Formation of Heterotrimers between the Membrane-Integrated and the Soluble Glycoproteins of Vesicular Stomatitis Virus Leads to Their Intracellular Cotransport

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BHK cells infected with vesicular stomatitis virus serotype Indiana generate intracellularly two different types of glycoproteins: the authentic membrane-integrated G protein of virions and a smaller soluble Gs protein lacking the transmembrane and cytoplasmic domains which is secreted into the growth medium. A Gs1 protein species which is formed during or shortly after translation in the endoplasmic reticulum lumen is modified in the same way as the G1 protein by endoglycosidase H-sensitive oligosaccharides of the high-mannose type. Both G1 and Gs1 are almost simultaneously transported, trimmed, and processed into G2 and Gs2 species which possess carbohydrate side chains of the complex type, making both glycoproteins resistant to endoglycosidase H cleavage. Secretion of Gs2 protein into the growth medium and arrival of G2 protein on the cell surface occur concomitantly. Membrane-integrated G protein and the soluble Gs protein molecules oligomerize intracellularly into heterotrimers which can be immunoprecipitated after chemical cross-linking. Gs protein seems to contain sufficient structural information for the formation of heterotrimers which are efficiently transported to the cell surface. Heterotrimer formation between G and Gs proteins explains the rapid secretion of Gs molecules.

Our understanding of the essential structural requirements for the intracellular transport of secretory and membrane glycoproteins from their site of biosynthesis in the endoplasmic reticulum (ER) to their final destinations, e.g., plasma membrane or cell supernatant, is still rather limited. In the last few years, it has become increasingly clear that newly synthesized polypeptides rapidly acquire defined secondary and tertiary structures after being translocated into the ER lumen (20, 39). Quaternary structure formation giving rise to homo- and heterooligomers occurs also in the ER. Correct folding and assembly of polypeptides is rigorously controlled because the proper three-dimensional structure seems to be important for the exit of the proteins from the ER (36). Misfolded, incorrectly assembled, or unassembled polypeptides are not transported but instead are retained in the ER and frequently become degraded. Misfolded or improperly assembled proteins tend to form aggregates which very often associate with immunoglobulin heavy-chain binding protein (19), a heat shock-related protein, into noncovalently linked complexes (4, 10, 16, 33, 36). The majority of plasma membrane glycoproteins are oligomeric, e.g., T-cell receptor (41), major histocompatibility complex class I and II (5), and viral antigens (20), which generally oligomerize in the ER, whereas the soluble secretory proteins are frequently monomeric, with some well-known exceptions, e.g., immunoglobulin G (2) and fibrinogen (44).

Structurally different membrane proteins, e.g., the histocompatibility antigens H-2K^k and H-2D^k (43), as well as secreted proteins, e.g., those in human HepG2 cells (31) and in hepatocytes (13), migrate from the ER to the Golgi complex at different rates and appear at the cell surface or in the supernatant at different times. For the histocompatibility antigens, Williams et al. (43) identified by subcellular fractionation the exit from the ER as the rate-limiting step.

Features of the proteins which could serve as signals controlling export have not yet been well characterized on the molecular level. Thus, it is necessary to find model systems in order to unravel the molecular events operating in the exocytotic pathway. In the past, G protein of vesicular stomatitis virus (VSV) has proved to be a very useful system for the elucidation of the functionally important properties for the transport of integral membrane proteins on the molecular (25), genetic (37, 38), biochemical (24), and immunoelectron microscopic (3) levels. VSV-infected tissue culture cells generate intracellularly, in addition to the authentic membrane-integrated G protein, a soluble glycoprotein called Gs protein which lacks the transmembrane and cytoplasmic domains completely (7, 8, 15, 17, 18). We chose this system to address the question whether the loss of the transmembrane and cytoplasmic domains has an influence on the rate and efficiency of Gs protein maturation and export. In addition, we analyzed the oligomeric state of both proteins in VSV-infected cells.

MATERIALS AND METHODS

Cell culture and virus growth. BHK 21 cells were grown as monolayers in Joklik's minimal essential medium supplemented with 5% calf serum. VSV serotype Indiana strain San Juan was grown on BHK 21 cells which were infected with a multiplicity of 0.1 PFU per cell. Virus was harvested from the cell supernatant approximately 18 h after infection by centrifugation for 1 h at 120,000 × g. The virus pellet was resuspended in 10 mM Tris-HCl (pH 7.5), purified, and assayed as described previously (27). The purified virus was stored at -80°C.

Metabolic labeling of VSV-infected BHK 21 cells. Confluent monolayers of BHK 21 cells (5 × 10⁶ cells, 6-cm-diameter dishes) were infected in 1.5 ml of serum-free medium containing 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.5) for 30 min at room tempera-

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ture. The inoculum was removed, and 4 ml of complete medium was added. After 3.5 h of infection at 37°C and 5% CO₂, the medium was removed, and cells were washed in prewarmed phosphate-buffered saline (PBS), pulse-labeled by the addition of 80 µCi of [³⁵S]methionine in 2 ml of Dulbecco's minimal essential medium without methionine at 37°C, and then chased with nonradioactive medium for the times indicated in the figure legends. The growth medium was collected, and cell debris were removed by sedimentation for 5 min at 4°C in an Eppendorf centrifuge. The samples were immunoprecipitated with monospecific or polyspecific rabbit antisera. Endoglycosidase H (endo H) treatment was done as described previously (17).

Immunoprecipitation of viral proteins. Antibodies against viral particles were raised in rabbits by standard procedures. Immunoprecipitation of growth medium after VSV infection of BHK cells was done by a modified immunoadsorbent procedure originally described by Kessler (21). Aliquots of the growth medium were adjusted to 1% sodium dodecyl sulfate (SDS) and heated to 95°C for 5 min. Samples were diluted with 10 volumes of TNET buffer (20 mM Tris-HCl [pH 7.5], 140 mM NaCl, 5 mM EDTA, 1% [wt/vol] Triton X-100, 2 mM methionine). Saturating amounts of antisera were added, and the samples were kept on ice for 2 h. The antigen-antibody complexes were incubated with 50 µl of 10% (wt/vol) *Staphylococcus* absorbent, and the suspension was centrifuged at 10,000 × *g* for 1 min at room temperature. The immunocomplexes adsorbed to protein A were washed with TNET, TNET plus 2.5 M KCl, and once again with TNET. The immunocomplexes were dissociated from the absorbent by boiling for 5 min in 60 µl of electrophoresis sample buffer.

Detection of viral G protein on the cell surface. BHK 21 cells were infected with VSV serotype Indiana (10 PFU per cell) and, after incubation for 3.5 h, were labeled with [³⁵S]methionine as described above. The growth medium was removed, the cells were washed three times in ice-cold PBS, and 2 ml of ice-cold PBS was added which contained 3 µl of antiserum against VSV. After 15 min of incubation at 4°C, the cell layer was washed five times with ice-cold PBS. The cells were resuspended in 2 ml of ice-cold PBS containing 50 µg of viral proteins in order to saturate the remaining free binding sites of the antibodies. Cells were collected by low-speed centrifugation and allowed to swell for 10 min in 500 µl of hypotonic buffer (10 mM Tris-HCl, 10 mM NaCl, 20 mM EDTA, pH 7.3) at 4°C. Triton X-100 was added to a final concentration of 1%, and the cytoplasmic extracts were freed of nuclei by centrifugation at 800 × *g* and 4°C for 5 min in a Haereus minicentrifuge. The antigen-antibody complexes were adsorbed to 15 µl of 10% *Staphylococcus aureus* as described above.

Trimerization assay. BHK 21 cells were infected with VSV serotype Indiana (40 PFU per cell) and labeled with 100 µCi of [³⁵S]methionine for 10 min and then chased with serum-free Joklik's minimal essential medium–20 mM HEPES for 10 min. Cells were washed with ice-cold PBS, scraped off, and collected in 1 ml of ice-cold PBS. After low-speed centrifugation, the cells were resuspended in 200 µl of Joklik's minimal essential medium and 200 µl of lysis buffer (100 mM phosphate, 200 mM NaCl, 1% Triton X-100, 2.5 mM EDTA, 2 mM EGTA, pH 5.5). A 100-µl sample of the cell lysate was loaded on an ice-cold 5 to 20% (wt/vol) continuous sucrose gradient in PNT buffer (50 mM phosphate, 100 mM NaCl, 1.25 mM EDTA, 1.0 mM EGTA, 0.1% Triton X-100 at pH 5.8 or pH 7.4). The samples were centrifuged in a Beckman SW50.1 rotor for 15 h at 195,000 ×

FIG. 1. Kinetics of oligosaccharide processing of the G and Gs proteins. BHK 21 cells were labeled with [³⁵S]methionine for 10 min at 3.5 h after infection by VSV serotype Indiana (40 PFU per cell) and then chased with an excess of nonradioactive methionine for up to 90 min (A) or 60 min (B). Cells were collected in ice-cold PBS, and cell extracts were prepared. The cell extracts were centrifuged in a Beckman 50 Ti rotor at 110,000 × *g* and 4°C for 1 h. The supernatants were immunoprecipitated with polyclonal antisera against purified G protein, and the samples were subsequently incubated with endo H as indicated in panel B. All samples were analyzed on a 10% polyacrylamide gel.

g and 4°C (11). Gradients were fractionated from the top. The proteins in each fraction were precipitated with 1 volume of ice-cold 20% trichloroacetic acid and analyzed on a 10% polyacrylamide gel (28).

Cross-linking of glycoproteins with DSP. A 0.7 volume of 20 mM phosphate–100 mM NaCl (pH 7.6) was added to 0.3 volume of the indicated gradient fractions. Samples were incubated with 1 mM dithio(succinimidylpropionate) (DSP) (Pierce) for 15 min at room temperature. The reaction was stopped by adding 100 mM glycine for 15 min at room temperature. The proteins were immunoprecipitated with the monoclonal antibody P5D4 (26).

SDS-polyacrylamide gel electrophoresis. Protein compositions were analyzed on 10% polyacrylamide gels by using the discontinuous buffer system of Laemmli (28). Electrophoretic separation was performed under reducing conditions at 15 mA for 11 h. Gels were fixed in 10% acetic acid–40% methanol–50% water, impregnated with 1 M sodium salicylate (6), vacuum dried, and exposed on Cronex 4 X-ray film at –80°C.

RESULTS

Kinetics of intracellular processing of G and Gs proteins. VSV-infected BHK cells were pulse-labeled with [³⁵S]methionine for 10 min, and the processing of G and Gs proteins was monitored during the chase for up to 90 min (Fig. 1A). At the end of the pulse and after a 10-min chase, Gs was still completely in its faster-moving derivative, which we called Gs1 on the basis of the report of Knipe et al. (22), indicating that this glycoprotein species still contained oligosaccharide side chains of the high-mannose type (Fig. 1A, lanes 1 and 2). At the end of the pulse and after a 10-min chase, a slower-migrating Gs protein band appeared in the polyacrylamide gel. Concomitantly, the radioactivity in the faster Gs1 band was reduced, indicating that there existed a precursor-product relationship between Gs1 and Gs2. The oligosaccha-

FIG. 2. Time of arrival of G protein on the cell surface. (A) VSV-infected BHK 21 cells were labeled with [35 S]methionine for 10 min and chased for 60 min. Cell surface-integrated G protein was immunoprecipitated as described in the Materials and Methods. The immunoprecipitates were analyzed on a 10% polyacrylamide gel. Lane 8, VSV proteins. (B) Fluorograms of five kinetic experiments described in panel A were quantified by the silver grain elution method (42).

ride side chains of the high-mannose type in the glycoproteins started to be processed after approximately 20 min into the complex type, giving rise to Gs2, which became endo H resistant. The intensity of the Gs2 band further increased at 30 min of chase (Fig. 1A, lane 4). Subsequently, the radioactivity of this band was continuously reduced until it completely disappeared at 90 min of chase (Fig. 1A, lanes 5 to 7). Concomitant with this mobility shift from Gs1 to Gs2, a similar modification of the authentic membrane-integrated G protein seemed to take place (Fig. 1A). Quantitation of the endo H sensitivity of the glycoproteins showed that there is a marginal time difference between the conversion of G1 to G2 and Gs1 to Gs2 (Fig. 1B). Endo H cleavage of the G1 species gave rise to a G1* species which migrated a little bit slower than Gs1 molecules (Fig. 1B, lanes 1 and 2) but was indistinguishable in its migration properties from Gs2 molecules (Fig. 1B, lanes 9 and 10).

Appearance of membrane-integrated G protein at the cell surface. The kinetics of G-protein appearance on the cell surface was analyzed to determine the period for the intracellular migration of the membrane-integrated G protein to the plasma membrane. After a 10-min pulse and a 20-min chase, G protein exposed on the plasma membrane gave only a faint band (Fig. 2A, lane 3), but it became easily

FIG. 3. Release of viral proteins into the growth medium. (A) Each growth medium of the experiment in Fig. 2A was collected at the times indicated, and cell debris were removed. Samples were immunoprecipitated with polyclonal and polyspecific antiserum, and the proteins were analyzed on a 10% polyacrylamide gel. Lane 8, VSV proteins. (B) Each Gs and G band of the fluorogram (A) was quantified by the silver grain elution method (42). The differential export of Gs ($\square$) and G (hatched) during each 10-min interval was calculated by subtraction and is shown for each interval.

detectable at 30 min after the beginning of the chase (Fig. 2A, lane 4). The radioactivity of this G-protein pool increased up to 40 min (Fig. 2A, lane 5) before continuously decreasing during the remaining chase period (Fig. 2A, lanes 6 and 7). By evaluating quantitatively several kinetics, the chase time at which the cell surface label has its half-maximal intensity was determined as $T_{1/2} = 27$ min (Fig. 2B). Next, we compared the time of G-protein arrival on the cell surface with the moment of Gs secretion into the growth medium.

Budding of virus-integrated G protein and secretion of soluble Gs protein into the growth medium. Concomitant with the arrival of virus-integrated G protein at the cell surface (Fig. 2A, lane 4), soluble Gs protein was released from the cells into the supernatant (Fig. 3A, lane 4). Monomeric Gs molecules were detected in the growth medium after sucrose gradient centrifugation at acidic pH (data not shown). Evaluating quantitatively five similar experiments, it became clear that there was no significant time lack between the appearance of G protein at the cell surface and the time of secretion of truncated Gs protein into the growth medium.

FIG. 4. Heterotrimer formation between G and Gs proteins. (A) BHK 21 cells were infected with VSV serotype Indiana and pulse-labeled for 3 min with [35 S]methionine and chased for 0, 2, and 4 min as described in the Materials and Methods. Cell extracts were centrifuged, and the protein composition in the gradient fractions was analyzed on a 10% polyacrylamide gel. (B) Fraction number 10 of a parallel gradient was incubated with 1 mM DSP to cross-link the oligomers. Trimers were immunoprecipitated with a monoclonal antibody (P5D4) against the C terminus of G protein. Cross-linked proteins were analyzed under nonreducing conditions on a 7.5% polyacrylamide gel. Protein bands I and II were cut out of the gel and reanalyzed under reducing conditions on a 10% polyacrylamide gel. A different degree of cross-linking might explain the appearance of two bands under nonreducing conditions.

Appearance of full-length G protein in the growth medium was delayed compared with Gs secretion (Fig. 3A, lane 4, 3B). Two facts might be responsible for the observed delay. (i) Maximal radioactive labeling of the cell surface pool of G-protein molecules took several minutes (Fig. 2B). (ii) Labeled G protein which is newly delivered to the cell surface has to be integrated into the viral bud before 35 S-labeled particles can be released from the plasma membrane. The rapid and efficient secretion of Gs protein is also apparent from Fig. 3B, in which the G and Gs yields exported during each 10-min interval were compared. These kinetic differences in the secretion behavior of G and Gs have been noted previously (17). Similarly, G and Gs as well as Gss protein (18) which were intracellularly formed after infection of BHK cells with VSV serotype New Jersey were cotransported through the intracellular compartments and almost simultaneously exported into the growth medium (data not shown).

Intracellular oligomers of G and Gs proteins identified by cross-linking. The nearly synchronous transport of both the membrane-integrated G protein and the truncated soluble Gs protein raised questions as to the underlying mechanism. A plausible explanation would be the intracellular assembly of heterooligomers, i.e., quaternary complex formation between G and Gs protein molecules. A pulse-chase experiment was done, and the intracellular extracts of VSV-infected BHK cells were analyzed by sucrose gradient centrifugation at acidic (Fig. 4A) and neutral (data not shown) pHs. Trimerization of G protein was observed as described previously (11). At the end of the pulse, G and Gs molecules were banding in the monomer and trimer regions of the gradients. Monomeric Gs protein in fractions 5 to 7 was chased after 4 min into trimers in fractions 9 to 12 (Fig. 4A). It is not clear from the sedimentation pattern (Fig. 4A) whether Gs protein is associated with the full-length G protein giving rise to heterotrimers or whether Gs is assembled into homotrimers. To answer this question, we did cross-linking experiments of glycoprotein fractions separated by sucrose gradient centrifugation using the reversible cross-linker DSP (Fig. 4B). Cross-linked G-protein complexes were immunoprecipitated with an antibody against the last 15 amino acids of the cytoplasmic tail of the G protein (25). Two oligomeric G-protein bands migrating with

the apparent molecular weight of trimers could be distinguished on gels under nonreducing conditions (Fig. 4B). Both bands contained G as well as Gs molecules when analyzed on polyacrylamide gels under reducing conditions.

DISCUSSION

Several different factors have been shown to influence the biosynthesis, transport, and insertion of VSV G protein into the plasma membrane. The role of the three structural domains of G protein in the transport and maturation process has been extensively studied: (i) the ectodomain starting with the amino terminus and consisting of the major body of the protein with both carbohydrate side chains; (ii) the transmembrane domain containing 20 amino acids with mostly hydrophobic side chains; and (iii) the cytoplasmic tail corresponding to the carboxy-terminal region which contains 28 amino acids in the Indiana serotype (40).

VSV-infected cells synthesize not only the authentic membrane-integrated G protein (Fig. 1) but also soluble glycoproteins which are secreted into the growth medium. A fragment of the ectodomain called Gs protein lacking approximately 70 amino acids was found to be released from infected cells into the extracellular medium (15, 17, 29, 30). Recent evidence points to an intracellular site of Gs generation (8, 15, 17, 18). In the presence of G protein, the soluble Gs protein is rapidly transported to the cell surface of VSV-infected cells (Fig. 1), while anchorless G protein lacking 79 amino acids expressed from cloned cDNA was slowly glycosylated and secreted (9, 37). Migration and processing of the membrane-integrated and soluble G-protein forms occurred almost simultaneously (Fig. 1). Thus, Gs protein corresponding to the ectodomain of the G protein seemed to contain sufficient information to fold into a transport-competent conformation.

Previous studies of the intracellular transport of the G protein from the VSV *tsO45* mutant in which a single serine residue was substituted for a phenylalanine (14) demonstrated the importance of the correctly folded ectodomain for the G-protein exit from the ER, because cells infected with these temperature-sensitive (*ts*) mutants at nonpermissive temperature produced full-length G protein, but the protein was defective in maturation and migration to the cell

surface (8, 23, 35). At nonpermissive temperature, G protein of *tsO45* was misfolded (10) and formed in the lumen of the ER high-molecular-weight aggregates which did not leave the ER (11).

The cytoplasmic domain of VSV G protein undergoes a conformational change in the Golgi complex which results in a higher accessibility to proteolytic cleavage (34). Antibodies to synthetic oligopeptides of the cytoplasmic tail when microinjected into VSV-infected cells blocked transport of VSV G protein to the cell surface (1, 26); Fab fragments, however, had no inhibitory effect (26).

Alteration of the cytoplasmic domain by deletion mutagenesis affected the intracellular transport of VSV G protein (9, 38) either by arresting G-protein transport to the cell surface at a stage prior to oligosaccharide processing or by reducing the rates of processing into complex carbohydrate chains. Taken together, these studies suggest that the amino acid sequence adjacent to the lipid bilayer and the resulting peptide conformation are important and can influence the rate of the vectorial transport of the G protein.

Folding and oligomerization of 12 mutant G proteins which had alterations in their cytoplasmic and transmembrane regions as well as in their ectodomains have been previously analyzed with the help of conformation-specific monoclonal antibodies (12). Mutant proteins not capable of undergoing correct initial folding did not trimerize, were not transported, and formed large aggregates. Mutations in the cytoplasmic domain generally had little effect on folding and trimerization. Changes in the transmembrane domain of the G protein were quite tolerable, although a drastic shortening in the transmembrane sequence affected the folding and trimerization of the G protein (12).

The loss of the transmembrane and cytoplasmic domains had little effect on the Gs transport rate (Fig. 1 and 3). Soluble Gs molecules formed heterotrimers intracellularly with the full-length G protein (Fig. 4) which were rapidly transported to the cell surface, where the Gs molecules were released into the supernatant (Fig. 3). In a recent report, heterotrimers were readily detected when G protein was coexpressed with mutant glycoproteins that formed trimers at the wild-type rate and were transported from the ER at the wild-type rate (45). Also, the dissociation of oligomeric VSV glycoproteins has been demonstrated *in vivo* (45) and *in vitro* (32). Heterotrimer formation by Gs protein in VSV-infected cells can explain the different transport rates compared with that of Gs homotrimers in transfection experiments (9, 37, 38). The high efficiency of the heterotrimer transport seems to depend on the presence of both the transmembrane and cytoplasmic domains of the full-length G protein. Since there is intracellularly an excess of G protein, we assume, because of the dynamic nature of trimer formation (32, 45), that all Gs molecules were assembled into heterotrimers. This assumption is consistent with the rapid export of the complete Gs-protein fraction (Fig. 3B), an event which has been previously described (15, 17).

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