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The presence of cysteine in the cytoplasmic domain of the vesicular stomatitis virus glycoprotein is required for palmitate addition

(fatty acid linkage/thioester/lipoprotein/glycoprotein transport)

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ABSTRACT **The transmembrane glycoprotein (G protein) of vesicular stomatitis virus (VSV) is known to contain 1–2 mol of covalently linked fatty acid (palmitate) per mol of protein. G protein is oriented in cellular membranes such that the carboxyl-terminal 29 amino acids protrude into the cytoplasm. We have obtained expression in eukaryotic cells of mutagenized cDNA clones that encode VSV G proteins lacking portions of this cytoplasmic domain. Labeling of these truncated proteins with [^{3}H]palmitate indicated that the palmitate might be linked to an amino acid residue within the first 14 residues on the carboxyl-terminal side of the transmembrane domain. Using oligonucleotide directed mutagenesis, we changed the single codon specifying cysteine in this domain to a codon specifying serine. Expression of this mutant gene results in synthesis of a G protein lacking palmitate. We suggest that linkage of palmitate to G protein is through the cysteine in the cytoplasmic domain and that such a linkage may occur in many viral and cellular glycoproteins. The G protein lacking palmitate is glycosylated and is transported normally to the cell surface.**

The modification of eukaryotic glycoproteins by covalent addition of fatty acid (palmitate) was first described in the glycoproteins of vesicular stomatitis virus (VSV) and Sindbis virus (1, 2). Since these initial reports, it has been determined that a number of cellular glycoproteins also contain covalently linked fatty acid (3). The linkage of fatty acid to the viral glycoproteins was determined to be an ester, based on its sensitivity to alkaline methanolysis (2). Although a definitive function for the fatty acid has not been identified, it has been suggested that it might help anchor proteins in the membrane or act as a signal in transport to the plasma membrane (reviewed in ref. 4).

We have been using the VSV glycoprotein (G protein) as a model system to study the signals involved in transport of membrane proteins from the rough endoplasmic reticulum to the plasma membrane. By expressing a cDNA clone encoding the G protein from a simian virus 40 vector in eukaryotic cells (5), we were able to show that the protein was synthesized, glycosylated, and transported to the plasma membrane just as in VSV-infected cells. In a subsequent study, we analyzed expression of truncated genes encoding G proteins lacking portions of the carboxyl-terminal cytoplasmic domain. The intracellular transport of these mutant proteins was either blocked or retarded at a point prior to acquisition of complex oligosaccharides in the Golgi apparatus (6). The further analysis of the fatty acid acylation in these mutant proteins described here allowed us to localize the site of acylation to the cytoplasmic domain, and to identify the probable site of acylation by using oligonucleotide-directed mutagenesis to change the protein sequence.

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MATERIALS AND METHODS

Oligonucleotide-Directed Mutagenesis. The method used for mutagenesis was modified from published procedures (7, 8). The cloned cDNA encoding the VSV G protein was modified with *Bam*HI linkers at both ends and was ligated into the single *Bam*HI site of the phage mp8 replicative form DNA (a gift from Daniel Cohen). A phage designated mp8-G4 carries the negative strand of the *G* gene in the phage particle. DNA purified from this phage was used for mutagenesis.

The synthetic oligonucleotide used here (17 mer) was purchased from Bruce Kaplan (City of Hope Research Institute, Duarte, CA). The DNA had been synthesized on a Systec automated DNA synthesizer using the phosphotriester chemistry (9) and was supplied in blocked form linked to polystyrene resin. Deblocking was accomplished according to published procedures (9). The full-length oligonucleotide was separated from incomplete chains by electrophoresis on a 12% polyacrylamide DNA sequencing gel (10). The 17 mer was eluted from the gel by soaking in water for 1 hr at 65°C and was precipitated with ethanol prior to use.

Priming with the oligonucleotide was carried out as follows. A 5′-phosphate group was added to the oligonucleotide using polynucleotide kinase and ATP. About 100 ng of primer was added to 2.5 μg of mp8-G4 DNA in 10 μl of water. After incubation at 65°C for 5 min and at 23°C for 2 min, 25 μl of buffer containing 0.2 M Hepes, pH 6.9/0.140 M KCl/0.01 M $MgCl_2$/0.02 M dithiothreitol was added. All four deoxynucleoside triphosphates were added to a final concentration of 0.2 mM each, and ATP was added to a final concentration of 0.4 mM. Finally, 1 unit of T4 DNA ligase (Collaborative Research, Waltham, MA) and 2.5 units of DNA polymerase I (Klenow fragment; New England Biolabs) were added, and the final volume was adjusted to 50 μl with water. The reaction mixture was incubated overnight at 14°C. The total reaction mixture was then electrophoresed on a 1.5% agarose gel. After the priming reaction the majority of the single-stranded DNA had been converted to a form that migrated with a marker of mp8-G4 nicked-circular replicative form DNA. This DNA was electroeluted from the gel and used to transfect *Escherichia coli* K-12 strain JM103. Two hundred plaques were picked with toothpicks onto fresh agar plates and allowed to grow overnight. The infected colonies were then transferred to a nitrocellulose filter and hybridized to 5′-^{32}P-labeled 17 mer (16 hr at 23°C; ref. 11). The filter was washed at 30°C, 35°C, and 40°C, and exposed to x-ray film for 1 hr after each wash. Hybridization to all colonies was observed after washing at 30°C and 35°C. After the wash at 40°C, only 12 colonies showed strong hybridization. Phage replicative form DNA was prepared from one of these colonies and the fragment containing the *G* gene was excised and ligated into the simian virus 40 expression vector JC119 (12).

Abbreviations: VSV, vesicular stomatitis virus; G protein, VSV glycoprotein.

Plasmid DNA (pCS2-17) containing the mutant gene in the appropriate orientation for expression was obtained and the nucleotide sequence at the site of the mutation was verified (10).

Transfection, Immunoprecipitation, and Immunofluorescence Microscopy. Expression of a cDNA clone encoding the VSV G protein under control of the simian virus 40 late promoter in COS cells has been described in detail (5, 6). Briefly, confluent monolayers of COS-1 cells were transfected with the appropriate plasmid DNA in the presence of DEAE dextran (Pharmacia; M_r, 2×10^6). After 40 hr the cells were labeled with either [^{35}S]methionine or [^{3}H]palmitate (New England Nuclear) as described (5, 13). Cells were lysed and proteins in the cytoplasmic fraction were subjected to immunoprecipitation with rabbit anti-VSV antiserum and fixed *Staphylococcus aureus* (Pansorbin, Calbiochem).

Indirect immunofluorescence was carried out using unfractionated rabbit or guinea pig anti-VSV antiserum diluted 1:200, and fluorescein-conjugated or rhodamine-conjugated IgG (Cappel Laboratories, Cochranville, PA; diluted 1:200) as the second antibody. Cells were observed with a Nikon Optiphot microscope equipped with Nomarski optics, fluorescence epi-illumination, and a Nikon 40× oil immersion plan apochromat objective (6).

RESULTS

Labeling of Truncated G Proteins with [^{3}H]Palmitate. We have constructed deleted genes encoding G proteins lacking carboxyl-terminal amino acids (6). Deletion mutations that affected only the sequence in the cytoplasmic domain of G protein either decreased the rate of transport or blocked transport of G protein to the plasma membrane (6). The sequences in the cytoplasmic domains of these proteins are shown in Fig. 1.

To determine if the altered transport of these proteins was due to the loss of the fatty acid acylation site, the G proteins encoded by the deletion mutants were labeled biosynthetically with [^{3}H]palmitate. Only three of the mutant proteins were labeled with [^{3}H]palmitate, while all were labeled with [^{35}S]methionine (Fig. 2). The two G proteins lacking the transmembrane domain and the cytoplasmic domain (1428 and 1429) were not labeled, nor was the mutant protein (1473) which retains the hydrophobic transmembrane domain and has a 12 amino acid cytoplasmic domain encoded by read-through into simian virus 40 sequences. In contrast, [^{3}H]palmitate labeling was observed for mutants 1513 and 1514, which encode proteins lacking one-half of the normal cytoplasmic domain, and mutant 1554, encoding a G protein lacking only the three carboxyl-terminal amino acids.

These results suggested the possibility that the fatty acid acylation site might lie in the segment of amino acids encoded between the end points of the deletions 1473 and 1513—namely, the first 14 amino acids on the carboxyl-terminal (cytoplasmic) side of the transmembrane domain. Alternatively, the G protein encoded by deletion mutant 1473 might not be labeled because it was not transported to the appropriate site for fatty acid addition. Initial reports suggested that the palmitate might be linked to serine in G protein (2), but the 14 amino acids potentially containing the

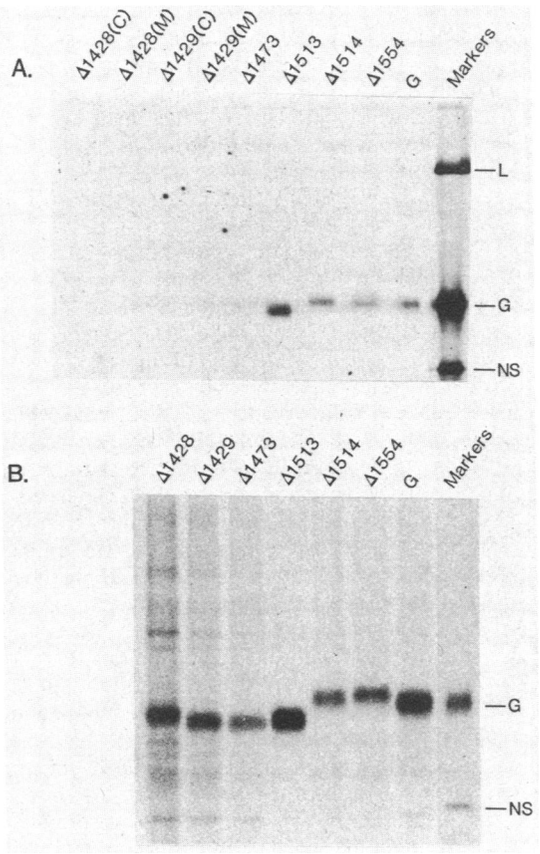

FIG. 2. Autoradiogram showing labeling of mutant G proteins with either [^{3}H]palmitate or [^{35}S]methionine. Semiconfluent monolayers of COS-1 cells on 6-cm dishes were transfected with 10 μg of simian virus 40 vector DNA specifying the indicated mutant G proteins. After 40 hr, the cells were labeled with 1 mCi of [^{3}H]palmitate for 6 hr (*A*) or 50 μCi of [^{35}S]methionine for 1 hr (*B*). Immunoprecipitated G proteins were electrophoresed on 15% polyacrylamide gels. The letters C and M indicate precipitation of cell-associated protein or protein in the medium. Fluorography of the dried gels was for 30 days (*A*) or 3 days (*B*). Positions of vsv protein markers (L, G, and NS) are indicated.

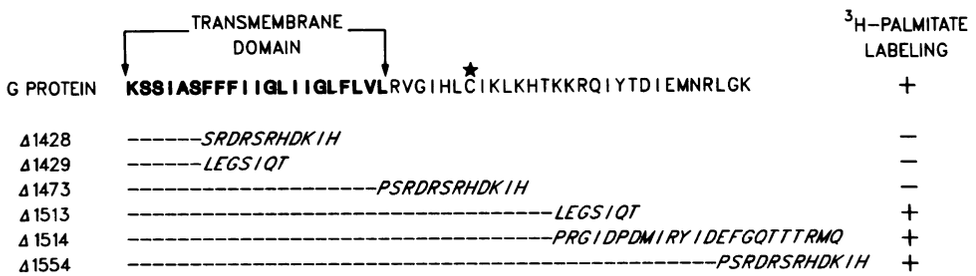

FIG. 1. Deduced amino acid sequences in the cytoplasmic domains of deleted G proteins. The amino acid sequence (indicated by standard one-letter abbreviations) of the carboxyl terminus of the wild-type VSV G protein is shown at the top, with the hydrophobic transmembrane segment indicated by boldface letters. The amino acid sequences of the carboxyl termini of altered G proteins encoded by the deletion mutants are shown such that a dashed line indicates the presence of normal G protein sequence and italic letters indicate the new amino acid residues encoded by simian virus 40 sequences (see ref. 6). All amino acid sequences are deduced from the corresponding DNA sequence. The right-hand column indicates which proteins can be labeled with [^{3}H]palmitate. The star indicates the single cysteine residue in the cytoplasmic domain.

acylation site did not contain any serine residues. There was, however, a single cysteine residue located seven amino acids from the transmembrane domain. A cysteine residue is a candidate for a thioester linkage to palmitate, or for a thioether linkage to a more complex structure such as the glyceryl palmitate which has been identified in the *E. coli* murein lipoprotein (14).

Mutagenesis of the Cysteine Codon. To test the possibility that this cysteine residue might be the site for fatty acid addition, we used oligonucleotide directed mutagenesis (7, 8) to change the triplet encoding the cysteine to one encoding a serine residue. The template for mutagenesis was the single-stranded DNA of the m13 coliphage mp8 carrying the gene for the G protein (8). The orientation of the *G* gene was such that the negative strand (anti-messenger sense) was incorporated into the single-stranded phage genome. We then used a synthetic oligonucleotide (17 mer) with two mismatches as a primer on this DNA template to introduce the appropriate changes (Fig. 3). After extension of the primer with the Klenow fragment of DNA polymerase I and ligation to form covalently closed double-stranded circles, we transfected the DNA onto *E. coli* to obtain plaques. The plaques were screened with 5′-^{32}P-labeled oligonucleotide as a hybridization probe to identify mutants that had incorporated the two base changes present in the primer. The melting temperature of the oligonucleotide hybridized to its perfect complement was at least 10°C higher than that of the oligonucleotide hybridized to the parental template.

After identification of phage carrying the desired mutation, the replicative form of the phage DNA was prepared, the *G* gene insert DNA was excised and inserted at the *Bam*HI site of the simian virus 40 expression vector JC119 (12). The presence of the double mutation in the DNA was verified by DNA sequence analysis (10) after cloning into the expression vector.

We then transfected the vector carrying either the mutant gene or the unmutated parental gene onto COS-1 cells and labeled with [^{3}H]palmitate or [^{35}S]methionine. The results (Fig. 4) show that the wild-type G protein is labeled with both [^{35}S]methionine and [^{3}H]palmitate, while the G protein with the single amino acid change of cysteine to serine labels only with [^{35}S]methionine.

Kinetics of Glycosylation. The G protein initially acquires two high-mannose or simple oligosaccharide chains that are added to the nascent polypeptide in the endoplasmic reticulum (15). These simple chains are susceptible to cleavage by endoglycosidase H (16). After transport to the Golgi apparatus (17), these oligosaccharides are converted to the complex type, which are resistant to cleavage by endoglycosidase H (18). The half-time for acquisition of endoglycosidase H resistance by normal G protein is 15–20 min in COS-1 cells (6). To determine if the rate of transport of the mutant G protein from the rough endoplasmic reticulum to the Golgi

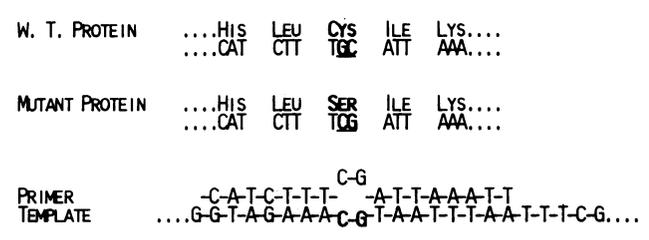

FIG. 3. Oligonucleotide-directed mutagenesis. The amino acid sequence (and corresponding nucleotide sequence) surrounding the single cysteine residue in the cytoplasmic domain of the wild-type VSV G protein is indicated. The synthetic oligonucleotide (17 mer) used as a primer on the mp8-G4 DNA is shown hybridized to the template DNA. Incorporation of the two mismatched bases into the G gene sequence will change the codon for cysteine (TGC) to that for serine (TCG).

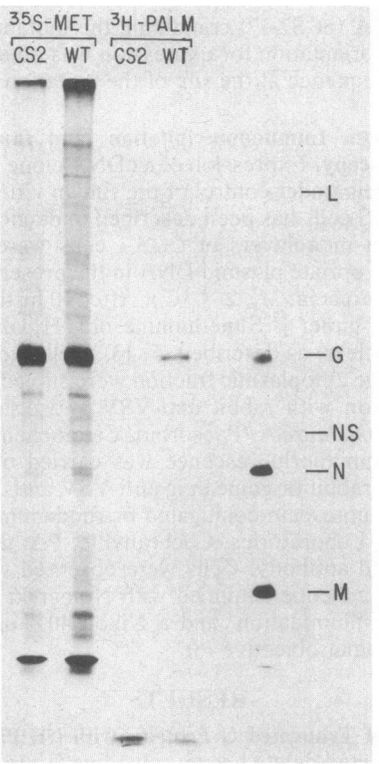

FIG. 4. [^{35}S]Methionine and [^{3}H]palmitate labeling of mutant and wild-type proteins. COS-1 cells were tranfected with vector DNA specifying the wild-type G protein (pSVGL; WT) or the mutant G protein (pCS2) in which the codon specifying the cysteine residue in the cytoplasmic domain had been changed to a serine codon. Transfections were carried out in parallel for both DNAs on either 5-cm plates for [^{35}S]methionine labeling or on 10-cm plates for [^{3}H]palmitate labeling. Immunoprecipitated proteins were electrophoresed on a 15% polyacrylamide gel and subjected to fluorography for 7 days. Positions of vsv protein markers (L, G, N, NS, and M) are indicated.

apparatus was normal, we carried out the pulse–chase labeling experiment shown in Fig. 5. About 30% of both the normal and mutant proteins have endoglycosidase H-sensitive oligosaccharides after a 20 min chase. Endoglycosidase H resistance is complete after 1 hr in both forms of G protein. These results indicate that the transport to the Golgi apparatus and glycosylation of the mutant protein occur at normal rates.

To determine if G protein lacking palmitate might be released from the cell surface, we also carried out a pulse–chase experiment and looked for release of G protein into the medium (data not shown). After 6 hr of chase, there was no immunoprecipitable G protein detectable in the medium, indicating that membrane anchoring was reasonably stable.

Cell Surface Expression of G Protein Lacking Palmitate. To determine if the G protein lacking palmitate could be transported to the cell surface and to analyze the intracellular localization of G protein, we carried out double-label indirect immunofluorescence on cells transfected with DNA encoding the mutant protein. Fig. 6 shows the results of this experiment. The cell surface labeling of the mutant protein (Fig. 6*A*) and the pattern of internal labeling in the same cell (Fig. 6*B*) are shown. Intense cell surface labeling of all cells showing internal labeling was observed just as for normal G protein (5, 6). Also, the pattern of internal labeling, showing intense labeling of the perinuclear Golgi region, is typical of normal G protein (6). These results indicate that transport and cell-surface anchoring of G protein lacking palmitate are normal.

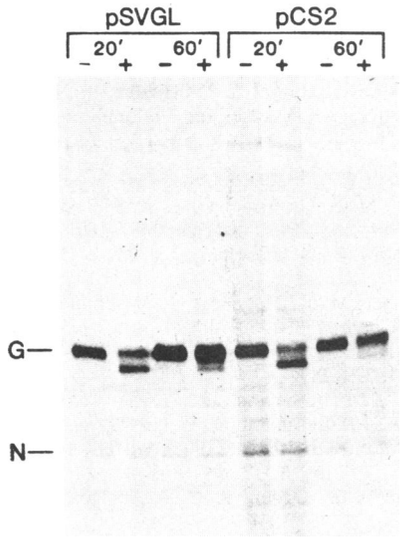

FIG. 5. Rate of acquisition of complex oligosaccharides in the mutant and wild-type proteins. COS-1 cells (two 5-cm dishes for each DNA) were transfected with vector DNA specifying the wild-type G protein (pSVGL) or the mutant G protein (pCS2) in which the codon specifying the cysteine residue in the cytoplasmic domain had been changed to a serine codon. After 40 hr, the cells were pulse-labeled for 15 min with 100 μCi of [^{35}S]methionine. The cells were then washed and transferred to medium containing 10 mM methionine. Cells were harvested at the indicated times. Immunoprecipitates of the G protein were divided in two equal portions and either digested with endoglycosidase H (+) or not digested (−) prior to electrophoresis on a 15% polyacrylamide gel.

DISCUSSION

The VSV G protein is inserted into the membrane of the rough endoplasmic reticulum and glycosylated as a nascent chain (15). At a time just prior to acquisition of complex oligosaccharides (which occurs in the Golgi apparatus) 1–2 molecules of fatty acid (palmitate) are esterified to the G protein (2). Fatty acylation is therefore believed to occur as a late event in the endoplasmic reticulum or as an early event in the Golgi apparatus (4). We and other laboratories have established that the fatty acid is attached to the G protein within the carboxyl-terminal 64 amino acid segment (4, 19). This segment includes the cytoplasmic domain, the transmembrane domain of 20 amino acids, and 14 amino acids on the NH_2-terminal side of the transmembrane domain. It was reported that the palmitate in G protein was probably linked to a serine residue (2), and all of the serine residues in the 64 amino acid fragment occur on the NH_2-terminal side of the transmembrane domain. We had therefore suggested previously that one or more of these residues might be the site(s) of palmitate addition (20).

In this study, we found that truncated G proteins, which retained the normal transmembrane domain and at least 14 residues of the normal cytoplasmic domain, could be labeled with [^{3}H]palmitate. However, a mutant that retained the normal transmembrane domain and had a new cytoplasmic domain of 12 amino acids specified by simian virus 40 sequences was not labeled with palmitate. This result suggested the possibility that the palmitate was added within the first 14 residues of the cytoplasmic domain. These 14 residues do not contain serine, but they do contain a single cysteine residue. The suggestion that this cysteine residue might be involved in fatty acid esterification came from comparison of the predicted amino acid sequences in the cytoplasmic domains of the G proteins of the Indiana and New Jersey serotypes. In contrast to the G protein from the Indiana serotype VSV (VSV_{IND}), the G protein from the New Jersey serotype of VSV (VSV_{NJ}) does not contain esterified palmitate (13). Although the amino acid sequences of these proteins show only about 50% homology, we noted that the cysteine residue present in the cytoplasmic domain of VSV_{IND} G protein was replaced by a serine residue in the corresponding position of the VSV_{NJ} G protein (13).

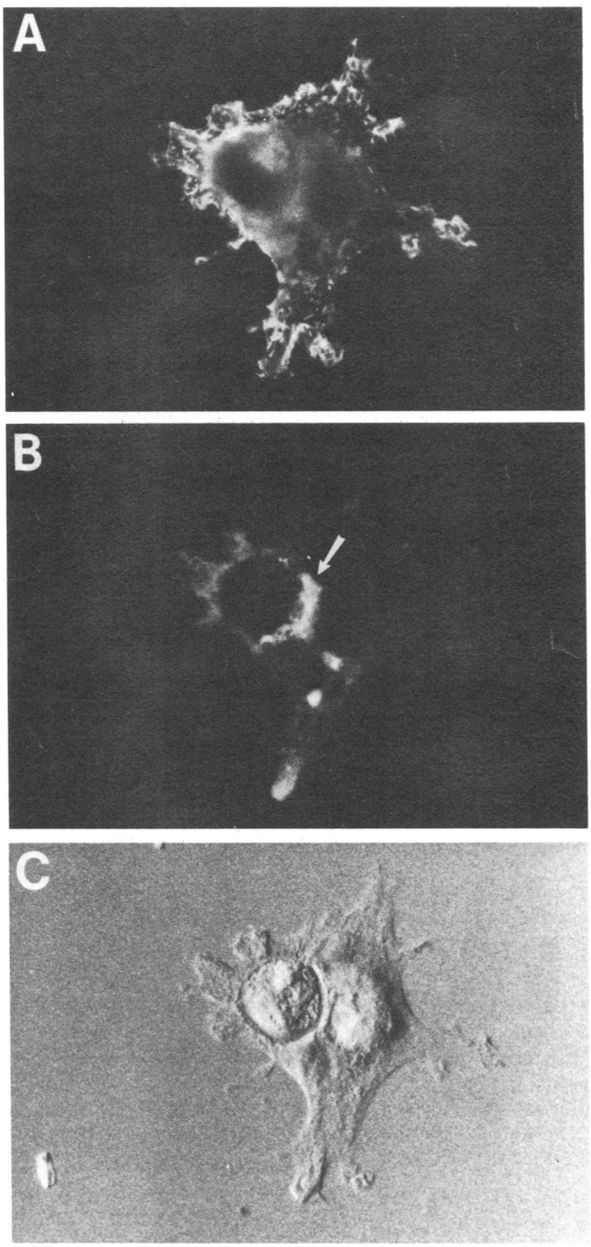

FIG. 6. Detection by double-label indirect immunofluoresence of the G protein lacking palmitate in a COS-1 cell transfected with pCS2-17 DNA. (*A*) Staining of cell surface G protein with rabbit anti-VSV antiserum and fluorescein-conjugated goat anti-rabbit IgG prior to permeabilization. (*B*) Staining of intracellular G protein in the same cell with guinea pig anti-VSV antiserum and rhodamine-conjugated goat anti-guinea pig antiserum after permeabilization. (*C*) Photomicrograph of the cell visualized with Nomarski optics.

To test the possibility that this cysteine residue was involved in fatty acid esterification, we used oligonucleotide-directed mutagenesis to change the codon for this cysteine residue to that for a serine residue. The mutated gene was then expressed in COS cells (5). This single amino acid substitution eliminated addition of palmitate to the G protein. Although this result does not prove that the fatty acid is linked to the cysteine residue, it seems very likely. It could

be postulated that the conversion of cysteine to serine changes the conformation of protein, preventing attachment of palmitate to some other amino acid. This possibility seems unlikely because replacement of more than half of the cytoplasmic domain with other amino acid sequences does not block fatty acid addition (Fig. 2).

Analysis of the kinetics of glycosylation indicated a normal rate of passage of the mutant protein to the Golgi apparatus, and indirect immunofluorescence showed that the protein was transported to the cell surface. Also, we did not observe any release of the nonacylated protein from the cell surface into the medium. These experiments would appear to leave the fatty acid acylation of G protein in search of a function. It is possible that palmitate might have an effect on the long-term stability of the protein in the viral membrane. Another possibility is that the fatty acid plays a role in the membrane fusion that occurs during virus entry into cells (21). Experiments have shown that cerulenin, an inhibitor of fatty acid synthesis, could block palmitate addition to G protein during a viral infection (22). The nonacylated G protein was transported to the cell surface, but it was incorporated poorly into virus particles. These results suggested that fatty acid acylation might play a role in virus budding, but the inhibition of budding might be due to other effects of cerulenin.

In the murein lipoprotein of *E. coli*, it is known that fatty acids are attached in ester linkage to a glycerol moiety in thioether linkage to cysteine (14). Such a linkage is unlikely in the VSV G protein because we and others (2) have attempted unsuccessfuly to label G protein with [2-^{3}H]glycerol. Recently, Schlesinger and co-workers (A. I. Magee, A. H. Koyama, C. Malfer, D. Wen, and M. J. Schlesinger, personal communication) have analyzed the stability of fatty acid on G protein to hydroxylamine. These results are consistent with at least some of the palmitate being in thioester linkage to G protein.

It seems possible that the involvement of cysteine in fatty acid linkage to proteins is very common. The HA glycoprotein of influenza virus, and the E2 and E1 glycoproteins of Sindbis and Semliki forest viruses all contain palmitate (reviewed in ref. 4). These proteins also contain one or more cysteine residues in their cytoplasmic domains or in the transmembrane domain near the cytoplasmic side (23, 24).

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