at pH 5.7 and lower (Fredericksen and Whitt, 1995). In this report we show that both D137-L and E139-L have reduced fusion activities relative to that of wild-type G protein at pHs ranging from 6.3 to 5.9. Replacing either of the two acidic residues with a hydrophobic amino acid may stabilize G protein in a fusion-inactive state and therefore slow G protein's progression toward a fusion-competent conformation. This hypothesis is further supported by the fact that D137-L, E139-L, and DE-SS were able to induce syncytia equivalent to wild-type G protein when exposed to pH 6.1 for 5 min at 37°.

In contrast to the effect seen with the leucine mutants, most of the other amino acid substitutions we examined

had little or no effect on the fusion activity of G protein. Substituting serine or threonine for either D137 or E139 may have provided sufficient polar character to the region that, in the presence of only one acidic residue, fusion activation occurred under conditions similar to those required for activation of wild-type G protein. However, when both acidic residues were replaced with serine, additional protonation was required to induce equivalent levels of membrane fusion. These results indicated that an acidic amino acid in this region is not absolutely required for membrane fusion, provided that proper folding and transport could occur. Interestingly, when both D137 and E139 were replaced with leucine very little of the protein was transported to the cell surface, suggesting that the protein could not acquire a transport-competent conformation and was retained in the ER. The observation that most of the mutants that we examined had slight to moderate transport defects indicated that D137 and E139 also play an important role either in the initial folding of G protein during its biosynthesis or in maintaining G in a conformation that allows transport to the cell surface.

If mutations that reduce the pH threshold for G protein-mediated membrane fusion act by stabilizing G in a fusion-inactive conformation, then there should have been detectable differences in the conformation of these mutants compared to wild-type G protein at pHs near the fusion threshold. To examine pH-dependent conformational changes in G protein we developed a protease sensitivity assay similar to that used for other pH-dependent fusion proteins except that in our assay cells transiently expressing G protein were first solubilized with nonionic detergent and then exposed to trypsin, rather than treating whole virus or water-soluble versions of the glycoprotein with proteases. For influenza hemagglutinin (HA) and the E2 glycoprotein subunit of alphaviruses, low-pH-induced conformational changes render these proteins more susceptible to digestion by proteinase K (Doms *et al.,* 1985; Edwards *et al.,* 1983; Skehel *et al.,* 1982). Similar results were observed with the G protein of rabies virus (Gaudin *et al.,* 1993), but unlike HA and the alphavirus glycoproteins, the conformational changes that result in protease sensitivity of rabies G do not appear to be those involved in membrane fusion (Gaudin *et al.,* 1995). In contrast to these results, we found that detergent-solubilized G protein became increasingly resistant to digestion by trypsin as the pH was lowered. This is similar to the E1 subunit of SFV, which gains resistance to trypsin after treatment with low pH and cholesterol (Kielian and Helenius, 1985). We interpret the decrease in protease sensitivity of G protein as the pH is lowered to be caused by pH-dependent conformational changes, although we do not know if these structural transitions are those required to activate membrane fusion. The observation that the pH dependence for trypsin resistance parallels that for membrane fusion suggests that the changes are biologically relevant (Fredericksen and Whitt, unpublished data). When compared to wild-type G protein, the mutants D137-L, E139-L, and DE-SS were more sensitive to digestion by trypsin at pH 6.5, suggesting that there was a difference between the conformation of the mutants and wild-type G protein at this pH. Although the only detectable difference in conformation between the mutant and wild-type G proteins occurred at pH 6.5, which is above the pH required for G protein-mediated membrane fusion, it seems likely that the altered fusion activities of the mutants result from an increased requirement for protonation to induce the necessary conformational changes needed to initiate membrane fusion. This hypothesis is further supported by the observation that a mutant with wild-type fusion activity (E139-S) was as resistant as wild-type G protein to digestion by trypsin at pH 6.5.

We also wanted to determine if the mutants D137-L, E139-L, and DE-SS had any effect on virus assembly or infectivity. Mutations in the putative internal fusion domain of the SFV E1 glycoprotein that either eliminated membrane fusion activity (G91-D) or caused an acid shift in the pH threshold for fusion activity (G91-A) also decreased the stability of E1–E2 dimers and affected virus assembly (Duffus *et al.,* 1995). Therefore to determine if amino acid substitutions at D137 and E139 had similar effects on virus assembly these mutations were cloned into the minigenome construct pBS-GMMG. We found that minigenomes encoding D137-L, E139-L, and DE-SS produced infectious particles; however, the infectivity of these particles appeared to be reduced compared to particles containing wild-type G protein. This attenuation could not be explained by reduced rates of transport of the mutant proteins since minigenome particles encoding E139-T, which was transported at a rate similar to that of the fusion-altered mutants, amplified like wild-type GMMG particles. Therefore, it appears that the mutations which caused a shift in the pH threshold for membrane fusion activity also produce a subtle yet detectable effect on virus spread.

The question still remains as to why D137 and E139 are conserved. The fact that we could recover infectious particles using the minigenome system indicates that these two residues are not absolutely required for virus viability; however, they may play a subtle role in maintaining viral fitness. Although we detected differences in pH-dependent conformational changes and slightly reduced fusion activities for some of the mutants, it is possible that residues 137 and 139 are involved in other biological functions of G protein, such as binding to the cell surface, which could explain the reduced infectivity seen with the minigenome particles. By introducing these mutations into full-length cDNA clones of VSV (Lawson *et al.,* 1995) and recovering infectious virus containing the mutant G proteins we should be able to better assess

the effects of these mutations on virus entry, replication, and assembly.

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REFERENCES

Blumenthal, R., Bali-Puri, A., Walter, A., Covell, D., and Eidelman, O. (1987). pH-dependent fusion of vesicular stomatitis virus with Vero cells. *J. Biol. Chem.* 262, 13614–13619.

Bonner, W. M., and Laskey, R. A. (1974). A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46, 83–88.

Crimmins, D. L., Mehard, W. B., and Schlesinger, S. (1983). Physical properties of a soluble form of the glycoprotein of vesicular stomatitis virus at neutral and acidic pH. *Biochemistry* 22, 5790–5796.

Doms, R. W., Blumenthal, R., and Moss, B. (1990). Fusion of intra- and extracellular forms of vaccinia virus with the cell membrane. *J. Virol.* 64, 4884–4892.

Doms, R. W., Helenius, A., and White, J. M. (1985). Membrane fusion activity of the influenza virus hemagglutinin: The low pH-induced conformational change. *J. Biol. Chem.* 260, 2973–2981.

Doms, R. W., Keller, D. S., Helenius, A., and Balch, W. E. (1987). Role for adenosine triphosphate in regulating the assembly and transport of vesicular stomatitis virus G protein trimers. *J. Cell Biol.* 105, 1957–1969.

Duffus, W. A., Levy-Mintz, P., Klimjack, M. R., and Kielian, M. (1995). Mutations in the putative fusion peptide of Semliki Forest virus affect spike protein oligomerization and virus assembly. *J. Virol.* 69, 2471–2479.

Durrer, P., Gaudin, Y., Ruigrok, R. W. H., Graf, R., and Brunner, J. (1995). Photolabeling identifies a putative fusion domain in the envelope glycoprotein of rabies and vesicular stomatitis viruses. *J. Biol. Chem.* 270, 17575–17581.

Edwards, J., Mann, E., and Brown, D. T. (1983). Conformational changes in Sindbis virus envelope proteins accompanying exposure to low pH. *J. Virol.* 45, 1090–1097.

Fredericksen, B. L., and Whitt, M. A. (1995). Vesicular stomatitis virus glycoprotein mutations that affect membrane fusion activity and abolish virus infectivity. *J. Virol.* 69, 1435–1443.

Fuerst, T. R., Niles, E. G., Studier, R. W., and Moss, B. (1986). Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* 83, 8122–8126.

Gallione, C. J., and Rose, J. K. (1983). Nucleotide sequence of a cDNA clone encoding the entire glycoprotein from the New Jersey serotype of vesicular stomatitis virus. *J. Virol.* 46, 162–169.

Gaudin, Y., Ruigrok, R. W., Knossow, M., and Flamand, A. (1993). Low-pH conformational changes of rabies virus glycoprotein and their role in membrane fusion. *J. Virol.* 67, 1365–1372.

Gaudin, Y., Tuffereau, C., Durrer, P., Flamand, A., and Ruigrok, R. W. H. (1995). Biological function of the low-pH, fusion-inactive conformation of rabies virus glycoprotein (G): G is transported in a fusion-inactive state-like conformation. *J. Virol.* 69, 5528–5534.

Kielian, M., and Helenius, A. (1985). pH-induced alterations in the fusogenic spike protein of Semliki forest virus. *J. Cell Biol.* 101, 2284–2291.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227, 680–685.

Lawson, N., Stillman, E., Whitt, M. A., and Rose, J. K. (1995). Recombinant vesicular stomatitis viruses from DNA. *Proc. Natl. Acad. Sci. USA* 92, 4477–4481.

Levy-Mintz, P., and Kielian, M. (1991). Mutagenesis of the putative fusion domain of the Semliki Forest virus spike protein. *J. Virol.* 65, 4292–4300.

Li, Y., Drone, C., Sat, E., and Ghosh, H. P. (1993). Mutational analysis of the vesicular stomatitis virus glycoprotein G for membrane fusion domains. *J. Virol.* 67, 4070–4077.

Matlin, K., Reggio, H., Helenius, A., and Simons, K. (1982). The pathway of vesicular stomatitis entry leading to infection. *J. Mol. Biol.* 156, 609–631.

Ohnishi, S.-I. (1988). Fusion of viral envelopes with cellular membranes. *Curr. Top. Membr. Transp.* 32, 257–298.

Puri, A., Winick, J., Lowy, J. R., Covell, D., Eidelman, O., Walter, A., and Blumenthal, R. (1988). Activation of vesicular stomatitis virus fusion with cells by pretreatment at low pH. *J. Biol. Chem.* 263, 4749–4753.

Rodriguez, J. F., Paez, E., and Estaban, M. (1987). A 14,000-Mr envelope protein of vaccinia virus is involved in cell fusion and forms covalently linked trimers. *J. Virol.* 61, 395–404.

Rose, J. K., and Bergmann, J. E. (1982). Expression from cloned cDNA of cell-surface and secreted forms of the glycoprotein of vesicular stomatitis virus in eucaryotic cells. *Cell* 30, 753–762.

Rose, J. K., and Bergmann, J. E. (1983). Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis virus glycoprotein. *Cell* 34, 513–524.

Rose, J. K., Buonocore, L., and Whitt, M. A. (1991). A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. *BioTechniques* 10, 520–525.

Skehel, J., Bayley, P., Brown, E., Martin, S., Waterfield, M., White, J., Wilson, I., and Wiley, D. (1982). Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. *Proc. Natl. Acad. Sci. USA* 79, 968–972.

Stillman, E. A., Rose, J. K., and Whitt, M. A. (1995). Replication and amplification of novel vesicular stomatitis virus minigenomes encoding viral structural proteins. *J. Virol.* 69, 2946–2953.

White, J. M. (1990). Viral and cellular membrane fusion proteins. *Annu. Rev. Physiol.* 52, 675–697.

Whitt, M. A., Buonocore, L., Prehaud, C., and Rose, J. K. (1991a). Membrane fusion activity, oligomerization, and assembly of the rabies virus glycoprotein. *Virology* 185, 681–688.

Whitt, M. A., Buonocore, L., Rose, J. K., Ciccarone, V., Chytil, A., and Gebeyehu, G. (1991b). TransfectACE Reagent: Transient transfection frequencies greater than 90%. *Focus* 13, 8–12.

Whitt, M. A., Chong, L., and Rose, J. K. (1989). Glycoprotein cytoplasmic domain sequences required for rescue of a vesicular stomatitis virus glycoprotein mutant. *J. Virol.* 63, 3569–3578.

Whitt, M. A., Zagouras, P., Crise, B., and Rose, J. K. (1990). A fusion-defective mutant of the vesicular stomatitis virus glycoprotein. *J. Virol.* 64, 4907–4913.

Wiley, D. C., and Skehel, J. J. (1987). The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu. Rev. Biochem.* 56, 365–394.

Zhang, L., and Ghosh, H. P. (1994). Characterization of the putative fusogenic domain in vesicular stomatitis virus glycoprotein G. *J. Virol.* 68, 2186–2193.