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NOTES

Amino-Terminal Mutation of the Vesicular Stomatitis Virus Glycoprotein Does Not Affect Its Fusion Activity

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Earlier studies demonstrated that synthetic peptides corresponding to the amino terminus of the vesicular stomatitis virus glycoprotein (G protein) have a pH-dependent hemolytic activity that is thought to be related to the fusion activity of G protein (R. Schlegel and M. Wade, J. Biol. Chem. 259:4691–4694, 1984; R. Schlegel and M. Wade, J. Virol. 53:319–323, 1985). A single amino acid change (lysine to glutamic acid at the amino terminus) abolishes the hemolytic activity of the peptide. Here we used oligonucleotide-directed mutagenesis to create a DNA encoding G protein with this same amino acid change at its amino terminus. The mutant protein encoded by this gene was expressed transiently in a monkey fibroblast cell line (COS) and was found to have a pH-dependent fusion activity indistinguishable from wild-type G protein. This result indicates that the hemolytic activity of the synthetic peptides was not related to the fusion activity of the G protein.

Glycoproteins present on the surfaces of membrane-enveloped animal viruses such as vesicular stomatitis virus (VSV) or on the surfaces of virus-infected cells can mediate fusion between the membranes in which they reside and target membranes. This activity is critical for infection because it allows the release of virus nucleocapsids into the cell (2, 8, 17). The single glycoprotein species encoded by VSV (G protein) has been expressed from cloned cDNA in animal cells and was found to be transported efficiently to the plasma membrane (3, 10). This protein, in the absence of other VSV proteins, can mediate a low-pH-induced fusion of cells (3, 10). The brief exposure to low pH probably causes a conformational change in the protein, resulting in fusion of membranes containing G protein with other membranes (17).

The VSV G protein (incorporated into liposomes) is known to hemolyze erythrocytes under acidic conditions, an activity that may be related to fusion (1). Recently, Schlegel and Wade (13) reported the interesting observation that a synthetic peptide (25 mer) corresponding to the amino terminus of the G protein also has a pH-dependent hemolytic activity. Further studies indicate that a peptide corresponding only to the first six amino acids of the G protein also displays hemolytic activity. The activity is sequence specific because changing the N-terminal amino acid from lysine to glutamic acid abolishes the hemolytic activity of the hexapeptide (14). In the present study we examined the effect of this same amino acid change on the fusion activity of mature G protein.

Analysis of fusion mediated by G protein in COS cells. Earlier studies demonstrated that G protein expressed from cloned cDNA is fusogenic in mouse C127 cells or mouse L cells (3, 10). G protein can also be expressed transiently from cloned DNA in COS-1 cells by using a simian virus 40-based vector (12). In this vector the DNA sequence encoding G protein is placed under control of the simian virus 40 late promoter (15). Transfection of COS cells with this vector results in a high level of G protein expression in about 5 to 10% of the cells (12). Expression is detectable by indirect immunofluorescence after about 20 h and is maximal after about 40 h.

To determine whether transient expression of G protein in COS cells (4, 12) was suitable for analysis of the fusion activity of G protein, we transfected COS cells with pSVGL DNA (which encodes the wild-type G protein) and then looked for low-pH-induced fusion after 40 h. In these initial experiments we found that there was fusion of more than half of the cells in the monolayer after a brief exposure to pH 5.5 (data not shown). Also, there was some G protein-dependent fusion evident even without exposure of the cells to the low-pH medium. We suspected that the extensive fusion resulted from the expression of large amounts of G protein at the cell surface. Because less extensive fusion and a low level of spontaneous fusion were desirable for quantitation, we performed all fusion assays at 24 h after transfection, when the level of G protein expressed at the cell surface was much lower.

Changing the N-terminal amino acid of G protein. Because the studies of Schlegel and Wade (13, 14) suggest that the amino terminus of G protein might be part of a domain crucial for the fusion activity, we decided to change the N-terminal amino acid of G protein from lysine to glutamic acid, the same change which abolishes the hemolytic activity of the hexapeptide corresponding to the N terminus of G protein (14). To accomplish this, we used oligonucleotide-directed mutagenesis (11, 18) to change the codon corresponding to the N-terminal lysine from AAG (lysine) to GAA (glutamic acid) in the cDNA clone encoding G protein (Fig. 1). These two nucleotide changes also introduced a cleavage site for the enzyme EcoRI and thus facilitated identification of the mutation. For unknown reasons, this particular mutation was extremely difficult to obtain by standard oligonu-

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Primer          GTGAATTGCGAATTCACCATAG (22-mer)
Template     ...CACTTAACGTTCAAGTGGTATC...

                        1
W.T.            ValAsnCysLysPheThrIle
                GTGAATTGCAAGTTCACCATA

Mutant          ValAsnGlyGluPheThrIle
                GTGAATTCCGAATTCACCATA
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FIG. 1. Oligonucleotide-directed mutagenesis. The primer-template combination used to generate the mutation changing the codon for the N-terminal amino acid of G protein from Lys to Glu is shown. The template was the single-stranded DNA of mp8-G4 containing the negative strand of the G cDNA clone in mp8 bacteriophage DNA. The procedures for mutagenesis and screening were modified from Zoller and Smith (18) as described previously (11). The sequence including and surrounding the mutation was determined by the procedure of Maxam and Gilbert (9) after subcloning into plasmid pJC119 (15). The three nucleotide and two amino acid changes from the wild-type sequences are underlined. The lysine (1) is the N-terminal amino acid of mature G protein.

cleotide-directed mutagenesis procedures, which usually yield other mutations at frequencies of 1 to 30% in this laboratory. We screened more than 1,000 plaques and found only 1 which contained the desired changes. This isolate also contained a second nucleotide change within the signal sequence, which changed a cysteine to glycine (Fig. 1). Fortunately, this unexpected mutation occurs naturally in other VSV variants and serotypes and does not affect the function or cleavage of the signal sequence (5, 6).

We excised the mutated gene from the replicative form of the mp8-G4 DNA and subcloned it into the expression vector pJC119 (15). To determine whether the mutated DNA encoded a glycosylated G protein of the correct size, we transfected COS cells with the plasmid DNA encoding wild-type G protein (pSVGL) or with DNA encoding the mutated protein (pKE-17). This designation of the vector indicates the specific amino acid change in the single letter code (lysine [K] to glutamic acid [E]) at residue 17 in the precursor to G protein. Cells were then labeled with [^{35}S] methionine with or without pretreatment with tunicamycin, a drug that blocks addition of all Asn-linked carbohydrate (16). Proteins were then immunoprecipitated from cell lysates and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins encoded by the mutated (KE) and wild-type (G) DNAs had indistinguishable mobilities and showed identical increases in mobility resulting from tunicamycin treatment (Fig. 2). These results indicated that the mutant protein was glycosylated and suggested that the 16-amino-acid signal sequence was cleaved from the mutant protein. We next used indirect immunofluorescence of fixed, unpermeabilized cells to determine whether the mutant protein was transported to the cell surface. The cell surface fluorescence of the mutant protein was indistinguishable from that of the wild-type protein (data not shown).

Cell-cell fusion mediated by the mutant and wild-type G proteins. We next examined the fusion activities of the mutant and wild-type G proteins in COS cells. Cells were transfected with DNA of pSVGL, pKE-17, or pJC119 (vector), and after 24 h they were given a 1-min treatment at pH 5.5 and returned to medium at pH 7.2. The low-pH treatment was repeated after 2.5 h. Control cells were not treated with fusion medium. After 4 h, cells were examined by phase-contrast microscopy. Repetition of the low-pH treatment enhanced the extent of fusion. These cells are shown in Fig. 3. Cells transfected with pSVGL or pKE-17 showed no significant fusion without low-pH treatment (Fig. 3A and C) but revealed several polycaryons per field after low-pH treatment (Fig. 3B and D). Cells transfected with the vector DNA alone with or without low-pH treatment did not show polycaryon formation (Fig. 3E and F). Quantitation of the fusion activity was obtained in a separate experiment by using pH shifts to 5.0, 6.0, or 6.5. The total number of polycaryons seen in 10 fields chosen randomly was determined for cells transfected with the pJC119, pKE-17, or pSVGL. The data are plotted in Fig. 4 and show that the fusion activities of the mutant and wild-type G proteins were indistinguishable.

From these results we suggest that the pH-dependent hemolytic activity of peptides corresponding to the NH_2 terminus of G protein is fortuitous and probably unrelated to the fusion activity of the G protein itself. Clearly, the hemolytic activities of synthetic peptides (or even of the mature G protein) should be interpreted with caution.

It should be noted that our results do not rule out involvement of the NH_2 terminus of G protein in the fusion activity. By analogy with fusion domains in other viral glycoproteins, it seems likely that the corresponding domain in G protein is hydrophobic (17). The NH_2 terminus of G protein is actually quite hydrophilic when analyzed by the method of Kyte and Doolittle (3, 7), although it does contain

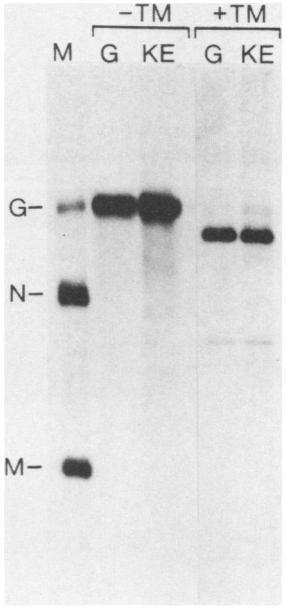

FIG. 2. Autoradiogram showing proteins produced in COS cells transfected with DNA of pSVGL or pKE-17. Approximately 6×10^5 cells were transfected with the indicated DNAs (G, pSVGL; KE, pKE-17) by procedures described previously (11). At 40 h after transfection, cells were labeled for 1 h with 100 μCi of [^{35}S]methionine in methionine-free Dulbecco-Vogt modified growth medium. Tunicamycin (Calbiochem-Behring) treatment (+TM) was for 2.5 h before labeling in growth medium containing 2.5 μg of tunicamycin per ml as indicated. G protein was precipitated from cell lysates with anti-VSV serum as described previously and subjected to electrophoresis on a 10% polyacrylamide gel (12). G, N, and M, Markers of VSV proteins from VSV-infected cells.

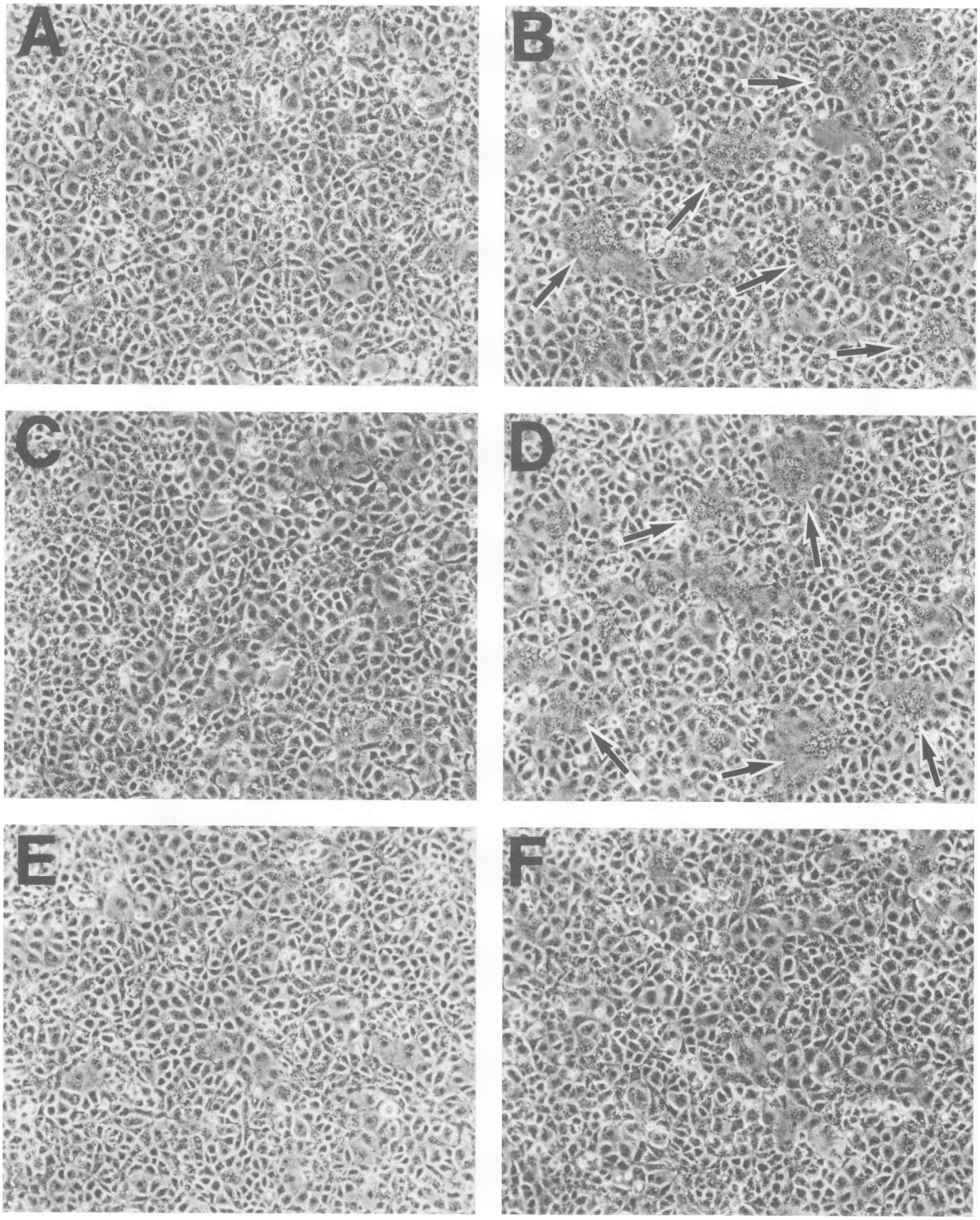

FIG. 3. Cell fusion induced by the G proteins specified by the plasmids pSVGL and pKE-17. Cells were transfected with DNA of pSVGL (A and B), pKE-17 (C and D), or pJC119 (E and F) and incubated for 24 h. Some cells (B, D, and F) were treated with fusion medium (pH 5.5) for 1 min and then returned to growth medium, incubated another 2.5 h, and given a second treatment with fusion medium for 1 min. These cells were returned to growth medium and photographed after an additional 1.5 h. Other cells (A, C, and E) were not treated with fusion medium. Photography was with a Nikon Diaphot microscope (10× phase-contrast objective). Fusion medium was prepared as described previously (3).

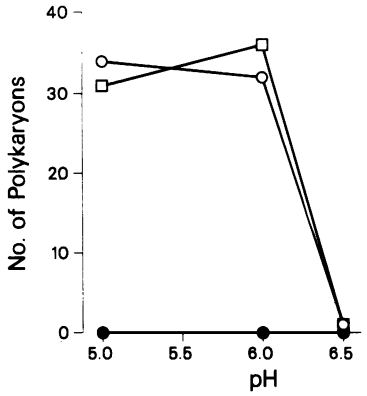

FIG. 4. Graph showing the amount of fusion induced by the G proteins specified by pSVGL or pKE-17 after treatment at pH 5.0, 6.0, and 6.5. Transfected cells were examined by microscopy, and the number of polycaryons containing at least five nuclei was determined for each of 10 fields chosen randomly. The total number of these polycaryons observed in 10 fields is plotted. Nontransfected COS cells contain occasional polycaryons, but these rarely have more than four nuclei. Symbols: ●, pJC119; ○, pSVGL; □, pKE-17.

the hydrophobic sequence Phe-Thr-Ile-Val-Phe after the NH_2-terminal Lys. We also used oligonucleotide-directed mutagenesis to generate a gene encoding a G protein with four amino acids (Phe-Thr-Val-Phe) of this sequence deleted. When this protein was expressed in COS-1 cells, it appeared to accumulate in the rough endoplasmic reticulum, and it was not transported to the cell surface (data not shown). Thus, it was not possible to measure the fusion activity of this protein. Future studies using in vitro mutagenesis of other regions of the protein may allow us to identify a domain involved in the fusion activity.

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