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Membrane Anchors of Vesicular Stomatitis Virus: Characterization and Incorporation into Virions

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Wild-type vesicular stomatitis virus-infected cells contained multiple carboxy-terminal fragments of the envelope glycoprotein G. They migrated in 16% polyacrylamide gels with two dominant apparent molecular weights, 14,000 and 9,000. Both fragments were immunoprecipitated by two antibodies, anti-G(COOH) and anti-G(stem), made against the last 15 amino acids at the carboxy terminus and against the first 22 amino acids of the ectodomain adjacent to the transmembrane region of G, respectively. Pulse-chase experiments in the presence and absence of tunicamycin indicated that the higher-molecular-weight fragment, Gal, was generated first, presumably in the rough endoplasmic reticulum, and then apparently chased into the faster-migrating, stable fragment, Ga2. Exposure of infected cells to radioactive palmitic acid labeled Ga2. Ga2 was detected in purified virions. These results show that a polypeptide approximately 71 amino acids long is transported and incorporated into budding virions. What signals are operative and whether this C-terminal fragment of G protein is transported as a complex with other viral or host cell proteins are presently unknown.

Viral transmembrane glycoproteins are often shed from cells in a soluble form. These truncated proteins could arise by several mechanisms. Studies on vesicular stomatitis virus (VSV) glycoprotein G suggests that the shed soluble glycoprotein, Gs, is generated by cleavage of the ectodomain from precursor G (7, 17).

Cells infected with the temperature-sensitive (*ts*) O45 mutant of VSV provide the best evidence for cleavage. They synthesize G at nonpermissive temperatures, but G is retained in the rough endoplasmic reticulum (RER) and cleaved to form a soluble product, Gs, which is then transported to the extracellular medium (7). A 13,000-molecular-weight (13K) polypeptide, including the carboxy terminus of G, can also be recovered from cells and shown by immunoblotting of aggregated material to be associated with spikeless virions (20). This C-terminal fragment of *ts*O45 G, however, is unstable. Also, the heat-labile phenotype of this mutant results in the cleavage of G protein at the cell surface as well as on virions (8).

Soluble Gs from wild-type VSV-infected cells has been identified for some time. Gs is derived from the ectodomain of G and contains all the same carbohydrates (5, 14, 15, 17). Unlike the mutant, wild-type Gs appears to be generated only in association with cells, most likely within the RER (6, 9, 11).

To differentiate whether this Gs was generated by cleavage or by translational termination, the polypeptide containing the anchoring domain was sought and characterized. Multiple C-terminal fragments were detected. They contained a portion of the ectodomain of G protein as well as the transmembrane and cytoplasmic domains. An unstable precursor of the 13K protein, Gal, appeared to chase into a 9K protein, Ga2. This stable Ga2 was covalently linked to the fatty acid palmitate and chased into purified virions. The functional role of small, presumably nonglycosylated, C-terminal fragments is discussed in relation to viral maturation and pathogenesis.

MATERIALS AND METHODS

Biologic reagents. The source of VSV (San Juan strain of the Indiana serotype) and Chinese hamster ovary cells has been described (27). Several antisera were used. Rabbit anti-G(COOH) and anti-G(stem) were made by glutaraldehyde linkage of peptides (made by the Children's Hospital Peptide Synthesis Facility) and inoculation into rabbits by the procedures of Kreis and Lodish (16). These antisera reacted against homologous peptides in an enzyme-linked immunosorbent assay at a dilution of ≥1:1,000. The amino acid sequences used for these peptides are from Rose and Gallione (23). Mouse monoclonal anti-G(ecto) was obtained from Herman Eisen and Mark Pasternak and has been described previously (7). Hyperimmune rabbit anti-VSV antisera were prepared as described elsewhere (13).

Labeling of cells and immunoprecipitations. All the procedures for labeling infected cells, harvesting, and immunoprecipitations have been detailed previously (7). When [^{3}H]leucine (140.8 Ci/mmol; New England Nuclear Corp.) was added with [^{35}S]methionine for labeling the C-terminal fragments of G, [^{3}H]leucine was used at a concentration of 50 μCi/ml. Monolayers were lysed with a modified RIPA buffer (24). To better separate the VSV proteins, including the C-terminal fragments, a sodium dodecyl sulfate–16% polyacrylamide gel was run. The composition of this gel was similar to that of the 7.5% gel described previously (7), except that 16% acrylamide was used. With each gel, either high- or low-molecular-weight markers (Bethesda Research Laboratories, Gaithersburg, Md.) were added and electrophoresed simultaneously.

RESULTS

Immunoprecipitation of a 9K polypeptide from VSV-infected cells. Although one of six glycoproteins synthesized in wild-type VSV-infected cells appears as Gs in the medium (17), C-terminal fragments of G containing just the trans-

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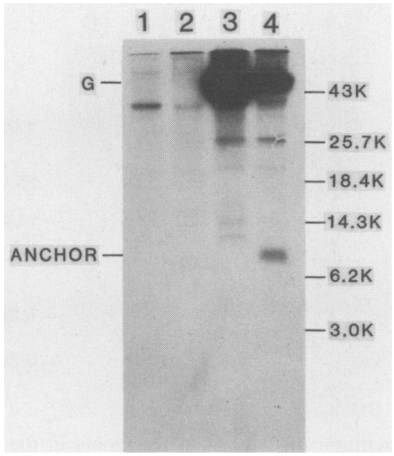

FIG. 1. Immunoprecipitates from mock- and wild-type VSV-infected cell lysates. Uninfected or infected cells labeled with [^{35}S]methionine and [^{3}H]leucine for 2.5 h were harvested at 5.5 h after infection. The cell-associated proteins were immunoprecipitated and separated on a 16% gel. Lane 1, Mock-infected, anti-G(ecto); lane 2, mock-infected, anti-G(COOH); lane 3, VSV-infected, anti-G(ecto); lane 4, VSV-infected, anti-G(COOH).

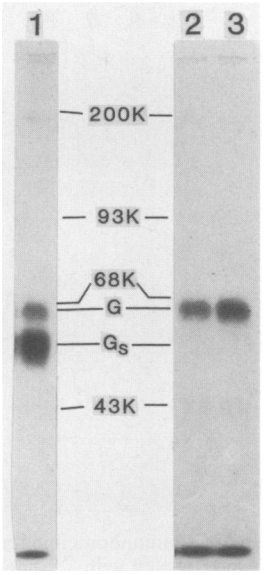

FIG. 2. Precipitation of the VSV glycoprotein with anti-G(stem). Infected cell cultures labeled with [^{35}S]methionine for 2 h were harvested for the total extracellular fraction. Proteins in this fraction were immunoprecipitated and separated on a 7.5% gel. Lane 1 Anti-G(ecto); lane 2, anti-G(stem), rabbit 1; lane 3, anti-G(stem), rabbit 2.

membrane and cytoplasmic domains have not been demonstrated as natural cleavage products. To determine whether a polypeptide with the carboxy-terminal domain of G is also generated during wild-type virus infection, cells were labeled with radioactive leucine and methionine for 2.5 h, and then cell lysates were reacted with either of two antisera: one was a monoclonal antibody against an epitope on the ectodomain of G, anti-G(ecto), and the other was against the last 15 amino acids at the carboxy terminus of G, anti-G(COOH). Only anti-G(COOH) precipitated a small ~9K polypeptide on the 16% gel (Fig. 1, lane 4). Precipitation of mock-infected cell lysates showed no <30K polypeptides (Fig. 1, lanes 1 and 2). Although the high gel concentration did not separate G and Gs well, the immunoprecipitated material above the 43K markers in lanes 3 and 4 were consistent with the precipitation of both G and Gs by anti-G(ecto) and only G by anti-G(COOH). The 9K polypeptide was presumably the C-terminal fragment generated when G was cleaved to Gs.

Failure of anti-G(stem) to precipitate Gs and its detection of the C-terminal fragment. From the molecular weights of G and Gs, it has been calculated that the difference could add up to 6,000 to 9,000 M_r (5, 14, 17). This accounts for the carboxy-terminal cytoplasmic domain and the transmembrane domain. To determine whether any part of the ectodomain of G was present in the C-terminal fragment, a peptide was synthesized that consisted of 22 amino acids in the stem region of the ectodomain, just adjacent to the putative transmembrane domain, sequenced by Rose and Gallione (23). Antibody to this peptide, anti-G(stem), was mixed with [^{35}S]methionine-labeled proteins from the medium of VSV-infected cells, and the immunoprecipitates were separated on a 7.5% gel. Antisera against the stem peptide from two different rabbits precipitated G but not Gs (Fig. 2, lanes 2 and 3). Anti-G(ecto) showed the relative amounts of G and Gs in this extracellular preparation (Fig. 2, lane 1).

Since anti-G(stem) failed to precipitate Gs, it would be expected to precipitate the C-terminal fragment. Figure 3 shows this result (lanes 3 and 4). In addition, anti-G(COOH) was used in these precipitations with and without prior incubation with its homologous peptide. As expected, the carboxy-terminal peptide effectively blocked the precipitation of the C-terminal fragment as well as of G (lane 2). Also, the stem peptide blocked precipitation of the C-terminal fragment by homologous sera (data not shown).

Generation of multiple C-terminal fragments of G. When VSV-infected cells were labeled for more than 30 min with [^{35}S]methionine, only one C-terminal fragment was routinely detected. However, with shorter labeling times, immunoprecipitation showed slower-migrating, more heterogeneous

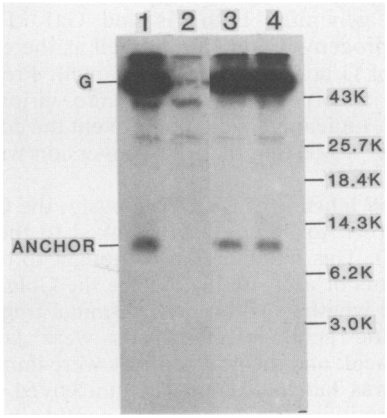

FIG. 3. Precipitation of C-terminal fragments by anti-G(stem) and immunocompetition with 15-mer carboxy-terminal peptide. Infected-cell lysates prepared as described in the legend to Fig. 1 were immunoprecipitated, and the proteins were separated on a 16% gel. For lane 2, the anti-G(COOH) serum was premixed with the carboxy-terminal peptide (final concentration, 1 mg/ml) at 4°C for 1 h before being added to the cell lysate. Lane 1, Anti-G(COOH); lane 2, anti-G(COOH) plus 15-mer peptide; lane 3, anti-G(stem), rabbit 1; lane 4, anti-G(stem), rabbit 2.

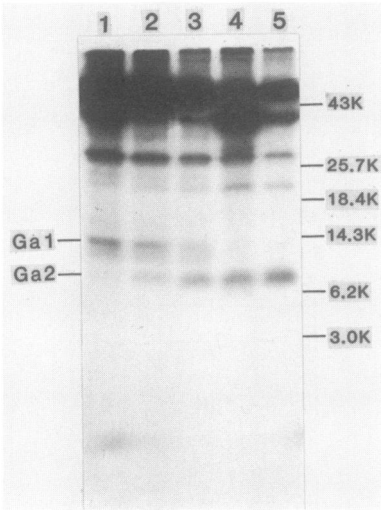

FIG. 4. Pulse-chase of immunoprecipitated C-terminal fragments. Infected cells were labeled with [^{35}S]methionine at 3 h after infection for 30 min and then chased for different times up to 3 h. The cell lysates were immunoprecipitated with anti-G(COOH) and separated on a 16% gel. Lane 1, Pulse, 30 min; lane 2, chase, 30 min; lane 3, chase, 1 h; lane 4, chase, 2 h; lane 5, chase, 3 h.

material around 14,000 M_r, Ga1 (Fig. 4, lane 1). Like the 9K fragment, Ga2, this material was immunoprecipitated by anti-G(stem) and specifically competed out by prior incubation of the antiserum with its homologous peptide (data not shown). As the chase period was increased, radioactivity decreased from Ga1 and increased in Ga2, suggesting that Ga1 is an unstable precursor of Ga2 (Fig. 4, lanes 2 to 5).

Generation of multiple C-terminal fragments in the presence of tunicamycin. Gs has been shown to be generated in the presence of inhibitors that prevent transport of glycoproteins form the RER (6). To see whether the C-terminal fragments of G were generated concomitantly, infected cells were treated with one of these drugs, tunicamycin, during the labeling and chase periods. Multiple C-terminal fragments were detected (Fig. 5), suggesting that cleavage of G in the RER generated both Gs and Ga1. Tunicamycin inhibited all progeny production as well as the extracellular appearance of G and Gs (data not shown). Presumably, it prevents the incorporation of Ga2 into virions as well. Nevertheless, tunicamycin did not prevent the conversion of Ga1 into Ga2, suggesting that the conversion was probably intracellular.

Palmitic acid labeling of Ga2. Previously, the G protein of VSV was shown to be covalently linked to the fatty acid palmitate (26). This modification is thought to occur in the Golgi apparatus or at a site just before the Golgi stage (25). To determine whether wild-type C-terminal fragments contained palmitic acid, infected cells were labeled with [^{3}H]palmitic acid, and the polypeptides were immunoprecipitated. Ga2 was labeled along with uncleaved G (Fig. 6). Immunocompetition with homologous peptide indicated that the radioactivity was specific to the C-terminal fragment (data not shown). These data support the transport of Ga2 through the Golgi or post-RER stages.

Incorporation of Ga2 into purified VSV. During previous pulse-chase experiments, Ga2 accumulated in the extracellular environment (data not shown). Because the medium contains both soluble antigens and virions, sucrose gradient-purified virions were examined to see whether any C-terminal fragments were incorporated directly into them. Figure 7 shows Ga2 immunoprecipitated from virions. Previously, it was shown that our purification procedures led to concentration of the infectivity (27) and purification of the virions with undetectable contaminating host proteins (4).

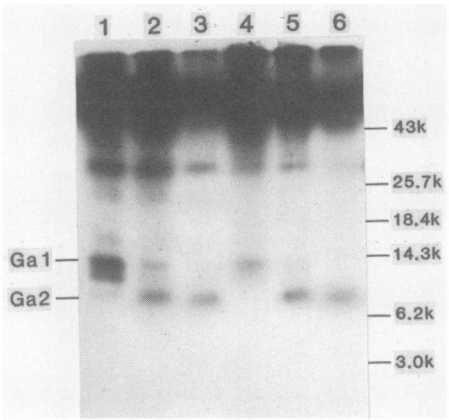

FIG. 5. Generation of C-terminal fragments in the presence of tunicamycin. Infected cells were incubated in the absence or presence of tunicamycin (2 μg/ml). At 3 h after infection, they were labeled for 30 min with [^{35}S]methionine and then chased for different times. Cell lysates were immunoprecipitated with anti-G(COOH) and separated on a 16% gel. Lane 1, pulse, 30 min; lane 2, chase, 1.5 h; lane 3, chase, 3 h; lane 4, tunicamycin, pulse, 30 min; lane 5, tunicamycin, chase, 1.5 h; lane 6, tunicamycin, chase, 3 h.

DISCUSSION

These experiments demonstrate the formation of a stable, functional C-terminal fragment, called the membrane anchor, during wild-type VSV infection of fibroblast cells. The anchor was defined operationally by its precipitation with antipeptide antibodies that spanned the last 71 amino acids at the carboxy terminus of G. This included the transmembrane and cytoplasmic domains as wells as 22 amino acids at the C-terminus of the ectodomain. Although the extent of the ectodomain remains undetermined, the molecular weight determination precludes any large additional amount. This region, just amino-terminal to the transmembrane region, has been called the stem region.

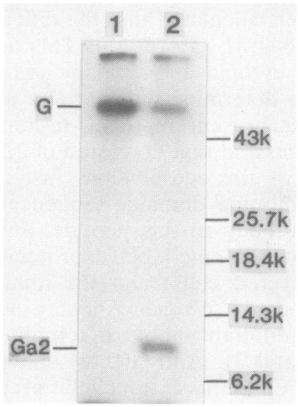

FIG. 6. Palmitic acid labeling of Ga2. Infected cells were labeled with 1 mCi of [^{3}H]palmitic acid (28.5 Ci/mmol) at 30 min after infection for 5.5 h. Samples of the cell lysate were immunoprecipitated and separated on a 16% gel. Lane 1, Anti-VSV; lane 2, anti-G(COOH).

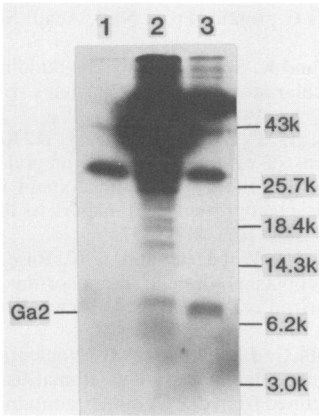

FIG. 7. Virion-associated Ga2. A large preparation of infected cells (3×10^8) was labeled with 1.25 mCi of [^{35}S]methionine in the suspension culture conditions described by Stampfer et al. (27), except that dialyzed fetal calf serum was used. Extracellular virions were purified by differential and rate-zonal centrifugations (27). Samples (100 μl) were taken from fraction 14, containing the virus peak, and immunoprecipated. Lane 1, Rabbit nonimmune serum; lane 2, anti-VSV; lane 3, anti-G(COOH).

The stem region may be another potentially important determinant for transport. Particularly in polarized cells, results with fusion proteins (19) or with prematurely terminated G protein (10) support a possible unrecognized role for the stem region in polar transport. However, a number of other studies with recombinant DNA and site-specific mutagenesis involving the VSV G protein focus on signals for transport within the cytoplasmic and transmembrane domains or through glycosylation (1, 2, 12, 18, 21, 22).

The identification of C-terminal fragments in wild-type VSV-infected cells supports a cleavage mechanism for the generation of Gs in the RER (6, 9). However, the generation of Gs and Ga1 occurred within 30 min of labeling, so it is not possible to completely rule out translational termination by ribosomal suppression and subsequent reinitiation. Terminal sequence determination of Ga1, which is in progress, may not show the cleavage site due to postcleavage processing.

The association of the anchor with fatty acids suggests that posttranslational modifications other than cleavage may result in migrational changes of Ga1 and Ga2. In fact, the palmitate labeling and kinetics suggest that Ga1 becomes Ga2, probably in the Golgi apparatus or at a site prior to the Golgi.

Finding the C-terminal fragment in purified virions suggests that after its intracellular generation, the anchor traveled to the plasma membrane and was incorporated into budding virions. The degree of incorporation of anchor into virions may explain the variable quantities of detectable G found in VSV preprations. More importantly, a polypeptide of 71 amino acids is transported and incorporated into virions. How this transport occurs is presently unknown. The polypeptide may form a complex with viral or cellular proteins. It would be interesting to determine whether Ga2 reaches the cell surface in the presence of tunicamycin, especially since extracellular G protein, Gs, and virions are not detected under these conditions. Unfortunately, such comparative determinations cannot be done because native Ga2 interacts weakly with anti-G(stem).

When these results are compared with those previously reported for a *ts* mutant of VSV (20), it is apparent that *ts*O45 has multiple *ts* phenotypes. *ts* G is blocked at the RER (3) and cleaved at 39°C (7, 8); in addition, its virion-associated transmembrane anchor aggregates and is unstable (20). Moreover, with *ts*O45, the pH-dependent cleavage of G (7) and variations in host cells lead to different results in different laboratories. Most likely, the 13K anchor of *ts*O45 corresponds to the unstable 14K anchors reported here. In contrast, wild-type VSV contains a stable ~9K anchor. Incubation of wild-type VSV at 37°C for several hours did not lead to any detectable cleavage of G (unpublished observations). Protease inhibitors added to the medium of wild-type VSV-infected cell cultures failed to significantly reduce the amount of Gs shed from cells (17). These data suggest that wild-type Gs and a C-terminal fragment were generated in the RER and not elsewhere.

Because the immunodominant epitopes, especially neutralizing ones, of G are found on the ectodomain, the incorporated C-terminal fragment reduces the chance that virions will be detected by the immune system (unpublished results). Although this loss also reduces the ability of VSV to attach to fibroblast cells, nonspecific uptake by macrophagelike cells in vivo could readily spread the infection. Therefore, the generation of Gs and anchors introduces both a smokescreen of soluble antigens and partially denuded virions that escape immune detection.

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