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Vesicular Stomatitis Virus Glycoprotein Mutations That Affect Membrane Fusion Activity and Abolish Virus Infectivity

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We have introduced amino acid substitutions into two regions of the extracellular domain of the vesicular stomatitis virus (VSV) glycoprotein (G protein) and examined the effect of these mutations on protein transport, low-pH-induced stability of G protein oligomers, and membrane fusion activity. We suggested previously that the region between amino acids 118 and 139 may be important for the membrane fusion activity of G protein, on the basis of the characterization of a fusion-defective G protein mutant (M. A. Whitt, P. Zagouras, B. Crise, and J. K. Rose, J. Virol. 64:4907–4913, 1990). It has also been postulated by others that this region as well as the region between amino acids 181 and 212 may constitute putative internal fusion domains of VSV G protein. In this report, we show that three different amino acids substitutions between residues 118 and 139 (G-124→E, P-127→D, and A-133→K) either altered or abolished low-pH-dependent membrane fusion activity. In contrast, substitutions between residues 192 and 212 resulted either in G proteins that had wild-type fusion activity or in mutant proteins in which the mutation prevented transport of G protein to the cell surface. Two of the substitutions between residues 118 and 139 (G-124→E and P-127→D) resulted in G proteins that were fusion defective at pH 5.7, although syncytia were observed after cells were treated with fusion buffer at pH 5.5, albeit at levels significantly less than that induced by wild-type G protein. Interestingly, when either G-124→E or P-127→D was incorporated into *ts*O45 virions, the resulting particles were not infectious, presumably because the viral envelope was not able to fuse with the proper intracellular membrane. These results support the hypothesis that the region between amino acids 118 and 139 is important for the membrane fusion activity of VSV G protein and may constitute an internal fusion domain.

Vesicular stomatitis virus (VSV) is an enveloped, negative-strand RNA virus which enters the host cell through receptor-mediated endocytosis (23). The glycoprotein (G protein) of VSV, which is located in the viral envelope, is responsible both for binding of virus to the cell surface and for fusion of the viral envelope with the endosomal membrane after endocytosis (9, 10, 26, 31). This fusion event releases the viral nucleocapsid into the cytoplasm of the cell and is required for VSV to initiate an infection. It is thought that G protein undergoes a pH-dependent conformational change (6), and this conformational change is believed to be required for G protein to catalyze fusion of the viral envelope with the endosomal membrane (2, 22). However, the regions of G protein involved in the conformational changes and in the actual membrane fusion event have not been definitively identified.

Although membrane fusion is required for all enveloped virus to enter cells and cause infection, only in the case of influenza virus hemagglutinin (HA) are the processes leading up to the fusion event understood at the molecular level. Membrane fusion is believed to result from the interaction of a specific region of HA, called the fusion peptide or fusion domain, with the target membrane (14). During transport, HA is proteolytically cleaved to generate two subunits, HA_1 and HA_2 (15, 17). The fusion peptide is located at the amino terminus of the HA_2 subunit and at neutral pH is sequestered within the HA trimer (40). Recently, it has been shown that HA undergoes a massive structural rearrangement after exposure to acidic pH and that this conformational change moves the fusion peptide over 100 Å (10 nm), positioning it so that it may interact with the target membrane (4).

For noncleaved fusion proteins, such as VSV G, the identification of a fusion domain has proven more difficult. In general, the amino termini of noncleaved fusogenic proteins are not particularly hydrophobic, and it is thought that these proteins have internal fusion domains. The current evidence for the existence of internal fusion domains is based on the characterization of mutant proteins that have altered fusion activities (19, 20, 38, 41). Internal fusion peptides are currently defined as regions of 16 to 17 apolar amino acids which are bounded by a positively charged amino acid on the N-terminal side and a negative or positively charged residue on the C-terminal side (33). These regions are relatively hydrophobic and are often rich in alanine and glycine residues. Lastly, internal fusion domains are thought to contain helix-breaking residues at their centers (1, 33).

We previously described the characterization of a G protein mutant that contained a single amino acid substitution which results in the addition of an extra glycosylation site at amino acid 117. This mutant (QN-1) was transported to the cell surface normally but did not induce membrane fusion (38). We suggested that the extra oligosaccharide side chain is located near a region important for the membrane fusion activity of G protein and that the additional oligosaccharide may block the interaction of this region with the host cell membrane. Because the region between amino acids 118 and 139 is conserved between VSV serotypes, we and others suggested that this region may be a potential internal fusion domain for VSV G protein (25, 38). Recent evidence using site-directed mutagenesis of this region supports this suggestion (41).

To further investigate the importance of the region between

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residues 118 and 139 in the membrane fusion activity of G protein, we constructed several new G protein mutants that have single amino acid substitutions within this region and then examined the effects of these mutations on the membrane fusion activity of G protein. We also constructed single amino acid substitutions in the region between amino acids 192 and 212, which others have postulated may also be important for the membrane fusion activity of VSV G protein (25).

The G protein mutants that are described in this report were grouped into four classes based on their fusion phenotypes. The first group of mutant G proteins had fusion activities similar to that of wild-type G protein. This class included all amino acid substitutions made in the region from amino acids 192 to 212 and many of the mutations in the region between amino acids 118 and 139. The second class of mutants had slightly lower pH thresholds for fusion but still fused like wild-type G protein at a pH of 5.7. The third class of mutants, which included G-124→E and P-127→D, had a significantly reduced pH threshold for fusion. Even at pH 5.2, these proteins had fusion activities significantly less than that of wild-type G protein. Despite the observation that G-124→E and P-127→D were able to induce membrane fusion to a limited extent, these mutant G proteins were unable to initiate infection of cells when incorporated into *ts*O45 particles. The last class, which consisted of a single mutant G protein (A-133→K), was completely defective for fusion activity at all pHs tested. Our results and those of Zhang and Ghosh (41) support our earlier hypothesis that the region between amino acids 118 and 139 is important for the membrane fusion activity of VSV G protein and may constitute an internal fusion domain.

MATERIALS AND METHODS

Cell culture, viruses, transfections, and rescue assay. HeLa cells were grown in high-glucose Dulbecco's modified Eagle medium (DMEM) containing 10% iron-supplemented calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Baby hamster kidney (BHK-21) cells were grown in DMEM containing 5% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Stocks of *ts*O45 and the recombinant vaccinia virus vTF7-3 (13) were prepared as described previously (36). Cell transfections were performed as described previously (35), using a suspension of liposomes composed of dimethyldioctadecyl ammonium bromide (DDAB) and L-α-dioleoylphosphatidylethanolamine (DOPE) at a weight ratio of 1:2.5 (29). Rescue assays were performed as described previously (36) except that all transfections were performed with DDAB-DOPE liposomes.

Plasmids and oligonucleotide-directed mutagenesis. The cDNA for the G protein of the Indiana serotype (San Juan strain) of VSV was recovered from pAR-G (36) and cloned into pBluescript-SK$^+$ (pBS-SK$^+$; Stratagene) between the *Xho*I and *Bam*HI sites such that G protein expression could be driven by the bacteriophage T7 promoter. This construct is called BS-G and contains *Eco*RI sites immediately flanking both ends of the G protein cDNA and an additional *Xho*I site adjacent to the *Eco*RI site at the 3′ end of the cDNA. These additional restriction sites were present in pSVGL1 (28), which was the parent plasmid of pAR-G. Therefore, the restriction sites in BS-G beginning at the 5′ *Xho*I site in the pBS-SK$^+$ polylinker are in the following order: *Xho*I-*Eco*RI-G protein cDNA-*Eco*RI-*Xho*I-*Bam*HI. All of the mutated G protein cDNAs described below were constructed by using either pBS-SK$^+$ or pBS-KS$^+$ as the vector. In addition, the mutant G protein cDNAs that were used in the rescue assay were also inserted into pAR-X (36), which is derived from the T7 expression vector pET-3 (30). All mutations that resulted in amino acid substitutions were introduced into the G protein cDNA by subcloning a PCR-amplified fragment into the wild-type G protein cDNA backbone. Sequences of the oligonucleotides and the conditions used for PCR amplification for the individual clones are available by request.

For substitutions at amino acids 119 to 127, PCR-amplified fragments were generated by using (i) antisense, mutagenic oligonucleotides which contained the appropriate nucleotide changes for the indicated amino acid substitutions and that spanned the unique *Sfa*NI site in the G protein cDNA and (ii) an oligonucleotide complementary to the T7 promoter. The template for these PCR amplifications was pAR-G. The amplified fragments were extracted with phenol-chloroform, precipitated with ethanol, and resuspended in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) prior to digestion with *Sfa*NI and *Bam*HI. The digested fragments were gel purified and then used to replace the corresponding fragment from the wild-type G protein cDNA clone in a three-way ligation. The components for each ligation were BS-KS$^+$ digested with *Bam*HI and *Xho*I, the PCR-amplified fragment digested with *Bam*HI and *Sfa*NI, and a fragment from the wild-type G protein cDNA clone containing sequences from the *Sfa*NI site to the *Xho*I site at the 3′ end of pAR-G. Mutations were confirmed by dideoxynucleotide sequencing of several individual clones for each of the mutant constructs, using Sequenase (United States Biochemical Corp.). The sequence of the entire region that was amplified and used in the ligation was determined for all of the mutants to confirm that the PCR-generated fragment contained the desired mutation only and that no other mutations were introduced during the amplification. Variations of the strategy described above were used to obtain cDNAs encoding the other amino acid substitutions listed in Table 1. The following descriptions detail these variations. For substitutions at amino acids 128 to 133, PCR-amplified fragments consisting of the 5′ portion of the G protein cDNA were used to replace the corresponding region from the wild-type G protein cDNA in a three-way ligation which consisted of pBS-SK$^+$ digested with *Eco*RI and *Bam*HI, the PCR fragment digested with *Eco*RI and *Sfa*NI, and the remaining 3′ region of the wild-type G protein cDNA from BS-G digested with *Sfa*NI and *Bam*HI. The mutation at amino acid 137 was introduced into the wild-type G protein cDNA backbone by amplifying a fragment by using BS-G as the template, a sense-strand mutagenic oligonucleotide that contained a new *Ava*I site at nucleotide 396, and an antisense, nonmutagenic oligonucleotide that overlapped the unique *Nco*I site in the G protein cDNA. The amplified fragment was digested with *Ava*I and *Nco*I and then used in a three-way ligation to regenerate the intact G protein cDNA. The 5′ portion of this clone was derived from the mutant G-131→A, which contained, in addition to the nucleotide changes at 421 (G to C) for the substitution of glycine 131 to alanine, nucleotide changes at positions 398 (A to C) and 401 (C to G) that resulted in the introduction of the new *Ava*I site. These nucleotide changes were silent and did not result in any additional amino acid changes. This strategy was necessary because the codon specifying amino acid 137 is part of the recognition/cleavage site for *Sfa*NI. For mutations at amino acids 139 and 147, the region between the *Sfa*NI and *Nco*I sites was amplified by using sense-strand, mutagenic oligonucleotides that overlapped the *Sfa*NI site and an antisense, nonmutagenic oligonucleotide that contained sequences overlapping the *Nco*I site. The PCR-generated fragments containing the mutations were digested with *Sfa*NI and *Nco*I and then used in ligations with BS-G digested with *Apa*I and *Nco*I plus an *Apa*I-to-*Sfa*NI fragment corresponding to the 5′ end of the wild-type G protein cDNA. The substitutions at amino acids 192 and 197 were generated by using an antisense, mutagenic oligonucleotide that overlapped the *Nco*I site and a nonmutagenic, sense-strand oligonucleotide that overlapped the *Sfa*NI site. The amplified fragments were used to replace the corresponding fragment in a three-way ligation containing BS-G digested with *Apa*I and *Nco*I, the *Apa*I-to-*Sfa*NI fragment also from BS-G, and the PCR-amplified region digested with *Sfa*NI and *Nco*I. Substitutions at amino acids 203 to 212 were introduced on a fragment generated by using a sense-strand, mutagenic oligonucleotide that spanned the *Nco*I site and an antisense, nonmutagenic oligonucleotide that overlapped the unique *Pst*I site in the G protein cDNA. The corresponding wild-type G fragment was replaced by using a 5′ *Apa*I-to-*Nco*I fragment from BS-G, the PCR-amplified *Nco*I-to-*Pst*I fragments that contained the appropriate mutations, and a vector derived from BS-G that was digested with *Apa*I and *Pst*I.

Surface expression and syncytium formation assays. Duplicate plates of HeLa cells were rinsed two times in serum-free DMEM (SF-DMEM) and infected with vTF7-3 at a multiplicity of infection of 10 for 30 min at 37°C. The inoculum was removed, and the cells were transfected with plasmids encoding either wild-type or mutant G proteins as described previously (35) or were incubated in SF-DMEM without DDAB-DOPE liposomes (mock transfected). At 7 h posttransfection, cells from one set of duplicate plates were processed for flow cytometry as described previously (38). To examine low-pH-induced membrane fusion activity, cells from the other set of duplicate plates were rinsed once with fusion medium [10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 150 mM NaCl, 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES); titrated to the indicated pH with HCl] and then bathed for 1 min in fresh fusion medium at room temperature. After 1 min of incubation, the fusion medium was replaced with DMEM containing 10% calf serum and the plates were returned to a 37°C, 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 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS$^+$) was added. The cultures were maintained at 4°C to prevent further cell-cell fusion until individual cultures could be examined for syncytium formation by 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 300 cells per field for each of the mutants and for parallel cultures of cells that were either mock transfected or were 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 protein 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 as a result of the activity of the vaccinia virus fusion protein (5, 27). The percentage

of fused to unfused cells was then determined by calculating the ratio of $fused_{corr}$ to unfused cells in each field. The percent cells fused represents the average value obtained from each of the fields.

Indirect immunofluorescence was performed essentially as described previously (28) except that the primary antibody was the G protein-specific monoclonal antibody I1 (18) and the secondary antibody was either fluorescein isothiocyanate- or lissamine rhodamine sulfonyl chloride-labeled, affinity-purified donkey anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc.).

Metabolic labeling, transport kinetics, and trimer stability assays. HeLa cells expressing either wild-type or mutant G proteins were rinsed once with methionine-free SF-DMEM (Met-free DMEM) at 3 to 4 h 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 of [^{35}S]methionine per ml. 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 mM Tris [pH 7.4], 66 mM EDTA, 1% Triton X-100, 0.4% deoxycholic acid, 0.02% sodium azide) or the labeling medium was replaced with DMEM containing 10% calf serum and 2 mM additional methionine (chase medium). Immunoprecipitation was performed essentially as described previously (28) 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°C. Digestion of immunoprecipitates with endoglycosidase (endo) H was performed as instructed by the manufacturer (New England Biolabs).

Trimer stability was examined by lysing radioactively labeled cells with 1% Triton X-100 in 2× MNT buffer [40 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 60 mM Tris, 200 mM NaCl, 2.5 mM EDTA] at the pH indicated as described previously (34). Cell lysates were centrifuged on 5 to 20% sucrose gradients as described previously (34). The fractions were immunoprecipitated with polyclonal anti-VSV serum and electrophoresed through 10% polyacrylamide gels containing SDS (16), and the resolved proteins were visualized following fluorography (3).

RESULTS

To identify regions that are important for the membrane fusion activity of G protein, we introduced single amino acid substitutions into the extracellular domain of G protein, using site-directed mutagenesis of the G protein cDNA. We chose two regions in the extracellular domain that have been suggested previously to be important for the membrane fusion activity of G protein to begin our mutagenesis study (25, 38). The first region includes amino acids 118 to 139, which we had proposed might constitute a putative internal fusion domain on the basis of the characterization of a fusion-defective mutant of G protein called QN-1 (38). The second region, which spans amino acids 181 to 212, was suggested by others because this region has properties expected for putative internal fusion domains (25). The rationale for the types of amino acid substitutions that were made was based on the premise that introduction of charged residues into a domain that is involved in membrane fusion should affect the ability of that domain to interact with a target membrane and subsequently alter or abrogate the fusion activity of the mutant G protein. Because several putative internal fusion domains of other fusion proteins have helix-breaking residues in the middle of the domain (1, 33), we also replaced proline residues with either alanine, glycine, or various charged amino acids. Table 1 lists the positions of the amino acids in G protein that were changed and the substitutions that were made.

Surface expression of mutant G proteins. Before we could examine whether the mutations listed in Table 1 alter the membrane fusion activity of G protein, we needed to first determine if the mutant proteins were transported to the cell surface. To assay for surface expression, we examined cells expressing each of the mutant proteins by indirect immunofluorescence. Most of the mutant G proteins were expressed on the cell surface, and the majority of these had signal intensities similar to that of cells expressing wild-type G protein (Table 1). One mutant (L-197→K) was found on the surface at levels lower than that of wild-type G protein, while five of the mutants were not detected on the cell surface by immunofluorescence. Because our assays for membrane fusion activity require transport to the plasma membrane, mutant proteins that were not detected on the cell surface were not examined further.

TABLE 1. Amino acid substitutions and surface expression of G protein mutants

Residue no.	Amino acid	Substitution	Surface expression[a]
119	Thr	Ala	++
119	Thr	Asp	++
121	Leu	Lys	++
121	Leu	Asp	++
123	Pro	Gly	++
123	Pro	Ala	++
124	Gly	Glu	++
125	Phe	Ala	−
125	Phe	Asp	−
127	Pro	Asp	++
128	Gln	Arg	++
131	Gly	Ala	++
133	Ala	Lys	++
137	Asp	Leu	++
139	Glu	Arg	−
139	Glu	Ile	++
139	Glu	Leu	++
139	Glu	Ser	++
139	Glu	Thr	++
147	Pro	Asp	++
192	Leu	Lys	++
192	Leu	Asp	++
197	Leu	Lys	+
203	Thr	Asp	−
209	Gly	Glu	−
212	Ser	Glu	++

[a] Determined by indirect immunofluorescence on HeLa cells that had been transfected with 5 μg of the indicated plasmids and fixed at 7 h posttransfection. ++, fluorescence intensity similar to that of cells expressing wild-type G protein; +, detectable surface expression, but signal is less than that for cells expressing wild-type G protein; −, no surface expression.

Membrane fusion activity of mutant G proteins. We initially examined the effects of the amino acid substitutions on the membrane fusion activity of G protein by using a syncytium formation assay. Cell fusion occurs when cells expressing wild-type G protein are incubated briefly in acidic pH buffer. The pH threshold for fusion activity of wild-type G protein is approximately 6.3 (11, 24, 31), and cells expressing G protein form extensive syncytia after treatment with pH 5.7. Cells expressing either wild-type G protein or individual G proteins mutants were treated with fusion buffer at pH 6.3, 5.7, or 5.2, and the cultures were examined for fused cells (Table 2). For some experiments, the amount of plasmid encoding wild-type G protein that was transfected was varied so that wild-type G protein was expressed on the cell surface at levels similar to those of the mutant G proteins. Fusion activities were determined by counting the number of syncytia formed after incubating cells expressing equivalent levels of either the mutant or wild-type G proteins at a given pH.

We found that the mutants could be grouped into four classes based on their ability to induce syncytia after incubation in fusion buffer at pH 6.3, 5.7, or 5.2. The first class are mutants which had fusion activities similar to that of wild-type G protein. All of the mutants in the region between amino acids 192 and 212 belonged to this class, as did most of the mutants having amino acid changes between residues 118 and 139. The second class of mutants consists of those that had slightly reduced fusion activities at pH 5.7. The mutant E-139→L represents this class of G protein, which appeared to have a

TABLE 2. Fusion activities of G protein mutants at pHs 6.3, 5.7, and 5.2

Residue no.	Amino acid	Substitution	Fusion activity[a] at pH:		
			6.3	5.7	5.2
119	Thr	Ala	−	+++	ND
119	Thr	Asp	−	+++	ND
121	Leu	Asp	−	+++	ND
121	Leu	Lys	−	+++	ND
123	Pro	Ala	+	+++	ND
123	Pro	Gly	+	+++	ND
124	Gly	Glu	−	−	++
127	Pro	Asp	−	−	++
128	Gln	Arg	+	+++	ND
131	Gly	Ala	+	+++	ND
133	Ala	Lys	−	−	−
137	Asp	Leu	−	+++	ND
139	Glu	Ile	−	+++	ND
139	Glu	Leu	−	++	ND
139	Glu	Ser	−	+++	ND
139	Glu	Thr	−	+++	ND
147	Pro	Asp	−	+++	ND
192	Leu	Asp	−	+++	ND
192	Leu	Lys	−	+++	ND
197	Leu	Lys	+	+++	ND
212	Ser	Glu	+	+++	ND

[a] Calculated by determining the ratio of fused to unfused cells in three to five separate fields containing at least 300 cells per field for each experiment. The percentage of fused cells for each mutant is represented as follows: −, <5% fused cells per field; +, 5 to 25% fused cells per field; ++, 25 to 70% fused cells per field; +++, >70% fused cells per field; ND, not determined.

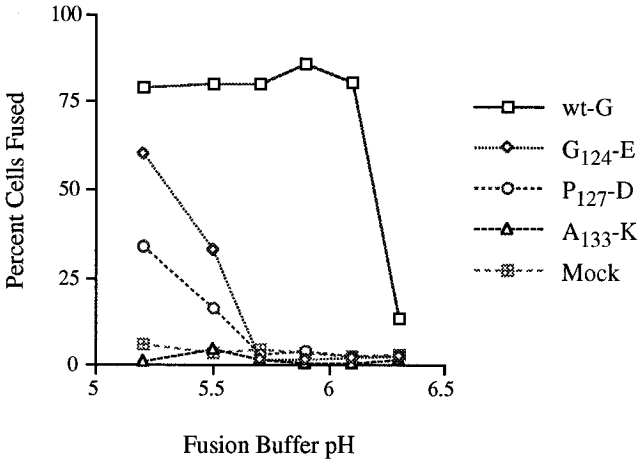

FIG. 1. pH dependence of G protein membrane fusion activity. HeLa cells expressing equivalent amounts of either wild-type G protein (wt-G) or mutant G proteins, or that were infected with vTF7-3 only (mock), were treated with fusion medium at the pH indicated, and the percentage of fused cells in the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

slightly reduced pH threshold for fusion. The third class of mutants is represented by G-124→E and P-127→D, which were fusion defective at pH 5.7. However, cells expressing G-124→E or P-127→D did form syncytia after treatment at pH 5.2, albeit at levels significantly less than that of wild-type G protein. These mutants were classified as pH-shift fusion mutants, indicating that they have a greatly reduced pH threshold for fusion activity. The fourth class consisted of the mutant A-133→K, which was fusion defective at all pHs tested.

To examine the pH dependence for fusion activity of the mutants G-124→E, P-127→D, and A-133→K in greater detail, we compared the fusion activities of these mutants with that of wild-type G protein at pHs between 6.3 and 5.2. Because the extent of syncytium formation in these cultures is dependent on both the number of cells expressing G protein and the amount of G protein on the cell surface, we used flow cytometry to determine the relative level of surface expression and the number of cells expressing each of the G protein mutants. Relative fusion activities are presented as the percentage of cells fused in the culture for each of the proteins at the pH indicated (Fig. 1). In all of these experiments, the cells were expressing equivalent levels of either wild-type or mutant G proteins. The results confirm that neither G-124→E nor P-127→D induces syncytium formation until the pH is below 5.7, which indicates that the pH threshold for both of the mutants is between 5.7 and 5.5. In addition, neither G-124→E nor P-127→D induces cell-cell fusion to the extent that G protein does. Cultures of cells expressing the mutant A-133→K had less than 5% fused cells after treatment with fusion medium at all pHs examined. This level of fusion was equivalent to the background fusion seen in cells that were infected with the recombinant vaccinia virus vTF7-3 and mock transfected only. Figure 2 shows examples of a typical cell fusion assay for the three fusion-defective mutants, for wild-type G protein and a nontransfected control culture at pH 5.7, and for G-124→E at pH 5.5.

Folding and transport of G protein fusion mutants. To determine the basis for the altered fusion activities exhibited by G-124→E, P-127→D, and A-133→K, we examined several different parameters that might affect the fusion activity of these mutant G proteins. These parameters include oligomerization, trimer stability, and transport kinetics. To examine whether G-124→E, P-127→D, and A-133→K were transported out of the endoplasmic reticulum (ER) properly, we determined the rate at which these proteins acquired endo H-resistant oligosaccharides. Acquisition of endo H-resistant sugars indicates that G protein has been transported to the medial compartment of the Golgi apparatus (8), and this assay allows us to measure the rate and extent of transport of G protein from the ER to the Golgi apparatus.

Both G-124→E and P-127→D were transported with kinetics similar to that of wild-type G protein (Fig. 3). These results indicated that neither of the substitutions adversely affected the folding or transport of the mutants. In contrast, A-133→K was transported to the Golgi apparatus more slowly than wild-type G protein, and only 75% of the pulse-labeled material became endo H resistant after 2 h. This result suggested that A-133→K may have difficulty attaining the proper conformation for transport; however, results from the flow cytometric analysis showed that A-133→K did accumulate on the cell surface, but to levels less than that of wild-type G protein when cells were transfected with equal amounts of plasmid DNA (data not shown).

pH-dependent trimer stability of G protein fusion mutants. Wild-type G protein trimers dissociate during centrifugation in sucrose gradients buffered to a pH of 6.3 or higher (6). Because the pH at which trimers dissociate correlates very closely with the pH threshold for the membrane fusion activity of G protein, trimer stability is thought to be a measure of the conformational change required for induction of the membrane fusion activity of G protein (6).

To examine the pH-dependent stability of the mutant G protein trimers, cells expressing either G-124→E, P-127→D, A-133→K, or wild-type G protein were detergent solubilized at the pH indicated, and the lysates were centrifuged through sucrose gradients having the same pH as the lysis buffer. The

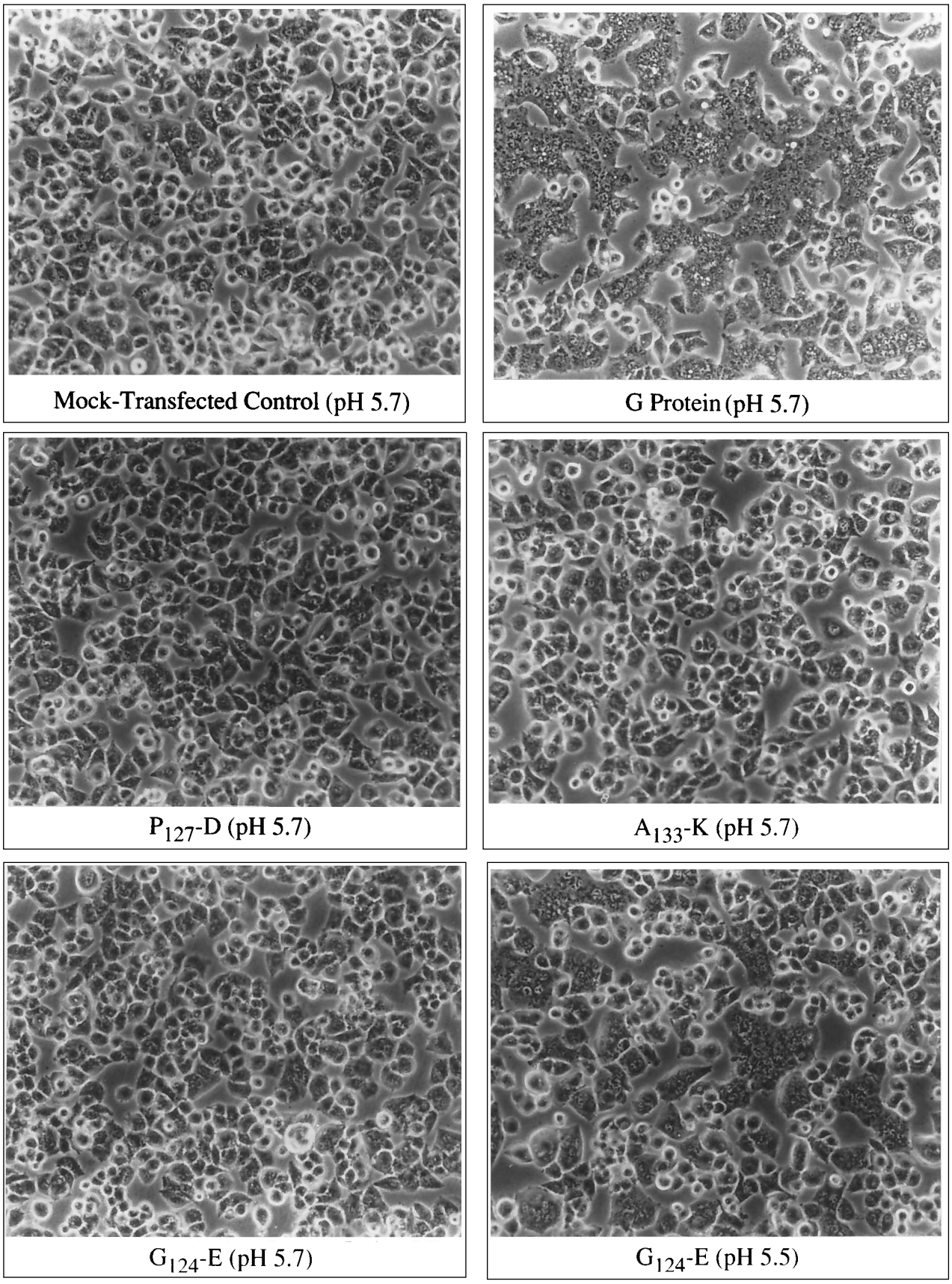

FIG. 2. Syncytium formation in cells expressing wild-type or mutant G proteins. HeLa cells from the experiment presented in Fig. 1 were photographed by using phase-contrast optics. The pH of the fusion medium is indicated in parentheses, and the G protein that was expressed is indicated below each photomicrograph. The mock-transfected control shows the amount of background fusion resulting from treatment of vTF7-3-infected cells with fusion medium at pH 5.7.

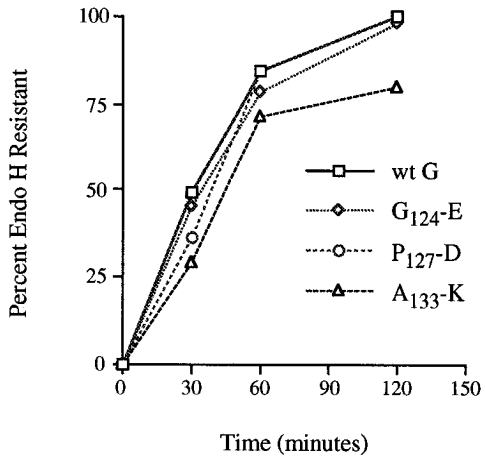

FIG. 3. Transport kinetics of wild-type and mutant G proteins. Cells expressing either wild-type G protein (wt G), G-124→E, P-127→D, or A-133→K were radioactively labeled with [^{35}S]methionine for 15 min at 3 h posttransfection and then chased for the times indicated in medium containing excess methionine. The G proteins were immunoprecipitated from cell lysates, and one-half of the immunoprecipitate was digested with endo H. Endo H-resistant and -sensitive forms of G protein were resolved by SDS-polyacrylamide gel electrophoresis and visualized following fluorography. The amount of G protein that had acquired endo H-resistant oligosaccharides was determined by image analysis of fluorograms, using a Millipore BioImage analyzer.

dissociation profiles for both P-127→D (Fig. 4) and A-133→K (not shown) were identical to that of wild-type G protein. This result indicated that P-127→D and A-133→K were properly folded and presumably undergo conformational changes similar to that of wild-type G protein. In contrast, trimers of G-124→E were significantly less stable to centrifugation in sucrose gradients at pH 6.3. Monomers were the predominate form that was recovered from gradients containing G-124→E at pH 6.3, whereas the majority of wild-type G and P-127→D were trimers at this pH (Fig. 4C). Even at pH 6.1, at which both wild-type G and P-127→D were predominantly trimers, G-124→E was found in the monomer fraction (Fig. 4E). These results suggested that G-124→E undergoes the conformational change required for trimer stability at a significantly lower pH that either wild-type G protein or P-127→D. Alternatively, the difference in trimer stability may simply reflect an increased requirement for protonation of the acidic residue at position 124 in order to prevent trimer dissociation during centrifugation.

Assembly and function of fusion-defective G proteins in virions. The second assay that we used to examine the fusion activity of mutant G proteins relies on the ability of the mutant proteins to assemble into virus particles. To accomplish this, we utilized an assay in which expression of either wild-type or transport-competent mutant G proteins is used to rescue the temperature-sensitive mutant *ts*O45 (34, 36–38). Because infectivity of the rescued particles depends on the fusion activity of the incorporated G protein, this assay provides a sensitive means for determining whether a mutant G protein is biologically active.

To examine whether G-124→E, P-127→D, or A-133→K could function in the viral envelope and bring about cell infection after complementation of *ts*O45, we first needed to determine if these proteins were transported to the cell surface at the nonpermissive temperature for *ts*O45. Flow cytometry was used to compare the relative levels of surface expression for each of the mutants with that of wild-type G protein at the restrictive temperature (40.5°C). We found that both G-124→E and P-127→D were expressed at levels equivalent to that of wild-type G protein at 40.5°C; however, A-133→K was not expressed on the cell surface at this temperature (data not shown). Because incorporation into virions requires expression of G protein on the cell surface, A-133→K could not be examined by this assay. We also examined whether the two mutants retained their low-level cell-cell fusion activities when expressed at the restrictive temperature. Although both G-124→E and P-127→D were expressed on the cell surface at 40.5°C, no syncytia were detected after treatment with fusion medium at either pH 5.5 or pH 5.2. This lack of fusion activity may be due slight differences in the conformation of the proteins when expressed at the restrictive temperature, although these differences were not significant enough to affect transport to the cell surface.

To determine whether G-124→E or P-127→D could assemble and function in virus particles, we used the *ts*O45 rescue assay described previously (36). We found that neither G-124→E nor P-127→D could rescue *ts*O45 infectivity when assayed by the ability to form plaques at the permissive temperature of 32°C (Table 3). The lack of infectivity was not due to the inability of either of the mutants to assemble into the viral envelope, since both G-124→E and P-127→D were incorporated into virions as efficiently as wild-type G protein (Fig. 5). These results demonstrated that both mutants were fusion defective when expressed and assembled into *ts*O45 virions at the restrictive temperature and showed that neither G-124→E nor P-127→D could induce fusion of the viral envelope with the proper intracellular membrane and initiate a productive infection.

DISCUSSION

The region between residues 118 and 139 as well as the region between amino acids 181 and 212 have been postulated to be involved in the membrane fusion activity of VSV G protein (20, 25, 38). Both of these regions have properties that are thought to characterize internal fusion domains (32), and mutations in both of these regions have previously been shown to alter the membrane fusion activity of G protein (20, 38, 41). To investigate the role of these regions in the membrane fusion activity of VSV protein further, we constructed several cDNAs for mutant G proteins that have single amino acid substitutions within both regions and then examined the effects of these substitutions on membrane fusion activity. The majority of the substitutions introduced charged residues into these regions. We postulated that if either of these regions interact directly with the host cell membrane as a prerequisite to or during the fusion event, the introduction of a charged residue may inhibit the ability of G protein to induce membrane fusion. Internal fusion domains have been postulated to contain helix-breaking residues near their centers (1, 33). The region between amino acids 118 and 136 contains prolines at amino acid 123, 126, and 127. We therefore replaced all three of these residues with either aspartic acid, glycine, or alanine. We also made several conservative substitutions in which residues were replaced with amino acids that have similar properties. These conservative substitutions were made to examine the possibility that a specific amino acid at a specific position is essential for membrane fusion activity.

All of the mutant G proteins were first examined for transport to the cell surface. Not surprisingly, some of the mutations resulted in G proteins which were not transported to the cell surface; therefore, these mutants could not be analyzed for defects in membrane fusion activity. The majority of mutants were transport competent and were grouped into four classes

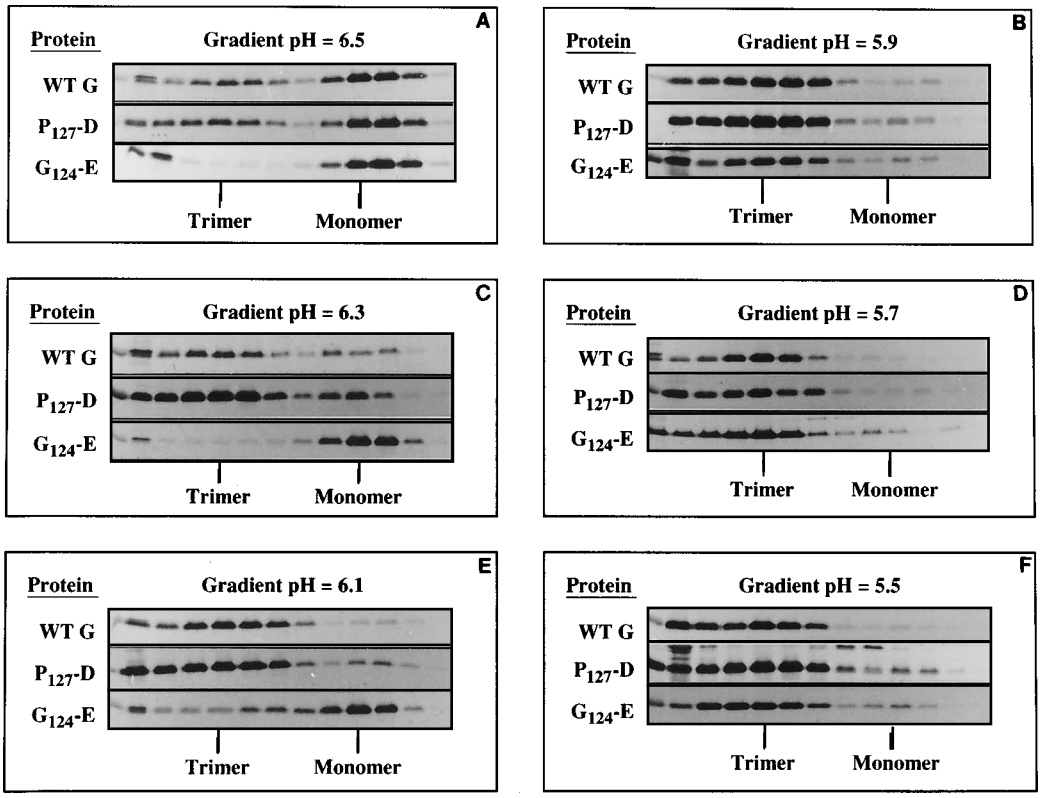

FIG. 4. Stability of G protein trimers to centrifugation through sucrose gradients as a function of pH. HeLa cells expressing either wild-type G protein (wt G), G-124→E, or P-127→D were radioactively labeled for 15 min and then chased for 1 h in the presence of excess methionine. Cells were lysed at the pH indicated, and the lysates were centrifuged through 5 to 20% sucrose gradients buffered to the same pH as the lysis solution. G proteins were recovered from fractions by immunoprecipitation, and each fraction was analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Only the relevant portion of the fluorogram is shown. The positions of G protein trimers and monomers are indicated for each set of gradients.

based on the effect that the amino acid substitution had on fusion activity of the mutant G protein. Class 1 mutants were defined as those substitutions which did not significantly alter the ability of G protein to induce syncytium formation compared with wild-type G protein. The majority of the mutants fell into this category. Importantly, all of the substitutions made between amino acids 192 and 212 that were expressed on the cell surface had wild-type G fusion activity. Although it was reported previously that a mutant G protein having an amino acid insertion between residues 194 and 195 appeared to have reduced fusion activity (20), it is unlikely that this region is directly involved in membrane fusion because one of the two conserved N-linked glycosylation sites in G protein is located near this region at amino acid 179. The addition of a hydrophilic oligosaccharide at N-179 would likely block this region of G protein from interacting with the host cell membrane. Our results showing that several amino acid substitutions between residues 192 and 212 did not alter the fusion activity of G protein support the conclusion that this region is probably not involved directly with the membrane fusion activity of G protein.

Members of the second class of mutant G proteins appeared to have a slightly reduced pH threshold for fusion but were still

TABLE 3. Titers of virus particles containing fusion-defective G protein mutants

Expressed protein	Titer (PFU/ml)[a]
Wild-type G	2.9×10^4–9.0×10^4
G-124→E	0–10^1
P-127→D	0–4×10^1
None (mock)	0–10^1

[a] Titers from four separate rescue experiments are given. Virus titers were determined on mouse L cells.

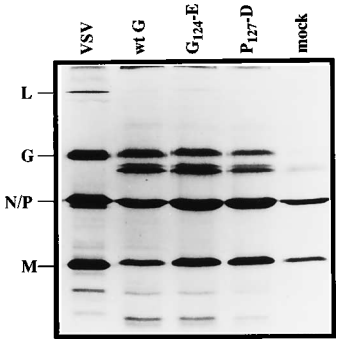

FIG. 5. Incorporation of G protein mutants into *ts*O45 virions. Labeling conditions for rescued *ts*O45 particles have been described elsewhere (36). Radioactively labeled virions were centrifuged through a 20% sucrose cushion, and the pellet fraction was resuspended and then immunoprecipitated with polyclonal anti-VSV serum. Viral proteins were resolved on 10% polyacrylamide gels containing SDS, and the gel was subjected to fluorography. Lanes from the left: VSV markers, particles from cells expressing wild-type G protein (wt G), particles from cells expressing G-124→E, particles from cells expressing P-127→D, and particles from mock-transfected cells.

capable of inducing syncytium formation equivalent to that of wild-type G protein at pH 5.7. The E-139→L substitution typifies this class of mutant G protein. The basis for the reduced fusion activity observed at a pH slightly below the threshold pH for wild-type G protein fusion activity was not fully explored. Protonation of acidic residues in internal fusion domains is thought either to contribute to the conformational change required for fusion activity or to act by increasing the hydrophobicity of the region. By replacing E-139 with L, we may have affected either one of these properties. A more likely explanation is that this mutation has altered the conformation of G protein slightly such that maximal fusion activity is not observed near the pH threshold.

The third class of mutant G proteins had significantly altered fusion activities compared with wild-type G protein. The proline-to-aspartic acid substitution at amino acid 127 (P-127→D) and glycine-to-glutamic acid substitution at amino acid 124 (G-124→E) belong to this class. Both of these G proteins did not induce syncytium formation at pH 5.7; however, syncytia were observed when cells expressing the mutants were treated with fusion buffer at pH 5.2. To determine whether substitution of other prolines also altered the fusion activity of G protein, we exchanged the proline at amino acid 147 with an aspartic acid and the proline at amino acid 123 with either alanine or glycine. In contrast to the P-127→D mutation, substituting the prolines at amino acids 123 and 147 had no effect on the fusion activity of G protein. These results indicate that the altered fusion activity of P-127→D is specific for the proline residue at amino acid 127. Our results for G-124→E and P-127→D support the results of Zhang and Ghosh, who also showed that substitutions at amino acids 124 and 127 altered the fusion activity of G protein (41). In addition, we obtained one mutant, an alanine-to-lysine substitution at amino acid 133 (A-133→K), that was completely fusion defective at all pHs tested. This protein constituted the fourth class of mutants in our study.

One explanation for the altered fusion activities G-124→E, P-127→D, and A-133→K is that the substitutions caused subtle changes in the folding or oligomerization the mutant G proteins. To indirectly examine the extent of misfolding of the mutant G proteins, we determined the rate at which the proteins were transported out of the ER. It has been previously shown that misfolded G proteins are retained in the ER until they achieve a transport-competent form (7). Therefore, by comparing the rate of transport of the mutant G proteins with that of wild-type G protein, we can indirectly determine the extent of misfolding of the mutant G proteins within the ER. We found that both P-127→D and G-124→E were transported at rates similar to that of wild-type G protein. These results suggest that neither the P-127→D nor the G-124→E substitution has a detrimental effect on the initial folding of the mutant proteins. In contrast, A-133→K was transported more slowly than wild-type G protein. This result indicated that A-133→K requires longer to achieve a transport-competent conformation and suggests that A-133→K may be slightly misfolded.

We were also interested in examining whether the mutant G proteins could undergo the pH-dependent conformational change that is thought to be required for induction of G protein's membrane fusion activity. Because there are no assays currently available to examine low-pH-induced conformational changes in G protein directly, we examined whether there was any difference in the stability of the mutant G protein trimers at different pHs. It has been shown previously that trimers of G protein dissociate to monomers when centrifuged through neutral pH sucrose gradients containing Triton X-100 unless cells lysates are maintained and centrifuged at acidic pHs (6, 21). There is also a strong correlation between the pH at which G trimers become stable to centrifugation on sucrose gradients and the pH required to induce the fusion activity of G protein (6). An explanation for this observation is that G protein undergoes a conformational change at low pH which stabilizes the trimers during centrifugation and this conformational change is required for activation of G protein's fusion potential (6). Because of this correlation, we used trimer stability to measure potential differences in the pH-dependent conformational changes of the mutant G proteins. Both A-133→K and P-127→D had trimer stability profiles identical to that of wild-type G protein. These results showed that the introduction of either acidic or basic amino acids at these positions did not alter the quaternary structure of G protein significantly. These results also suggested that trimers of P-127→D and A-133→K appear to undergo the conformational changes required to stabilize trimers. On the basis of these results, we suggest that the altered fusion activities of both of these mutants are due to the introduction of a charge into a domain directly involved in membrane fusion. In the case of P-127→D, it appears that this effect can be partially reversed by lowering the pH below 5.5, although decreasing the pH to 5.2 is not sufficient to restore wild-type fusion activity. The lower pH may partially neutralize the charge on the side chain of aspartic acid, allowing interaction between G protein and the host cell membrane.

The G-124→E mutant had a pattern of trimer stability much different from that of wild-type G protein. The majority of G-124→E trimers were not stable until pH 5.9, suggesting that G-124→E does not undergo the conformational change required for stabilization of trimers and possibly for membrane fusion activity until pH 5.9 or lower. However, our observation that G-124→E is still not capable of inducing syncytium formation until pH 5.5 indicates that this substitution has an additional effect on fusion activity. As we postulated for P-127→D, the addition of a charged amino acid at this position may interfere with the interaction of this region with target membranes, and as with P-127→D, when the charge is partially neutralized at lower pHs, syncytium formation does occur to a limited extent. An alternative explanation is that both G-124 and P-127 are essential for fusion activity, possibly by providing a necessary structural motif important for interaction of the fusion domain with target membranes, and that no other amino acids are allowable at these positions. As we had shown previously (12) and as reported by Zhang and Ghosh (41), both G-124 and P-127 are amino acids that are conserved between the putative internal fusion domains of Semliki Forest virus and many of the vesiculoviruses. Therefore, replacing either of these amino acids may affect the fusion activity of G protein. This interpretation appears plausible in light of the results obtained by Zhang and Ghosh, who showed that substitution of G-124 with alanine and P-127 with glycine resulted in G proteins that had altered fusion activities (41).

The initial assay that was used to characterize the mutants described in this study relied on the ability of the mutant G proteins to induce cell-cell fusion. However, the inability to induce syncytium formation does not necessarily correspond to a complete lack of fusion activity (32). In other virus systems, it was shown that some mutant viral fusion proteins which were not capable of inducing syncytium formation were still able to mediate infection of a host cell (39). To examine whether mutant G proteins that do not efficiently induce syncytium formation can function in viral entry, we assembled mutant G proteins into *ts*O45 particles and examined whether the resulting virions were infectious. When transiently expressed in *ts*O45-infected cells, both P-127→D and G-124→E were incorporated into *ts*O45 particles, but neither was capable of

rescuing *ts*O45 infectivity. The lack of biological activity, with respect to infectivity, is most likely due to an inability of the mutant G proteins to induce fusion of the viral envelope with an intracellular membrane, since neither of the mutant proteins induced cell-cell fusion after incubation at pH 5.5 or lower when expressed at 40.5°C. Alternatively, the lack of infectivity could result from the inability of the mutant G proteins to bind to the cell surface. Although this is formally possible, our previous results showing that an additional N-linked oligosaccharide at amino acid 117 had no effect on binding to cells (38) suggest that this region can tolerate significant changes without affecting the binding properties of G protein.

The identification of single amino acid substitutions within the domain defined by amino acids 118 to 136 that alter the low-pH-induced fusion activity of VSV G protein and that prevent virus infectivity strongly suggests that this region is important for the membrane fusion activity of G protein. Although we have shown that two of the substitutions appear to be folded properly, we cannot exclude the possibility that these mutations simply alter the conformation of another region that is directly responsible for the fusion event. Until there is a direct demonstration that a single domain in G protein mediates membrane fusion, the mechanism responsible for this event will remain uncertain.

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