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Characterization of the Soluble Glycoprotein Released from Vesicular Stomatitis Virus-Infected Cells

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Vesicular stomatitis virus-infected Chinese hamster ovary cells release into the extracellular medium a soluble form of the vesicular stomatitis virus glycoprotein (G protein) termed G_s (Kang and Prevec, Virology 46:678–680, 1971). The properties of this molecule and the cellular site at which it is generated were characterized. By comparing the sizes and the peptide maps of the unglycosylated forms of G and G_s, we found that between 5,000 and 6,000 daltons of the carboxy-terminal end of the G protein is cleaved to generate the G_s molecule. This truncated molecule contains no fatty acid. G_s released from cells grown at 39°C migrated on polyacrylamide gels slightly slower than G_s released at 30°C. The unglycosylated form of G_s also showed this size difference. Furthermore, unglycosylated G_s was resolved into two species upon isoelectric focusing; the relative amounts of the two species depended upon the temperature at which infected cells were incubated. Full-sized unglycosylated virus-associated G also was resolved into two species, but the more basic form predominated at both 30 and 39°C. The appearance of G_s in the extracellular medium depended upon the presence of stable, full-sized G at the cell surface. The amount of G_s released was quantitated in seven different situations in which the migration of G to the cell surface was inhibited. In all cases, the amount of G_s released was also decreased. In addition, incubation of cells surface labeled with ^{125}I resulted in the release of ^{125}I-labeled G_s protein, as well as full-sized G protein. These results suggest that G_s is generated primarily by proteolytic cleavage of plasma membrane-associated G at a site in the molecule just amino terminal to the membrane-spanning region of the molecule.

The release of soluble macromolecules from the cell surface, termed shedding, appears to be a general phenomenon of viable cells (1). Shedding of soluble molecules, which is not to be confused with secretion of cellular products by exocytosis (1), is the release of cell surface molecules into the extracellular environment. Shedding occurs from normal, virus-infected, and malignant cells (1, 2, 11).

A possible model system for studying glycoprotein shedding involves vesicular stomatitis virus (VSV)-infected cells. Not only are virions containing glycoprotein (G protein) released from infected cells, but also a truncated form of G (G_s) is found in the extracellular medium (11, 21, 22). Little and Huang (21, 22) suggested that G_s may be derived from G protein by proteolysis. These authors reported that G_s is approximately 10,000 to 12,000 daltons smaller than G, but that accurate molecular weight determinations could not be made due to anomalous migration of glycoproteins in polyacrylamide gels (32).

Previous experimental results concerning the cellular location of the cleavage of G to produce G_s have been contradictory. Although Little and Huang (22) suggested that G_s is derived from G protein by proteolysis at the cell surface, they reported that cells infected with the group V or glycoprotein (17) mutant *ts*O45 released more G_s at the nonpermissive temperature (38°C) than cells infected at the permissive temperature (31°C) (21). It has been demonstrated that *ts*O45 G protein does not reach the cell surface at 38°C (15). Therefore, if G_s is released by *ts*O45-infected cells at the nonpermissive temperature, then (i) the temperature-sensitive block in migration to the cell surface may be relieved if one end of the molecule is lost or (ii) intracellular cleavage may be responsible for the generation of the G_s protein or both. Alternatively, premature termination of translation of the G protein mRNA could generate G_s. In contrast, Schnitzer et al. (31) found no G_s in the supernatant of *ts*O45-infected cells at the nonpermissive temperature.

The experiments described here more precisely characterized the position of the cleavage site in the G polypeptide used to generate the G_s protein. To explore further the cellular location of this cleavage, the release of G_s protein was quantitated under numerous conditions where G protein migration to the cell surface was inhibited. In all cases, the amount of G_s released varied with the amount of G detected at the cell surface. Furthermore, radiolabeled G_s was generated from surface-labeled glycoprotein. These results suggest that G_s is generated primarily from plasma membrane-associated G protein.

MATERIALS AND METHODS

Cells and virus. The cells which we used were Chinese hamster ovary (CHO) cells. VSV was propagated and purified as previously described (33). *ts*O44, *ts*O45, and *ts*O110 were kindly provided by P. Marcus and M. Sekellick. *ts*O57 was kindly provided by J. Lenard. The prototype strain of VSV was kindly provided by D. Summers, and the San Juan strain was obtained from H. Lodish.

Preparation of cytoplasmic extracts. Confluent monolayers containing 2×10^6 CHO cells were infected with virus at a multiplicity of 5 PFU/cell.

(i) Labeling with [^{35}S]methionine. After 4 h of incubation the medium was removed, and the monolayers were washed three times with methionine-free minimal essential medium supplemented with nonessential amino acids and 7.5% dialyzed fetal calf serum. [^{35}S]methionine (20 μCi/ml; 500 Ci/mmol; Amersham Corp.) was added to the monolayers for 4 h.

(ii) Labeling with [^{3}H]palmitic acid. [9,10-^{3}H(*N*)]-palmitate (23.5 Ci/mmol; New England Nuclear Corp.) in 80% ethanol was desiccated in a glass tube. Dialyzed fetal calf serum was added to the tube, and the preparation was sonicated for 30 s and then placed at 37°C for 30 s. This procedure was repeated three times. The sonicated fetal calf serum containing [^{3}H]palmitate was added to supplemented minimal essential medium (final concentrations, 7.5% fetal calf serum and 100 μCi of [^{3}H]palmitate per ml). After 4 h of incubation, the monolayers were washed three times with serum-free minimal essential medium supplemented with nonessential amino acids. Then 1 ml of the [^{3}H]palmitate labeling medium described above was added to each culture, and labeling was continued for 4 h.

After incubation, medium from both the [^{35}S]methionine-labeled and [^{3}H]palmitate-labeled cell cultures was removed. The monolayers were washed once with NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-hydrochloride, pH 7.4) and then lysed in NET buffer containing 1% Triton X-100.

Growth, labeling, and purification of virus. Confluent monolayers containing 2×10^6 CHO cells were infected with virus at a multiplicity of 5 PFU/cell. After 4 h of incubation at the appropriate temperature (30, 37, or 39°C) the medium was removed, and the monolayers were washed with methionine-free minimal essential medium supplemented with nonessential amino acids and 7.5% dialyzed fetal calf serum. [^{35}S]methionine (25 μCi/ml; 500 Ci/mmol) was added to the monolayers, and incubation was continued for 4 h. The medium was then removed, and the virus in the extracellular supernatant was gradient purified in 9 to 40% (wt/vol) continuous sucrose gradients with an 80% sucrose pad (sucrose solutions were prepared in Dulbecco phosphate-buffered saline [7]). Each gradient was centrifuged for 1 h in a Beckman SW27 rotor at 22,000 rpm and 4°C.

Immunoprecipitation. Cell extracts or extracellular supernatants from infected cells were incubated with rabbit anti-VSV antiserum, and immune complexes were precipitated with Immunobeads (Bio-Rad Laboratories) to which goat anti-rabbit antiserum was coupled. Before use, Immunobeads were washed once in NET buffer containing 1% Nonidet P-40 and 5 mg of bovine serum albumin per ml and twice in NET buffer containing 1% Nonidet P-40 and 1 mg of bovine serum albumin per ml. The concentrations of antibody and Immunobeads used in the immunoprecipitation experiments were the concentrations necessary to precipitate all VSV proteins from the cell extracts and extracellular supernatants.

Anti-VSV antiserum raised against the San Juan strain of VSV was equally efficient in precipitating G_s and viral proteins from the prototype strain, the San Juan strains, and the Orsay strains at the concentrations used.

Polyacrylamide gel electrophoresis. Polypeptides were resolved in 10 and 12.5% polyacrylamide slab gels (14 by 22 by 0.15 cm), which were prepared and run as described by Laemmli (16). The gels were then fixed and stained with Coomassie brilliant blue as described by Clinkscales et al. (6), dried, and subjected to autoradiography (X-Omat AR X-ray film; Eastman Kodak Co.). The resulting autoradiograms were scanned with an Ortec microdensitometer. The gels containing ^{3}H-labeled proteins were impregnated with the water-soluble fluor sodium salicylate (1 M; Malinckrodt), exposed to preflashed film, and stored at −70°C as described by Chamberlin (3).

Fractionation of infected cell cultures. After the end of the radioactive labeling period described above, infected CHO cell cultures incubated at 30, 37, or 39.5°C were divided into the following three fractions: a cell-associated fraction (cytoplasmic extract), extracellular virions, and a soluble fraction. The cytoplasmic extract was prepared as described above. Virions were gradient purified as described above. The soluble fraction was defined as those proteins which remained in the extracellular culture medium after removal of the cells and virions.

Tryptic peptide analysis. Trypsin digestion of individual polypeptides was done as described previously (23–25). Tryptic peptides were resolved by paper electrophoresis at pH 3.5 (23).

Isoelectric focusing. Virion-associated and soluble proteins were fractionated as described above, acetone precipitated, and suspended in buffer containing 9.5 M urea, 2% (wt/vol) Nonidet P-40, 2% ampholine solution, and 5% β-mercaptoethanol. Isoelectric focusing and sodium dodecyl sulfate gel electrophoresis were carried out as described by O'Farrell (26). To determine the pH gradient, the isoelectric focusing gel was cut into 5-mm sections, and these were placed in vials with 2 ml of degassed water. The vials were sealed and incubated with shaking for 10 min at room temperature. The pH of the solution was then measured with a Radiometer/Copenhagen pH meter.

Lactoperoxidase iodination of cell surfaces. A total of 2×10^6 infected CHO cells were washed three times in phosphate-buffered saline and suspended in 0.4 ml of ice-cold phosphate-buffered saline containing lactoperoxidase (20 μg; 50 U/mg; Miles Laboratories), NaI (2.0 μM), and $Na^{125}I$ (100 μCi/0.1 ml; New England Nuclear Corp.). Samples of hydrogen peroxide (50 μM) were added at 1-min intervals for 10 min. The reaction was then allowed to proceed for 5 min at 4°C and was made 2.5 mM in NaI. The cells were then washed three times in cold phosphate-buffered saline. Cells were lysed in 100 μl of NET buffer containing 1% Triton X-100. The samples were immunoprecipitated.

RESULTS

Physical characterization of G_s. (i) Molecular weight determination of the G_s protein. Previous studies suggested that the molecular weight of the G_s protein is 54,000 to 57,000, whereas the molecular weight of full-sized virion and cell-associated G protein is 67,000 (9, 22). However, a precise molecular weight determination could not be made with certainty because of the oligosaccharide component of the G_s protein. Thus, the unglycosylated forms of G and G_s were compared. Tunicamycin is an antibiotic which inhibits the glycosylation of glycoproteins containing asparagine-linked carbohydrates, such as the VSV G protein. Inhibition of glycosylation by tunicamycin did not inhibit the release of G_s protein from VSV prototype strain-infected cells incubated at 30°C (Fig. 1, lane 4). To compare the sizes of unglycosylated G and G_s, these two proteins were subjected in parallel to electrophoresis in 10% polyacrylamide gels, and an apparent molecular weight difference of 5,000 to 6,000 was observed (Fig. 1, lanes 3 and 4). Although the release of unglycosylated G_s from tunicamycin-treated cells infected with VSV San Juan strain at 30°C was low (see below), the G_s released was also 5,000 to 6,000 daltons smaller than the full-sized G protein (Fig. 1, lanes 5 and 6). Thus, the size of G_s relative to G protein is not a feature unique to a particular strain of VSV.

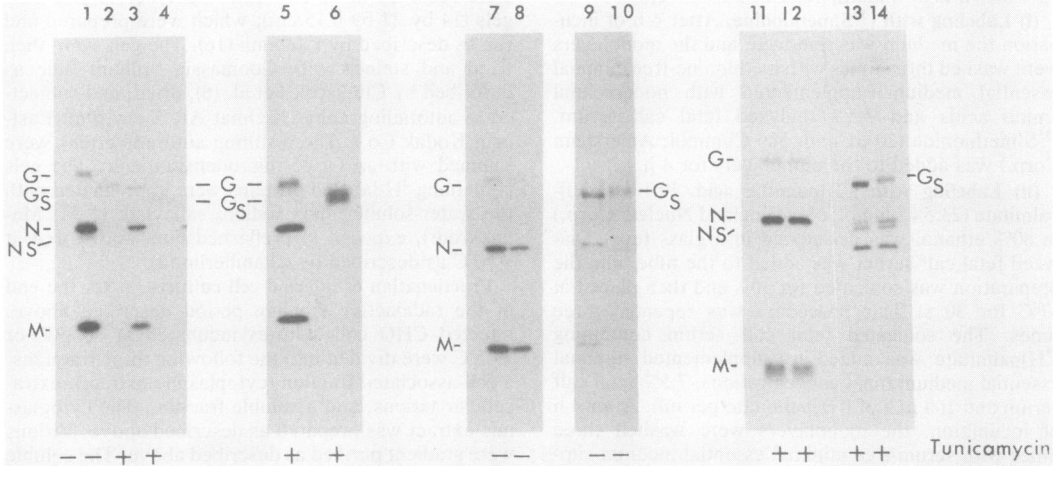

FIG. 1. Polyacrylamide gel electrophoresis of VSV virion-associated and extracellular soluble polypeptides. Cultures containing 2×10^6 CHO cells were infected, labeled, and fractionated as described in the text. The gradient-purified [^{35}S]methionine-labeled virus particles released from 2×10^5 cells were acetone precipitated, and the proteins were suspended in gel sample buffer and analyzed by polyacrylamide gel electrophoresis as described in the text. Equal volumes of extracellular supernatant were immunoprecipitated, and the precipitated proteins were placed in sample buffer and analyzed by polyacrylamide gel electrophoresis as described in the text. The figure shows an autoradiogram of a fixed, dried, 10% polyacrylamide gel. Lanes 1 and 3, VSV prototype strain virions released from untreated cells (lane 1) and tunicamycin-treated infected cells (lane 3) incubated at 30°C; lanes 2 and 4, immunoprecipitated soluble VSV proteins released from untreated cells (lane 2) and tunicamycin-treated infected cells (lane 4) incubated at 30°C; lane 5, incubated at 30°C; VSV San Juan strain virions lane 6, G_s protein released from tunicamycin-treated cells, incubated at 30°C; lanes 7 and 8, *ts*O44R virions released from infected cells incubated at 30°C (lane 7) and 39°C (lane 8); lanes 9 and 10, *ts*O44R G_s protein released from infected cells incubated at 30°C (lane 9) and 39°C (lane 10); lanes 11 and 12, *ts*O44R virions released from tunicamycin-treated infected cells incubated at 30°C (lane 11) and 39°C (lane 12); lanes 13 and 14, soluble *ts*O44R proteins released from tunicamycin-treated infected cells incubated at 30°C (lane 13) and 39°C (lane 14). Lanes 1 through 4 were exposed for 14 h, lanes 5 and 6 were exposed for 24 h, lanes 7 through 10 were exposed for 20 h, and lanes 11 through 14 were from a gel which was impregnated with 1 M sodium salicylate and exposed to preflashed film for 24 h. Molecular weights were determined by co-electrophoresis with molecular weight standards.

In the course of these studies, we found that the glycosylated G_s protein released from cells incubated at 39°C migrated more slowly than the G_s protein released from cells incubated at 30°C (Fig. 1, lanes 9 and 10). To determine whether this small size difference was due to an actual protein difference and not a carbohydrate difference, the sizes of the two G_s proteins synthesized in the presence of tunicamycin in cells incubated at 30 and 39°C were compared. For this experiment, VSV *ts*O44R-infected cells were used as a source of unglycosylated G_s synthesized at 39°C. *ts*O44R is a spontaneous pseudorevertant of *ts*O44 which, like its prototype parent, can release virus at the high temperature, but, unlike its parent, does not require carbohydrate for efficient particle release at 39°C (5). The unglycosylated G_s proteins released from *ts*O44R-infected cells incubated at 30 and 39°C were subjected in parallel to electrophoresis in 10% polyacrylamide gels, and an apparent molecular weight difference of 500 to 1,000 was observed (Fig. 1, lanes 13 and 14). This size difference was not reflected in virion-associated full-sized glycosylated or unglycosylated G (Fig. 1, lanes 7 and 8 and 11 and 12, respectively); full-sized glycosylated G molecules synthesized at high and low temperatures were the same size, and full-sized unglycosylated G molecules synthesized at 30 and 39°C also were the same size. It should be noted that these results were not unique to *ts*O44R; they were also typical of wild-type virus (data not shown).

(ii) Isoelectric focusing of G_s protein. Since G_s shed at a high temperature migrated more slowly in polyacrylamide gels than G_s shed at 30°C, the two forms of G_s were analyzed by isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis to determine whether there was a charge difference between unglycosylated G_s molecules shed at 30 and 39°C. [^{35}S]methionine-labeled G_s protein and virion-associated polypeptides were separated first by isoelectric focusing as described by O'Farrell (26) and then in 10% sodium dodecyl sulfate-polyacrylamide gels. The regions of the gel containing the G_s proteins shed at 30 and 39°C are shown in Fig. 2A through D. Unglycosylated G_s protein was resolved toward the acidic end of the isoelectric focusing dimension and was always clearly resolved into two species with isoelectric points of 5.5 and 5.2. However, the relative ratios of these two species varied depending upon the temperature of incubation of the infected cells. G_s shed at 30°C consisted of a major species (88% of the total) with a pI of 5.5 and a minor species (12% of the total) with a pI of 5.2 (Fig. 2A). However, G_s shed at 39°C consisted of a major species with a pI of 5.2 (88% of the total) and a minor species with a pI of 5.5 (12% of the total) (Fig. 2B). The results with mixtures of G_s molecules shed at 30 and 39°C are shown in Fig. 2C (equal amounts of [^{35}S]methionine) and Fig. 2D (equal volumes of extracellular supernatant). Unglycosylated G protein found in virions synthesized at either 30 or 39°C was also resolved into predominantly two species with isoelectric points of 6.1 and 6.0. In contrast to G_s, the ratios of the two species did not change with temperature; 85% of the virion-associated G protein had an isoelectric point of 6.1 in virus harvested at 30°C (Fig. 2E-1) or 39°C (Fig. 2E-2).

(iii) Ratio of extracellular G to extracellular G_s. The relative amounts of extracellular G and G_s also changed with the temperature at which the cells were incubated (Fig. 3). At 30°C, conditions which favored the more basic forms of G_s, the ratio of extracellular G (predominantly virus associated) to G_s was 1:1.5 (Fig. 3, lanes 1 and 3), a result similar to the finding of Schnitzer et al. (31). However, at 39°C the ratio of G to G_s was 1:8 (Fig. 3, lane 2), and at 37°C the ratio of G to G_s was 1:6 (Fig. 3, lane 4). The reason for the large excess of G_s at the higher temperature was not clear. The ratios of G to G_s were not affected by proteolytic enzyme inhibitors. In addition, labeling was done in the absence of serum in order to reduce extracellular proteases. Little and Huang reported an increased amount of G_s at a high temperature after infection with *ts*G31, an M protein mutant (22). These differences in the ratio of G to G_s occurred with the San Juan, prototype, and Orsay strains of VSV (data not shown).

(iv) Peptide analysis of the G_s. G_s protein probably results from proteolytic cleavage of G protein (22). To determine which end of the G protein molecule is cleaved to generate the G_s protein, we compared the methionine-containing tryptic peptides of the G and G_s proteins. The methionine-containing tryptic peptides derived from the carboxy- and amino-terminal regions of the G protein have been identified previously (4). Peptide 5 (Fig. 4) was identified as the most amino-terminal methionine-containing tryptic peptide, whereas peptide 1 was located near the carboxy terminus. It has also been shown that trypsin digestion of membrane vesicles isolated from pulse-labeled, infected cells removes the carboxy-terminal region of the molecule (4). The truncated molecule left in membranes is missing peptide 1 (Fig. 4D). When the methionine-containing tryptic peptides of the G (Fig. 4A and C) and G_s proteins (Fig. 4B) were compared, we found that the G_s protein was missing the carboxy-terminal peptide (peptide 1) of the G protein.

(v) Labeling of G protein with [^{3}H]palmitate. The VSV G protein has one or two molecules of

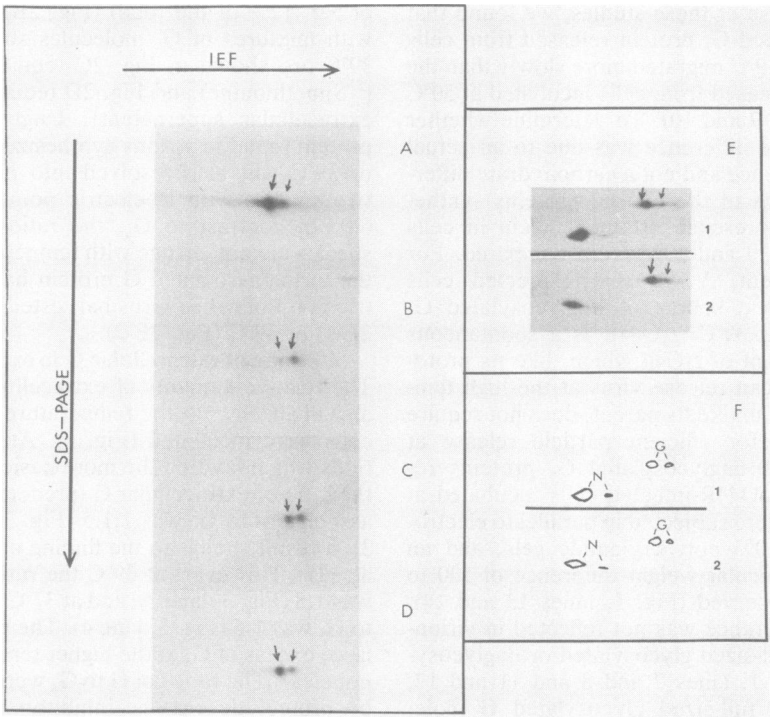

FIG. 2. Isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis of G_s protein. [^{35}S]methionine-labeled G_s protein and virions released from tunicamycin-treated *ts*O44R-infected cells incubated either at 30°C for 17 hours or at 39°C for 11 h were analyzed by isoelectric focusing (IEF) in the first dimension and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. Autoradiograms of the regions of the slab gels between pH 6.5 and 4.5 (from left to right) and between 65,000 and 45,000 daltons (from top to bottom) are shown. (A) G_s shed at 30°C (B) G_s shed at 39°C. (C) Mixture of G_s shed at 30 and 39°C (1.0×10^{-4} cpm of [^{35}S]methionine in each sample). (D) Mixture of G_s shed at 30 and 39°C (G_s released from 5×10^5 cells incubated at 30 and 39°C). (E) Virions released at 30°C (E-1) and 39°C (E-2). (F) Diagram identifying the proteins in (E). The sodium dodecyl sulfate-polyacrylamide slab gels were impregnated with sodium salicylate (3) and exposed to X-ray film for 24 h (A through D) or 48 h (E).

fatty acid attached to the protein during maturation (30) (Fig. 5, lanes 3 and 4). However, G_s protein released from VSV-infected cells incubated at 30 or 39°C in the presence of [^{3}H]palmitate contained no [^{3}H]palmitate label (Fig. 5, lanes 5 and 6). [^{35}S]methionine-labeled virion-associated and soluble polypeptides from parallel cultures were also examined (Fig. 5, lanes 1 and 2).

Generation of the G_s protein. If G_s shedding results from proteolysis of the G protein at the cell surface, then G_s shedding should not occur under conditions where G protein does not reach the cell surface. There are numerous situations which result in the failure of the G protein to reach the cell surface. First, it has been shown previously (5, 20) that in the presence of tunicamycin, significant amounts of the G protein do not reach the cell surface in (i) VSV strain San Juan-infected cells incubated at 30, 37, and 39°C, (ii) VSV prototype strain-infected cells incubated at 39°C and, (iii) group V mutant, *ts*O110-infected cells incubated at 30°C. Second, Johnson and Schlesinger (10) have demonstrated that in the presence of the ionophore monensin G protein does not reach the cell surface. Third, the G proteins of group V temperature-sensitive mutants, such as *ts*O45, do not reach the cell surface at nonpermissive temperatures (15). Therefore, we looked for the release of G_s under all of these conditions where G migration to the cell surface does not occur.

(i) G_s release in the presence of tunicamycin. At 30 and 39°, VSV San Juan-infected cells treated with tunicamycin released 1 to 5% of the yield of particles released from untreated infected cells (5, 20), and a small amount of G was detectable at the cell surface (up to 6.8% of the amount in untreated VSV San Juan-infected cells). At 30 and 39°, a correspondingly small amount of G_s was shed into the extracellular medium (Table 1).

Tunicamycin also blocks the release of virus from VSV prototype-infected cells incubated at

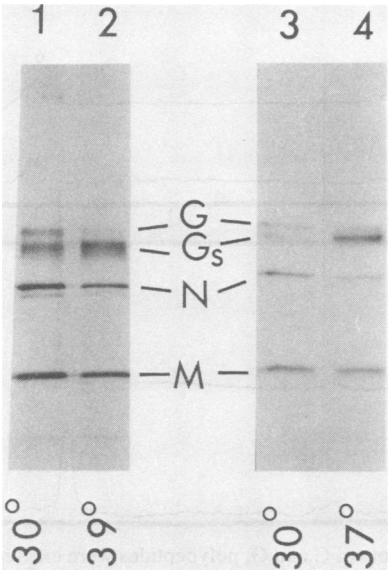

FIG. 3. Ratio of G to G_s released from cells. Monolayers containing 2×10^6 CHO cells were infected and radioactively labeled with [^{35}S]methionine from 4 to 8 h postinfection at 30, 37, or 39°C as described in the text, except that fetal calf serum was omitted during the labeling period. The extracellular supernatants (containing both virions and soluble proteins) were subjected to centrifugation to remove contaminating, detached cells, and then samples of the supernatant were diluted in gel sample buffer. The proteins present in the supernatants were electrophoresed on 10% polyacrylamide gels. Equal counts were applied to lanes 1 and 2 and to lanes 3 and 4. Lanes 1 and 3, viral proteins released from cells incubated at 30°C; lane 2, protein released from cells at 39°C; lane 4, proteins released from cells at 37°C. The supernatants used for lanes 1 and 2 were prepared in parallel infections, whereas the supernatants used for lanes 3 and 4 were prepared in separate parallel infections.

39°C (5, 20). Under these conditions, very little G protein is detectable at the cell surface (5), and there was little detectable G_s shed into the culture medium (Table 1). In contrast, at 30°C nearly normal amounts of the G protein are detectable at the cell surface in the presence of tunicamycin (90% of the amount found at the surface of untreated VSV prototype-infected cells [5]), and G_s was released into the culture medium (50% of the amount released from untreated VSV Prototype-infected cells [Table 1]).

Cells infected by the group V mutant *ts*O110 incubated at 30°C release virus particles (18), the G protein is detectable at the cell surface, and G_s is shed into the medium. However, tunicamycin-treated *ts*0110-infected cells incubated at 30°C do not release virus particles (5), the G protein is barely detectable at the cell surface (5), and there was little G_s shed into the extracellular medium (less than 1% of the amount released from untreated *ts*O110-infected cells [Table 1]).

(ii) G_s release in the presence of monensin. Monensin is an ionophore which has been shown to prevent the migration of G protein from the Golgi apparatus to the cell surface (10). As previously reported, the yield of virions from monensin-treated cells is inhibited by 90% compared with untreated cells. The release of G_s was inhibited by 98% compared with untreated cells (Table 2).

(iii) G_s release from temperature-sensitive mutant-infected cells. Cells infected with the group V mutants *ts*O110, *ts*O45, *ts*O44, and *ts*O57 and the group III mutant *ts*O23 do not release virus particles at 39°C (17, 18). As has been shown previously, there is no G protein on the surface of *ts*O45-infected cells incubated at 39°C (Fig. 6, lane 7) (15). Similarly, the G proteins of *ts*O44, *ts*O57, and *ts*O110 were not detectable at the surface of infected cells incubated at 39.C (Fig. 6, lanes 3, 5, and 9, respectively). However, G proteins of these mutants were detectable at the cell surface at 30°C (Fig. 6, lanes 2, 4, 6, and 8). Group V mutant-infected cells released G_s into the soluble fraction only when the G protein reached the cell surface. At 39°C, G protein migration to the cell surface was inhibited by at least 99%, and the release of G_s was inhibited by 93 to 99% (Table 3). In contrast, the G proteins of the prototype strain and the group III mutant reached the cell surface at both 30 and 39°C (5) (Fig. 6, lanes 10 and 11). G_s was released into the soluble fraction of infected cell cultures at both temperatures (Table 3). Therefore, our results suggest that G_s is released into the extracellular environment only when the G protein reaches the cell surface.

(iv) G_s release from surface-labeled cells. To test directly the idea that G_s is derived from plasma membrane-associated G protein, we examined whether ^{125}I-labeled G_s could be detected in the supernatant from surface-labeled, infected cells. Surfaces of infected cells were radioactively labeled by lactoperoxidase-mediated iodination. No internal proteins were labeled by this procedure. After the iodination reaction monolayers were incubated for 1 h at 37°C in growth media, the supernatants were removed, and the monolayers were disrupted. Labeled proteins in the cell supernatant and in the cell extract were immunoprecipitated, and the precipitated proteins were resolved on 10% polyacrylamide gels. Proteins released into the supernatant from [^{35}S]methionine-labeled cells were co-electrophoresed as markers. Only full-sized G protein was detected in extracts derived from the infected cells (Fig. 7, lane 2). However, the supernatants contained both full-sized G protein and G_s protein (Fig. 7, lane 3). These

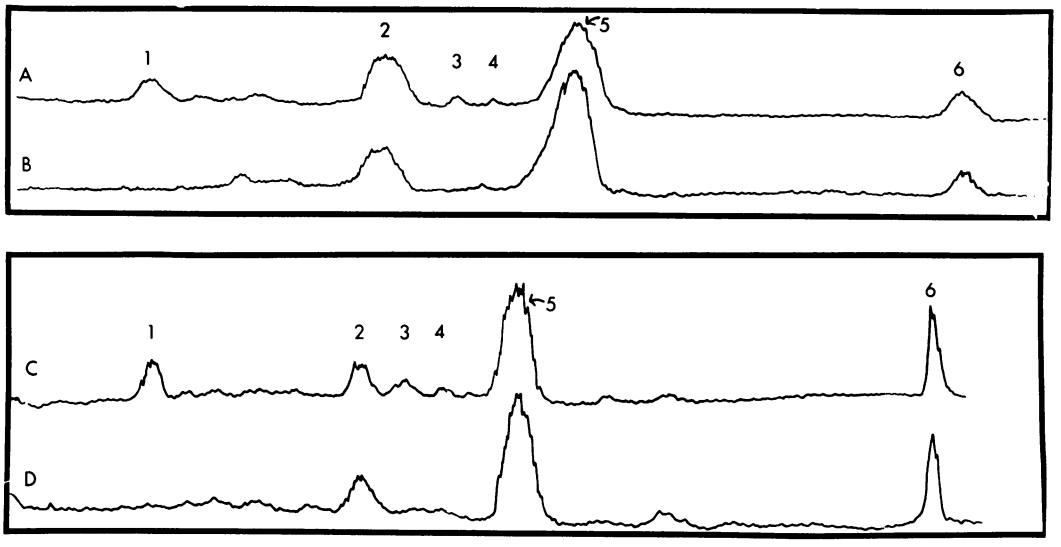

FIG. 4. ^{35}S-labeled methionyl tryptic peptides of G and G_s protein. G and G_s polypeptides were excised from fixed, dried, 10% polyacrylamide gels and digested with trypsin, as described in the text. The methionyl tryptic peptides were resolved by paper ionophoresis at pH 3.5. After electrophoresis, the paper was exposed to X-ray film, and the resulting autoradiogram was scanned with an Ortec microdensitometer. (A) Tryptic peptides of virion-associated G protein. (B) Tryptic peptides derived from G_s protein. For comparison, pulse-labeled intracellular membrane-associated G was digested with trypsin as previously described (4) in order to remove the carboxy-terminal end of the protein. (D) Tryptic peptides derived from truncated G. (C) Peptides derived from full-sized, pulse-labeled G. Electrophoresis of the material shown in (A) and (B) was for 3 h, and electrophoresis of the material shown in (C) and (D) was for 2.5 h.

results clearly showed that G_s can be derived from cell surface G protein.

DISCUSSION

Cells infected with several different viruses release soluble antigens into extracellular supernatants. The best-characterized of these cells are arenavirus-infected cells, VSV-infected cells, RNA tumor virus-infected cells, and herpesvirus-infected cells (1, 2, 11, 12). In 1971, Kang and Prevec (11) made the intriguing observation that VSV-infected cells release a truncated form of glycoprotein, which they called G_s. In subsequent discussions, this molecule has been widely assumed to be missing the hydrophobic carboxy-terminal end. In addition, the mechanism of formation of G_s has been unclear, although the appearance of G_s has been assumed to be the result of proteolytic cleavage of cell surface glycoprotein. However, the results of Little and Huang (21) argued against this mechanism. These authors showed that *ts*O45-infected cells released more G_s at the nonpermissive temperature, conditions under which full-sized G does not reach the cell surface (15). Schnitzer et al. (31), however, were unable to repeat these observations. The results described above support the idea that G_s is generated by proteolytic cleavage of cell surface glycoprotein.

The molecular weight of G_s has been variously estimated at 54,000 to 57,000 (9, 22). Migration of glycoproteins in polyacrylamide gels depends upon the ratio of protein to carbohydrate (32), and it is likely that the ratio of protein to carbohydrate of G_s protein is different from that of the full-sized G protein. To eliminate this potential problem in molecular weight determinations, the addition of carbohydrate to the G_s molecule was inhibited by treating infected cells with tunicamycin (19). The unglycosylated G_s migrated with a molecular weight of 57,000 to 58,000, whereas the unglycosylated G migrated with a molecular weight of 63,000, suggesting that 50 to 60 amino acids were missing from one end of the molecule. An analysis of the [^{35}S]methionine-containing tryptic peptides of the G_s molecule clearly showed that the amino acids missing from the molecule were derived from the carboxy-terminal end. Similar results have been reported recently by Irving and Ghosh (9).

Numerous laboratories have shown that approximately 3,000 daltons of the carboxy-terminal end of G is on the cytoplasmic side of the membrane (4, 13, 34). After sequencing DNA clones from cDNA copies of the glycoprotein mRNA, Rose et al. (29) suggested that the carboxy-terminal 29 amino acids of the G pro-

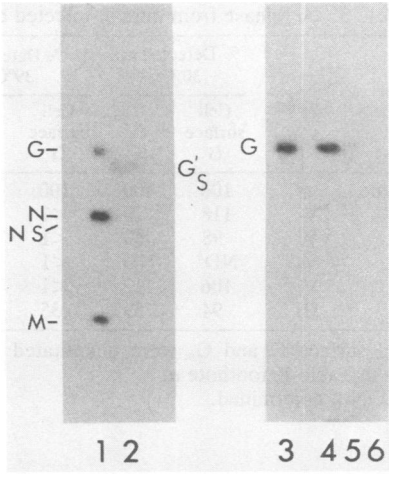

FIG. 5. Polyacrylamide gel electrophoresis of [^{3}H]palmitate-labeled proteins. The figure shows an autoradiogram of a fixed, dried, 10% polyacrylamide gel containing [^{35}S]methionine-labeled virion-associated polypeptides (lane 1), [^{35}S]methionine-labeled nonvirion-associated G_s (lane 2), [^{3}H]palmitate-labeled polypeptides from cell-associated (lane 3) and virion-associated fractions (lane 4), and [^{3}H]palmitate -labeled polypeptides found in the soluble, virus-free fraction from cells incubated at 30 and 39°C (lanes 5 and 6, respectively). Material derived from 5×10^5 cells was loaded into lanes 1 and 3 through 6. Material derived from 10^5 cells was loaded into lane 2. The gels containing ^{3}H label were impregnated with sodium salicylate as described in the text. Lanes 1 and 2 were exposed for 24 h, lane 3 was exposed for 48 h, and lanes 4 through 6 were exposed for 7 days.

tein are located on the cytoplasmic side of the membrane, whereas the next 20 amino acids are quite hydrophobic and probably constitute the membrane-spanning region of the molecule. If G_s is missing 50 to 60 amino acids at the carboxy-terminal end, then the carboxy-terminal end of G_s corresponds to the region of full-sized G just amino terminal to the membrane-spanning region.

This interpretation is supported by the finding that G_s contains no fatty acid. Fatty acid acylation is a recently discovered post-translational modification of the VSV glycoprotein (30). It has been suggested that the fatty acid palmitate is covalently attached to the molecule near, but on the amino-terminal side of, the membrane-spanning region. From the work of Petri and Wagner (27) and Rose and Gallione (28), it seems likely that palmitate is attached to amino acid 48, 49, 52, or 53 from the C-terminal end. If we assume that G_s is derived from palmitate-containing G protein, then the carboxy-terminal end of G_s corresponds to a position on the G protein that must be just amino terminal to these residues.

In the course of our studies of G_s, we discovered that G_s released from cells incubated at 39°C migrates on polyacrylamide gels slightly slower than G_s released from cells incubated at 30°C. In a perhaps related observation, we also found that unglycosylated G_s consists of two charged species and that the ratio between these two species depends upon the temperature at which the cells are incubated. Full-sized, unglycosylated G also contained two charged species,

TABLE 1. G_s release in the presence of tunicamycin[a]

Virus	Tunicamycin	% Detected at 30°C		% Detected at 39°C	
		Cell surface G	G_s	Cell surface G	G_s
Prototype	−	100	100	100	100
	+	90	50	<1	4.5
*ts*O110	−	110	80	ND[b]	ND
	+	<1	<1	ND	ND
San Juan	−	115	106	150	99
	+	6.8	5.0	<1	<1

[a] To quantitate the amounts of G on the surfaces of cells, 2.0×10^6 cells were iodinated as described in the text at 6 h after infection at 30°C and at 4.5 h after infection at 39°C. ^{125}I-labeled polypeptides were immunoprecipitated as previously described (5), suspended in gel sample buffer, and subjected to electrophoresis on 10% polyacrylamide gels. The amount of ^{125}I-labeled G protein at the cell surface was determined by scanning autoradiograms of the polyacrylamide gels. The values obtained with prototype infections at 30 and 39°C were defined as 100%. To quantitate the amount of G_s released from infected cells, 2×10^6 cells were radioactively labeled with [^{35}S]methionine. Virus particles were removed by centrifugation from the extracellular supernatant, and the proteins present in the virus-free supernatant were resolved on 10% polyacrylamide gels. The amount of G_s present was determined by scanning the resulting autoradiograms. The amounts of [^{35}S]methionine-labeled G_s released from prototype-infected cells at 30 and 39°C were defined as 100%. When used, tunicamycin was added at a concentration of 1 μg/ml and was present from the beginning of infection to the end of the labeling period.

[b] ND, Not determined.

TABLE 2. G_s release in the presence of monensin[a]

Monensin	% Release of:	
	Virion-associated G	G_s
−	100	100
+	9.5	2

[a] Cells were infected and radioactively labeled as described in Table 1, footnote *a*. The amount of G_s released was quantitated as described in Table 1, footnote *a*. To quantitate the amount of virion-associated G, [^{35}S]methionine-labeled particles released from 2×10^6 cells were gradient purified as previously described (5). The virion-associated polypeptides were resolved on 10% polyacrylamide gels, and the amount of G present was quantitated by scanning with a microdensitometer. The amount of virion-associated G protein released from untreated VSV San Juan-infected cells was defined as 100%. When used, monensin was added at a concentration of 1 μM and was present from 30 min postinfection to the end of the labeling period. San Juan strain was used for all experiments.

but the majority of the protein was in a more basic form at 30 and 39°C. At present, it is not clear what is responsible for these differences in charge and apparent size. It is possible that there are two sizes of G_s molecules released from cells, one of which has a few more amino acids at the carboxy-terminal end than the other. The ratio of the amounts of the two types of molecules might depend upon the temperature of incubation. Alternatively, it is possible that

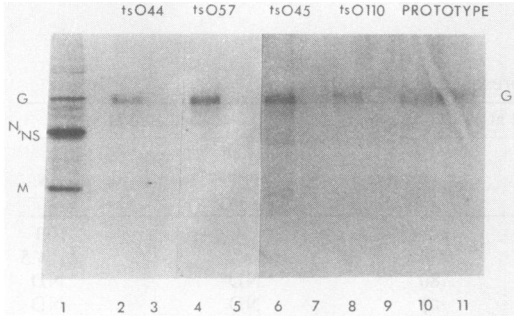

FIG. 6. Lactoperoxidase-catalyzed iodination of infected CHO cells. A total of 2×10^6 infected CHO cells were iodinated as described in the text. The ^{125}I-labeled polypeptides were immunoprecipitated, suspended in gel sample buffer, and subjected to electrophoresis on 10% polyacrylamide gels as described in the text. ^{125}I-labeled immunoprecipitated polypeptides from 5×10^5 cells were loaded into each lane. The figure shows an autoradiogram of the fixed dried gel containing cell surface radiolabeled G protein at 39°C (lanes 3, 5, 7, 9, and 11) and 30°C (lanes 2, 4, 6, 8, and 10). Lane 1 contained [^{35}S]methionine-labeled polypeptides from a parallel wild-type infection. The autoradiogram was exposed to X-ray film for 24 h.

TABLE 3. G_s release from mutant-infected cells[a]

Virus	Group	% Detected at 30°C		% Detected at 39°C	
		Cell surface G	G_s	Cell surface G	G_s
Prototype	ts^+	100	100	100	100
*ts*O45	V	118	70	<1	<1
*ts*O110	V	98	80	<1	<1
*ts*O57	V	ND[b]	ND	<1	<1
*ts*O44	V	106	112	<1	5.7
*ts*O23	III	94	59	35	26

[a] Cell surface G and G_s were quantitated as described in Table 1, footnote *a*.
[b] ND, Not determined.

there is an uncharacterized temperature-sensitive, post-translational modification which might produce differences in apparent size and charge. This possibility seems more likely in light of the fact that full-sized, unglycosylated G also contains two charged species. It should be noted that Kingsford et al. (14) have shown that the G protein may be glycosylated not only by asparagine-linked complex mannose-containing oligosaccharides, but also presumably by simpler glucosamine- or sialic acid-containing structures or both. These glycosylations may be tunicamycin resistant and perhaps temperature sensitive. However, we have not detected any labeling of G or G_s by glucosamine in the presence of tunicamycin, nor are the size differences in G_s affected by neuraminidase digestion (data not shown).

The virion-associated G protein also contains two charged species, but the more basic form predominates at both 30 and 39°C. The acidic form of G_s predominates at 39°C. Significantly, much more of the extracellular glycoprotein occurs in the G_s form at higher temperatures. Perhaps a difference in post-translational modification in G protein at high temperatures results in generation of the acidic form of the protein. This form perhaps is in a conformation which favors the generation of G_s rather than the incorporation of the protein into virus.

The mechanism for the generation of G_s is unclear, although it has been widely assumed that G_s results from proteolytic cleavage of plasma membrane-associated G. It seems possible that G_s could result in proteolytic cleavage of intracellular G; thus, G_s would emerge from the cell in a fashion similar to that of a secreted protein. It also seems possible that G_s results in premature termination of translation. Of the three possible mechanisms for G_s formation, the latter two require the formation of G_s intracellularly. Therefore, we quantitated the amount of

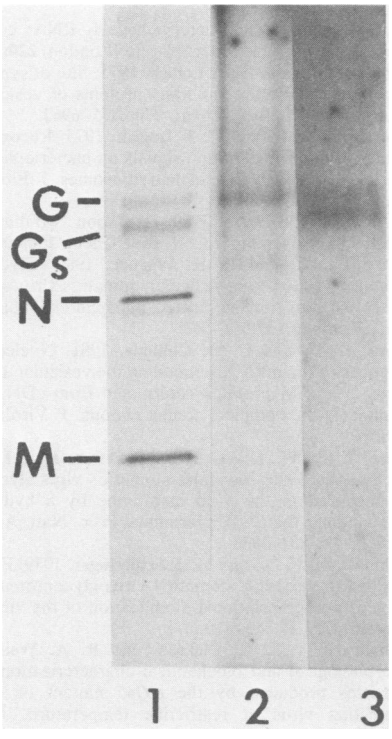

FIG. 7. Generation of G_s from cell surface-labeled G protein. Monolayers containing 2×10^6 infected CHO cells were iodinated as described in the text. After labeling, 1 ml of minimal essential medium containing nonessential amino acids but no fetal calf serum was added to the monolayers, and incubation at 37°C was continued for 1 h. Supernatants were removed and centrifuged to removed detached cells. Monolayers were lysed with Triton X-100. Samples of supernatants and cell extracts were immunoprecipitated as described in the text, and the precipitated proteins were electrophoresed on 10% polyacrylamide gels. ^{35}S-labeled proteins were generated as described in the legend to Fig. 3. Lane 1, [^{35}S]-methionine-labeled viral proteins; lane 2, immunoprecipitated, cell-associated G protein present in cell extracts; lane 3, immunoprecipitated, labeled protein in the cell supernatant.

G_s released from cells in numerous situations in which the migration of intracellular G to the cell surface was blocked. We reasoned that if G_s was generated intracellularly, we should be able to find conditions under which G_s could be detected in the absence of cell surface G protein.

There are several conditions under which failure to glycosylate the G protein blocks the migration of G to the cell surface (5, 8). Under all of these conditions, the amount of G_s released corresponds to the amount of G detected at the cell surface. There are also several glycoprotein mutants, including *ts*O45 (15), which, upon infection of cells at nonpermissive temperatures, fail to express G at the cell surface. In all cases (*ts*O57, *ts*O110, *ts*O44, and *ts*O45) inhibition of cell surface G resulted in inhibition of G_s release. The ionophore monensin blocks the migration of the VSV G protein from a site in the Golgi apparatus to the plasma membrane (10). Under these conditions, we also found a block in the release of G_s protein.

It could be argued that factors responsible for the failure of G to reach the cell surface could also block the release of intracellular G_s. However, under all circumstances described above, we failed to find any evidence of cell-associated G_s protein (data not shown). These results strongly support the idea that at least the majority of G_s is generated by proteolytic cleavage of G at or close to the cell surface. That G_s can be generated from cell surface G protein is clearly shown by the fact that radioactively labeled G_s protein can be generated from G protein labeled only at the cell surface.

In summary, we have characterized the shedding of G_s protein from VSV-infected CHO cells. Our results suggest that G_s results from proteolytic cleavage of cell surface G protein at a region in the molecule just amino terminal to the membrane-spanning region of the protein.

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