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Oligomerization of Glycolipid-Anchored and Soluble Forms of the Vesicular Stomatitis Virus Glycoprotein

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The vesicular stomatitis virus glycoprotein forms noncovalently linked trimers in the endoplasmic reticulum before being transported to the Golgi apparatus. The experiments reported here were designed to determine if the extracellular domain of the glycoprotein contains structural information sufficient to direct trimer formation. To accomplish this, we generated a construct encoding G protein with the normal transmembrane and anchor sequences replaced with the sequence encoding 53 C-terminal amino acids from the Thy-1.1 glycoprotein. We show here that these sequences were able to specify glycolipid addition to the truncated G protein, probably after cleavage of 31 amino acids derived from Thy-1.1. The glycolipid-anchored G protein formed trimers and was expressed on the cell surface in a form that could be cleaved by phosphoinositol-specific phospholipase C. However, the rate of transport was reduced, compared with that of wild-type G protein. A second form of the G protein was generated by deletion of only the transmembrane and cytoplasmic domains. This mutant protein also formed trimers with relatively high efficiency and was secreted slowly from cells.

The vesicular stomatitis virus (VSV) glycoprotein (G) has been an important tool for studying membrane protein biogenesis. It consists of a single polypeptide chain with an N-terminal, extracellular domain of 446 amino acids, a membrane-spanning domain of 20 amino acids, and a 29-amino-acid cytoplasmic domain (26). In VSV particles, this is the single protein species that is responsible for virus entry via membrane fusion (10, 22). Previous studies of the VSV G protein have shown that it is inserted across the membrane of the endoplasmic reticulum (ER) as a nascent chain (27). Although G protein is initially detected in the ER as a monomeric species, it undergoes initial folding steps and then forms noncovalently associated timers prior to transport to the Golgi apparatus and cell surface (4, 17).

Correct folding and oligomeric assembly are required for transport of many proteins from the ER to the cell surface (for a review, see reference 25). VSV G protein provides a clear example of this principle. Several mutated VSV G proteins with alterations in the ectodomain or transmembrane domain do not form trimers and accumulate as aggregates in the ER (4, 5, 17, 19), suggesting that trimer formation may be a prerequisite for G protein transport.

Studies of mutated forms of G protein suggested that the determinants required for trimer formation might reside in the extracellular domain, because deletion or replacement of the cytoplasmic domain or deletion of up to six residues from the transmembrane domain did not disrupt trimer formation (5). Because a quantitative assay for trimer formation was recently developed for G protein (4), we were able to undertake the studies reported here to determine if structural information sufficient for trimer formation resides in the extracellular domain.

For these studies, we generated two new mutant forms of the VSV G protein. The first form has the normal transmembrane and cytoplasmic domains substituted with a C-terminal sequence from the glycoprotein Thy-1.1 which signals glycolipid anchor addition to the hybrid protein. Thy-1.1 is a membrane protein which is attached to the membrane by a glycosyl-inositol phospholipid anchor (18, 29). The glycolipid-anchored G–Thy-1.1 hybrid protein forms trimers and is transported to the plasma membrane. The second G protein mutant is a secreted form which has a complete deletion of the transmembrane and cytoplasmic domains. This protein also forms trimers prior to exit from the ER.

MATERIALS AND METHODS

Construction of plasmids. A cDNA clone encoding Thy-1.1 (pThy1.1) was a gift from Ihor Lemischka (Princeton University) and Ralph Greenspan (Roche Institute). It was generated from a genomic clone (14) which was passaged in a retrovirus vector to remove the introns (I. Lemischka, unpublished data). We determined the complete sequence of the cDNA insert and verified that the introns had been removed correctly. A cDNA clone, encoding VSV G protein but with the new *Ava*I site illustrated in Fig. 1 (pGA1), was generated by oligonucleotide-directed mutagenesis (A. Shaw, K. Amrein, C. Hammond, D. Stern, B. Sefton, and J. Rose, Cell, in press). pBSGThy1.1 was constructed by ligating a *Xho*I-*Ava*I fragment (from pGA1) encoding the entire extracellular domain of VSV G protein to an *Ava*I-*Bgl*II fragment encoding the C-terminal 53 amino acids of the Thy-1.1 precursor. The ligated product was inserted into the polylinker of Bluescript SK$^+$ vector (Stratagene) after the vector had been cleaved with *Xho*I and *Bam*HI. The resulting plasmid was designated pBSGThy1.1. To generate a construct encoding the truncated G protein containing only the extracellular domain, a *Hpa*I linker (GTTAAC), which provides an in-frame translation-termination codon, was ligated at the *Ava*I site after filling in with Klenow fragment of DNA polymerase I (Fig. 1). The starting plasmid for this construction was pGCD4 (Shaw et al., in press), in which sequences from CD4 had been substituted that followed the new *Ava*I site in the Bluescript vector. This construct was generously provided by Carolyn Machamer and was designated pBSGA$^-$.

Expression, radiolabeling, and immunoprecipitation. HeLa cells (5×10^5 cells on 6-cm dishes) were infected with a recombinant vaccinia virus encoding T7 RNA polymerase

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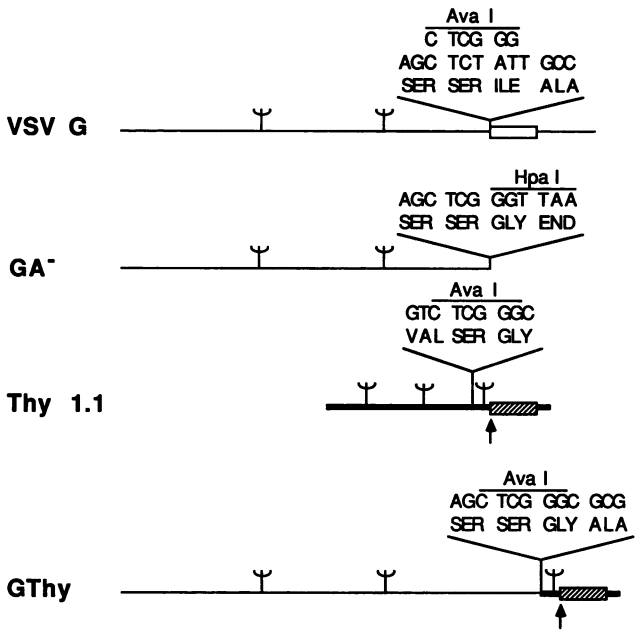

FIG. 1. Diagram illustrating the proteins employed. The top line (VSV G) represents the VSV G protein with glycosylation sites (ψ) and the transmembrane domain beginning with the amino acid sequences Ser-Ser-Ile-Ala (▭). The nucleotide sequence illustrates the three nucleotide changes introduced to generate the *AvaI* site. This change converts the Ile codon to a Gly codon. The G protein lacking the transmembrane and cytoplasmic domains (GA^-) is illustrated to show the nucleotide and protein sequence changes introduced after addition of a *HpaI* linker at the *AvaI* site to generate a stop codon. The Thy-1.1 protein is illustrated with its three glycosylation sites and hydrophobic C-terminal sequence (▨) which is cleaved at the site indicated by the arrow prior to glycolipid attachment. The nucleotide sequence at the naturally occurring *AvaI* site in the Thy-1.1 cDNA is indicated with the protein sequence. The G–Thy-1.1 hybrid is illustrated with the nucleotide sequence at the junction of the two cDNAs.

(vTF7-3 [12]) at a multiplicity of 10 to 25 in 1 ml of Dulbecco modified Eagle medium (DME) without serum for 30 min at 37°C. The inoculum was then removed, and cells were transfected with 5 μg of plasmid DNA in 1 ml of DME lacking serum by a liposome-mediated procedure (L. Buonocore et al., manuscript in preparation) similar to that described by Felgner et al. (8). Transfected cells were incubated for 3 h, washed, and subsequently labeled with [^{35}S]methionine in DME lacking methionine. Alternatively, cells were labeled with 125 μCi of [^{3}H]ethanolamine (Amersham Corp.) in 1 ml of DME containing 10% tryptose phosphate broth, 5% fetal calf serum, nonessential amino acids, and sodium pyruvate (GIBCO Laboratories) for 4.5 h at 37°C.

Prior to immunoprecipitation, cells were washed once in phosphate-buffered saline (10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 150 mM NaCl [pH 7.2]) and lysed with 1 ml of a solution (lysis buffer) containing 1% Nonidet P-40, 0.4% deoxycholate, 66 mM EDTA, and 10 mM Tris hydrochloride, pH 7.4. Nuclei were removed by centrifugation at 10,000 × *g* for 1 min, sodium dodecyl sulfate (SDS) was added at a concentration of 0.25% to the supernatant, and 3 μl of rabbit anti-VSV serum was added. After incubation at 37°C for 15 min, antibody-antigen complexes were precipitated with fixed *Staphylococcus aureus* (Calbiochem-Behring), washed three times, and subjected to SDS gel electrophoresis. Immunoprecipitation from the medium was performed by first removing particulate material by centrifugation at 10,000 × *g* for 1 min. Supernatants were added to an equal volume of 2× lysis buffer, and the total volume was adjusted to 0.25% SDS before addition of anti-VSV serum. Endoglycosidase H treatment of the immunoprecipitates was performed as described previously (24).

PI-PLC treatment of intact cells. Cells expressing either G–Thy-1.1 or G proteins were labeled with [^{35}S]methionine (described above) for 1 h at 37°C and incubated in chase medium containing an excess of unlabeled methionine for 1 h. The labeled cells were then treated at room temperature for 10 to 15 min with phosphate-buffered saline containing 25 mM EDTA to remove the cells from the tissue culture plates. The cells were transferred to 1.5-ml Eppendorf tubes, rinsed in DME, suspended in 100 μl of DME containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4, and treated with 1 μl of *Bacillus thuringiensis* phosphoinositol-specific phospholipase C (PI-PLC) for 1 h at 37°C. PI-PLC (activity with [^{3}H]phosphatidylinositol, 325 μmol/min per ml) was a generous gift from Martin Low (Columbia University). The cells were collected by centrifugation, and both the cells and media were recovered. Cell lysis and immunoprecipitation of lysates and medium with anti-VSV serum were done as described above.

Sucrose gradient sedimentation. The oligomeric state of G protein, G–Thy-1.1, and GA^- was determined by sucrose gradient centrifugation essentially as described previously (4, 5). Briefly, HeLa cells were labeled as described above for 10 min with [^{35}S]methionine at 200 μCi/ml. The cells were then rinsed in DME and subsequently incubated in chase medium containing excess unlabeled methionine for the times indicated. Cells were lysed with 300 μl of 1% Triton X-100 in a solution of 80 mM MES (morpholineethanesulfonic acid), 120 mM Tris, 400 mM NaCl, 4 mM EDTA, 4 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid] (4× MNT buffer), pH 5.7. The lysates were placed on ice and centrifuged for 1 min (10,000 × *g*) to remove nuclei. Portions (200 μl) were loaded onto 5 ml of continuous 5 to 20% (wt/wt) sucrose gradients in 2× MNT buffer with 0.1% Triton X-100. The gradients were centrifuged in an SW 50.1 rotor (Beckman Instruments, Inc.) for 16 h at 47,000 rpm. Sixteen fractions were collected from the bottoms of the tubes in 300-μl portions and immunoprecipitated with anti-VSV serum after the fractions were diluted 1:1 with lysis buffer, and SDS was added to a concentration of 0.25%.

RESULTS

Construction of DNAs encoding glycolipid-anchored and soluble VSV G proteins. To construct a DNA that might encode a VSV G protein with a glycolipid anchor instead of the normal transmembrane anchor, we started with a cDNA clone of G protein containing a unique *AvaI* restriction enzyme site introduced by oligonucleotide-directed mutagenesis at the position indicated in Fig. 1 (Shaw et al., in press). The Thy-1.1 gene contains a natural *AvaI* site which permits fusion of DNA encoding the C-terminal 53 amino acids of Thy-1.1 to DNA encoding G protein which lacks the normal transmembrane and cytoplasmic domains (Fig. 1). We anticipated that this construct would specify a glycolipid-anchored G protein because earlier studies have shown that the signal for glycolipid anchor addition is contained within the C-terminal peptide of glycolipid-anchored proteins and can be transferred to other proteins (2, 30).

We also used the *Ava*I site to generate a G protein lacking only the transmembrane and cytoplasmic domains. This was accomplished by introduction of a *Hpa*I DNA linker (which contains a translation-termination codon) at this site as indicated in Fig. 1. We have previously described other constructs encoding soluble, secreted G proteins with larger deletions of C-terminal amino acids and with extra amino acids encoded by vector sequences (23, 24). However, for the purposes of studying trimer formation, we felt it was best to use a construct encoding the complete extracellular domain. The cDNA constructs expected to encode the G–Thy-1.1 fusion protein and the soluble G protein (GA^-) were placed under control of the T7 promoter in the plasmid Bluescript to allow expression in animal cells. These plasmids were designated pBSGThy1.1 and pBSGA$^-$.

Expression of G–Thy-1.1 and GA$^-$. To obtain expression of G–Thy-1.1 and GA^-, we used the system described by Fuerst et al. (12). The pBSGThy1.1 and pBSGA$^-$ DNAs were transfected into HeLa cells that had first been infected with recombinant vaccinia virus vTF7-3. This virus encodes bacteriophage T7 RNA polymerase which transcribes the cytoplasmic plasmid DNA containing a T7 promoter, leading to high-level expression of RNA and protein.

Cells infected with vTF7-3 were transfected with pGThy1.1, pGA$^-$, or an additional plasmid (pARG) encoding wild-type G protein (31). Infected and transfected cells were labeled with [^{35}S]methionine, and cell lysates were immunoprecipitated with polyclonal anti-VSV serum and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2). The G-related protein encoded by pBSGA$^-$ migrated slightly faster than did the wild-type G protein, consistent with the removal of the 46 amino acids from GA^-. The protein encoded by pBSGThy1.1 was a single species migrating slightly slower than wild-type G protein (Fig. 2). This protein has a glycolipid anchor and was presumably processed like Thy-1.1, being cleaved at the cysteine preceding the hydrophobic sequence (Fig. 1) prior to glycolipid addition (29). Without considering the contribution of the glycolipid anchor, the predicted molecular weight of cleaved G–Thy-1.1 protein would be slightly greater than that of wild-type G protein because of the addition of an extra site of N-linked glycosylation and 22 amino acids from Thy-1.1. Thus, the slower mobility of G–Thy-1.1 relative to G protein was as expected.

G–Thy-1.1 contains a glycolipid anchor. To determine if the signal directing glycolipid anchor addition to Thy-1.1 had been transferred from Thy-1.1 to G–Thy-1.1, we employed two techniques. First, we attempted to specifically label the glycolipid moiety. The glycolipid anchor of Thy-1.1 contains ethanolamine (15) and can be labeled with [^{3}H]ethanolamine (7, 29). HeLa cells expressing either G–Thy-1.1 or G protein were labeled either with [^{3}H]ethanolamine or with [^{35}S]methionine. Immunoprecipitates from the cells were then analyzed by SDS-PAGE. Both proteins were labeled with [^{35}S]methionine, while only G–Thy-1.1 was labeled with [^{3}H]ethanolamine, suggesting that G–Thy-1.1 contained the glycolipid anchor (Fig. 3A).

Our second approach was to ask if the G–Thy-1.1 protein could be cleaved from the cell surface by PI-PLC, since the glycolipid anchor of Thy-1.1 is known to be cleaved by this enzyme (18). This is a common method for identifying glycolipid-anchored proteins (for a review, see reference 9). Our initial studies with indirect immunofluorescence (data not shown) revealed that the G–Thy-1.1 protein was expressed on the cell surface. Therefore, if G–Thy-1.1 were glycolipid anchored, incubation with PI-PLC should release G–Thy-1.1 into the medium.

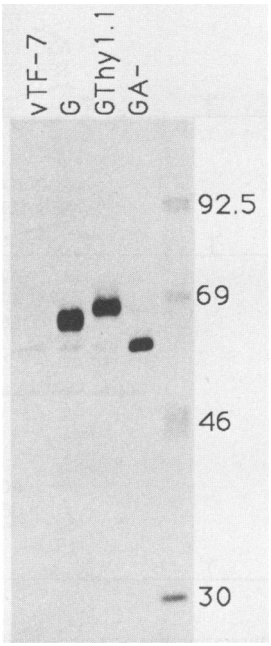

FIG. 2. Gel electrophoresis showing expression of VSV G, G–Thy-1.1, and GA$^-$. HeLa cells infected with a recombinant vaccinia virus, vTF7-3, were transfected with cDNAs encoding VSV G, G–Thy-1.1, or GA$^-$, or were left untransfected (vTF-7). Cells were pulse-labeled for 10 min with 50 μCi of [^{35}S]methionine in 0.5 ml of methionine-free medium and incubated in chase medium containing an excess of unlabeled methionine for 60 min. Cell lysates were immunoprecipitated with rabbit anti-VSV G serum, and immunoprecipitates were analyzed by SDS-PAGE. Molecular weight markers are shown on the right.

HeLa cells expressing G–Thy-1.1 or wild-type G protein were labeled for 1 h in media containing [^{35}S]methionine and then were incubated for 1 h in chase medium containing excess unlabeled methionine. The cells were removed from the tissue culture dish after treatment with EDTA and then were treated with PI-PLC. Released and cell-associated proteins were then immunoprecipitated separately and analyzed by SDS-PAGE. The results showed that the G–Thy-1.1 protein was released from the cell surface after PI-PLC treatment while the wild-type G protein was not (Fig. 3B). Also, there was a clear decrease in the amount of cell-associated G–Thy-1.1 after cleavage while the amount of cell-associated G protein was unaffected. Quantitation of this and other experiments suggested that only about one-third of the total G–Thy-1.1 was released; however, this probably represents a large fraction of the cell surface protein (see Discussion).

Rate of transport of G–Thy-1.1 and GA$^-$. The fact that G–Thy-1.1 could be cleaved from the cell surface with PI-PLC indicated that the protein was being transported through the exocytic pathway. Other mutant G proteins with truncated cytoplasmic tails are transported slowly relative to wild-type G protein, suggesting that the normal cytoplasmic tail might allow rapid transport from the ER to the Golgi apparatus (24). Because G–Thy-1.1 lacks the normal transmembrane and cytoplasmic domains, it was of interest to examine its rate and efficiency of transport. To do this, we determined the rate at which the N-linked oligosaccharides

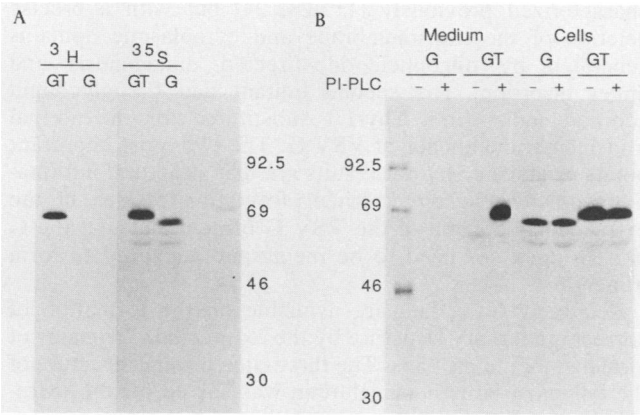

FIG. 3. Electrophoretic analysis of specific labeling and cleavage of the glycolipid anchor of G–Thy-1.1. (A) HeLa cells (5×10^5) expressing VSV G (G) or G–Thy-1.1 (GT) were labeled for 4.5 h with 125 μCi of [^{3}H]ethanolamine or 50 μCi of [^{35}S]methionine in 0.5 ml of the appropriate labeling medium (as described in Materials and Methods). Cell lysates were immunoprecipitated with rabbit anti-VSV G serum. Positions of molecular weight markers are indicated. (B) HeLa cells (5×10^5) expressing VSV G (G) or G–Thy-1.1 (GT) were labeled for 1 h with 50 μCi of [^{35}S]methionine in 0.5 ml of methionine-free medium and then were incubated in chase medium containing unlabeled methionine for 1 h. Cells were then removed from the tissue culture plate and divided into two equal portions for treatment with PI-PLC (+) or mock treatment (−). The cells and medium were immunoprecipitated separately. All of the immunoprecipitates from the medium and one-fourth of the immunoprecipitates from the cells were analyzed by SDS-PAGE. Positions of molecular weight markers are indicated.

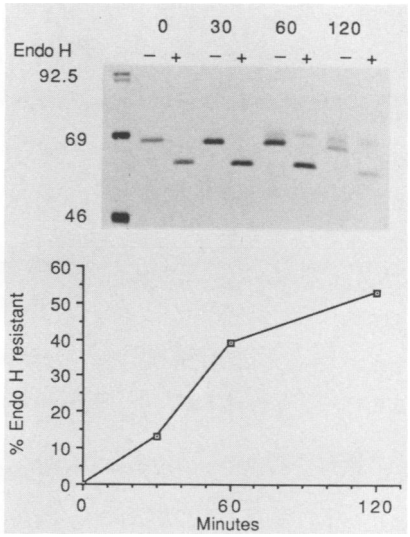

FIG. 4. Acquisition of endoglycosidase-H (endo H)-resistant oligosaccharides on the G–Thy-1.1 protein. HeLa cells (5×10^5) were labeled for 15 min with 50 μCi of [^{35}S]methionine in 0.5 ml of methionine-free medium and then were incubated in chase medium containing unlabeled methionine for 0, 30, 60, or 120 min. Immunoprecipitates from each time point were incubated in the presence (+) or absence (−) of endoglycosidase H (24) and analyzed by SDS-PAGE (gel shown at top). The amount of endoglycosidase-H-resistant G–Thy-1.1 at each time point was quantified by scanning densitometry of a fluorographed gel (graph shown at bottom).

on G–Thy-1.1 became resistant to treatment with endoglycosidase H (28). High-mannose core oligosaccharides added in the ER are susceptible to cleavage by endoglycosidase H and become resistant to cleavage only after processing, probably in the medial Golgi compartment (6, 16).

Cells transfected with pBSGThy1.1 were pulse-labeled with [^{35}S]methionine and then incubated for the indicated times in chase medium containing excess unlabeled methionine. Immunoprecipitates from cell lysates were divided and digested or mock-digested with endoglycosidase H (Fig. 4). The results showed that G–Thy-1.1 acquired endoglycosidase H resistance much more slowly than did wild-type G protein. Quantitation of the gels showed only about 50% resistance at 2 h. In contrast, wild-type G protein acquires 50% endoglycosidase H resistance after about 15 min (24). This slow transport rate is similar to that of G protein mutants with deletions in the normal cytoplasmic tail (21, 24, 31).

We have previously observed that truncated, soluble forms of G protein lacking the transmembrane and cytoplasmic domains are secreted with a half time of about 2 h when expressed in transfected COS cells (24) or in mouse C127 cells (10). A similar value was obtained by Moore and Kelly (20) in AtT20 cells. Although the GA$^-$ construct employed here was slightly different from those described earlier, we found that this protein was also secreted slowly, with a half time of 2 to 3 h (data not shown).

Trimerization of G–Thy-1.1 and GA$^-$. The assay developed by Doms et al. (4) to follow trimerization involves lysis of cells at a mildly acidic pH (pH 5.8) and separation of trimeric and monomeric forms of G protein on a 5 to 20% sucrose velocity gradient. Although the VSV G protein is a trimer at neutral pH, the trimeric form is stabilized at pHs below 6.0 and can then be analyzed by velocity gradient sedimentation. With this assay, the half time of trimer formation by wild-type G protein has been determined in a variety of cell types and is in the range of 7 to 10 min (4, 5).

We initially found that both G–Thy-1.1 and GA$^-$ proteins showed a high efficiency of trimer formation when analyzed on sucrose velocity gradients at pH 5.8. To determine the kinetics of trimer formation, we performed the experiment shown in Fig. 5. Transfected HeLa cells expressing each protein were pulse-labeled with [^{35}S]methionine and then were lysed immediately or incubated for the indicated times. Detergent lysates of cells were fractionated on sucrose gradients, and gradient fractions were immunoprecipitated with anti-VSV serum and analyzed by SDS-PAGE. The relevant portions of the gels are shown for wild-type and mutated G proteins in Fig. 5. The percentage of protein in the trimer peak was quantitated for each protein and is shown in the graph (Fig. 5). All of the G proteins formed trimers, although the G–Thy-1.1 and GA$^-$ proteins appeared to be somewhat slower and less efficient at trimer formation than the wild-type protein. The estimated half times for wild type, G–Thy-1.1, and GA$^-$ are 10, 30, and 15 min, respectively.

We also analyzed the GA$^-$ protein secreted into the medium to determine if it remained trimeric. All of the secreted GA$^-$ protein appeared to be monomeric when analyzed on sucrose gradients at pH 5.8 (data not shown). A possible explanation for this result is that the soluble form dissociates in dilute solution. The gradients shown for the GA$^-$ protein were not run in parallel with those for G and G–Thy-1.1 and are therefore not directly comparable for peak positions. In other experiments where the GA$^-$ gradients were run with wild-type G markers for monomers and trimers, we found that the presumed monomer and trimer peaks of GA$^-$ displaced one fraction above the G peaks.

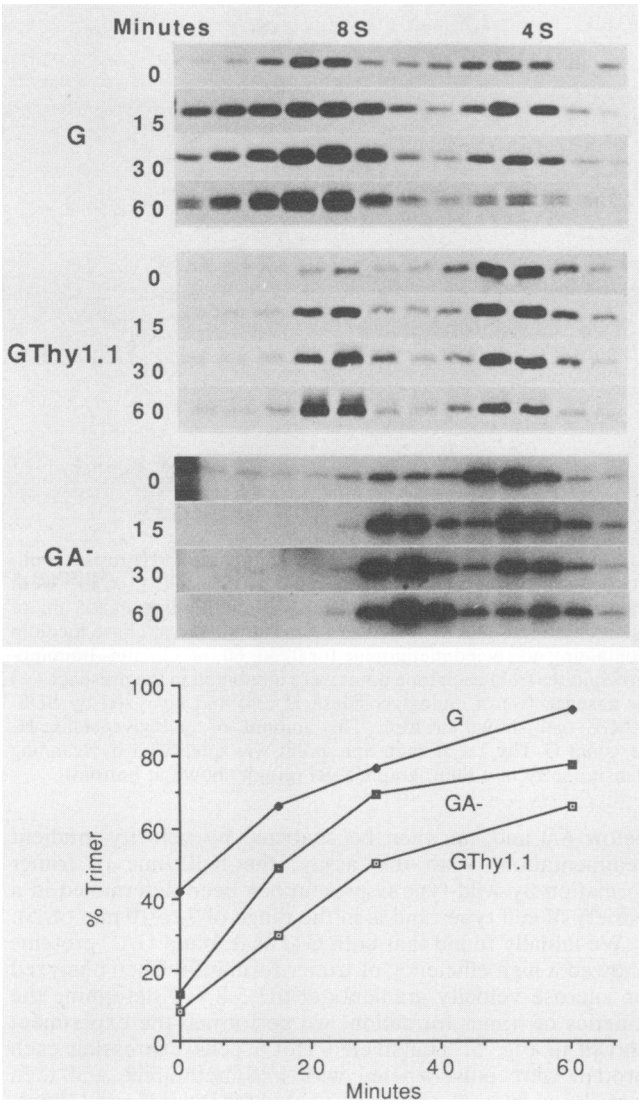

FIG. 5. Kinetics of trimer formation of VSV G, G–Thy-1.1, and GA$^-$ proteins. HeLa cells (5×10^5) expressing each indicated protein were pulse-labeled for 10 min with 100 μCi of [^{35}S]methionine in 0.5 ml of methionine-free medium and were incubated in chase medium containing unlabeled methionine for various times before lysis. Lysates were applied to 5 to 20% sucrose gradients and centrifuged as described in Materials and Methods. Gradient fractions were immunoprecipitated with rabbit anti-VSV serum and analyzed by SDS-PAGE followed by fluorography. The area from each fluorograph showing the precipitated G protein or G-related protein is shown (top). The bottom of the gradient is on the left, and the approximate 8S and 4S positions are shown. The percentage of total G protein in the trimer peak at each time point was determined by scanning densitometry of fluorographed gels and plotted in the graph shown at the bottom.

This is as expected from the deletion of approximately 10% of the protein.

DISCUSSION

In this study, we generated two mutant forms of the VSV G protein lacking the transmembrane and cytoplasmic domains. One was a soluble form similar to soluble forms characterized previously (11, 23, 24) but with a precise deletion of the transmembrane and cytoplasmic domains generated by oligonucleotide-directed mutagenesis and linker insertion. The second mutant had the glycolipid addition signal from Thy-1.1 substituted for the normal transmembrane anchor of VSV G. The two most important points established by our results are that structural information sufficient to direct trimer formation resides in the extracellular domain of the VSV G protein and that the G protein does not need to be membrane anchored to form trimers.

Relatively little data are available on the formation of correct quaternary structure by the extracellular domains of viral membrane proteins. The three-dimensional structure of the influenza virus hemagglutinin was solved for the extracellular domain, which was crystallized after cleavage from the virus surface (33). This cleaved form retains the trimeric structure. It has been reported also that at least a fraction of a soluble, anchor$^-$ form of influenza virus hemagglutinin synthesized in cells can be cross-linked to a trimeric form (13). Thus, the influenza virus hemagglutinin appears similar to the VSV G protein in not requiring membrane anchoring to form trimers.

The half times for trimer formation by glycolipid-anchored and soluble forms of the VSV G protein are faster than those for transport from the ER. These results would be consistent with a requirement for trimer formation prior to transport of these proteins from the ER. However, these results, like earlier results obtained with G proteins having deletions in the cytoplasmic domain (5), suggest that trimer formation is not sufficient for rapid transport. If trimer formation were sufficient to direct rapid transport out of the ER, then we would expect that the rate of acquisition of endoglycosidase-H-resistant oligosaccharides would be only slightly slower than the rate of trimer formation. This is, in fact, the case for wild-type G protein, where the half time for trimer formation is 7 to 10 min and the half time for the acquisition of endoglycosidase-H-resistant oligosaccharides is 15 min (4, 5, 24). In contrast, the half time for trimer formation by G–Thy-1.1 is about 30 min, while 50% endoglycosidase H resistance is acquired between 1 and 2 h. Similarly, the GA$^-$ protein forms trimers with a half time of about 15 min, but acquires endoglycosidase H resistance and is secreted with a half time of 2 to 3 h. We have also examined trimer formation by one of the more severely truncated forms of VSV G protein (TG [23]), lacking 30 C-terminal amino acids from the extracellular domain. Analysis by sucrose gradient sedimentation showed that this protein also formed trimers with at least 50% efficiency (data not shown).

A possible explanation for the lag in transport after trimer formation is that the truncated proteins lack signals for transport that are present in the wild-type protein. These proteins might then move at a slow rate of bulk flow with newly synthesized membrane. If this were the case, then bulk flow would have to be considerably slower than was suggested by Wieland et al. (32). Alternatively, one could argue that movement of these proteins is retarded for some other reason.

Our studies also localize the signal for glycolipid addition to the C-terminal 53 amino acids of Thy-1.1, since these residues specify glycolipid addition to VSV G protein. Related studies have shown that the signal for glycolipid addition to the decay-accelerating factor is contained within the C-terminal 37 amino acids (2). In our construct, assuming that cleavage occurs at the normal cysteine residue in

Thy-1.1, we would have an extra 22 amino acids from the C terminus of Thy-1.1 appended to G protein.

Polypeptide cleavage and glycolipid anchor addition are known to take place during or immediately after synthesis of the protein (1, 3). Because we detected only a single species of the G–Thy-1.1 protein after a 10-min pulse label, it is likely that this hybrid protein is also processed and modified rapidly. The fact that we were only able to cleave about 35% of the total G–Thy-1.1 protein from the cell surface in the experiment shown in Fig. 3 is probably due to the slow (and perhaps inefficient) transport of G–Thy-1.1 as well as its accessibility to PI-PLC cleavage. Thy-1 itself is only cleaved with about 50% efficiency (18).

We anticipate that this or other glycolipid-linked forms of the VSV G protein will be useful tools for future studies on sorting and on physical properties of glycolipid-linked proteins. They may also be useful for large-scale preparation of the extracellular domain itself.

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LITERATURE CITED

1. **Bangs, J. D., D. Hereld, J. L. Krakow, G. W. Hart, and P. T. Englund.** 1985. Rapid processing of the carboxyl terminus of a trypanosome variant surface glycoprotein. Proc. Natl. Acad. Sci. USA **82**:3207–3211.
2. **Caras, I. W., G. N. Weddell, M. A. Davitz, V. Nussenzweig, and D. W. Martin.** 1987. Signal for attachment of a phospholipid membrane anchor in decay accelerating factor. Science **238**: 1280–1283.
3. **Conzelmann, A., A. Spiazzi, and C. Bron.** 1987. Glycolipid anchors are attached to Thy-1 glycoprotein rapidly after translation. Biochem. J. **246**:605–610.
4. **Doms, R. W., D. S. Keller, A. Helenius, and W. E. Balch.** 1987. Role for adenosine triphosphate in regulating the assembly and transport of vesicular stomatitis virus G protein trimers. J. Cell Biol. **105**:1957–1969.
5. **Doms, R. W., A. Ruusala, C. Machamer, J. Helenius, A. Helenius, and J. K. Rose.** 1988. Differential effects of mutations in three domains on folding, quaternary structure, and intracellular transport of vesicular stomatitis virus G protein. J. Cell Biol. **107**:89–99.
6. **Dunphy, W. G., and J. E. Rothman.** 1985. Compartmental organization of the Golgi stack. Cell **42**:13–21.
7. **Fatemi, S. H., R. Haas, N. Jentoft, T. L. Rosenberry, and A. M. Tartakoff.** 1987. The glycophospholipid anchor of Thy-1. J. Biol. Chem. **262**:4728–4732.
8. **Felgner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and G. A. Danielsa.** 1987. Lipofection: highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. USA **84**:7413–7417.
9. **Ferguson, M. A. J., and A. F. Williams.** 1988. Cell-surface anchoring of proteins via glycosyl-phosphatidylinositol structures. Annu. Rev. Biochem. **57**:285–320.
10. **Florkiewicz, R., and J. Rose.** 1984. A cell line expressing the vesicular stomatitis virus glycoprotein fuses at low pH. Science **225**:721–723.
11. **Florkiewicz, R. Z., A. Smith, J. E. Bergmann, and J. K. Rose.** 1983. Isolation of stable mouse cell lines that express cell surface and secreted forms of the vesicular stomatitis virus glycoprotein. J. Cell Biol. **97**:1381–1388.
12. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesized bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83**:8122–8126.
13. **Gething, M. J., and J. Sambrook.** 1982. Construction of influenza haemagglutinin genes that code for intracellular and secreted forms of the protein. Nature (London) **300**:598–603.
14. **Giguere, V., K. I. Isboe, and F. Groveld.** 1985. Structure of the murine Thy-1 gene. EMBO J. **4**:2017–2024.
15. **Homans, S. W., M. A. J. Ferguson, R. A. Dwek, T. W. Rademacher, R. Anand, and A. F. Williams.** 1988. Complete structure of the glycosyl phosphatidylinositol membrane anchor of rat brain Thy-1 glycoprotein. Nature (London) **333**:269–272.
16. **Kornfeld, R., and S. Kornfeld.** 1985. Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem. **54**:631–664.
17. **Kreis, T. E., and H. F. Lodish.** 1986. Oligomerization is essential for transport of vesicular stomatitis viral glycoprotein to the cell surface. Cell **46**:929–937.
18. **Low, M. G., and P. W. Kincade.** 1985. Phosphatidylinositol is the membrane-anchoring domain of the Thy-1 glycoprotein. Nature (London) **318**:62–64.
19. **Machamer, C. A., and J. K. Rose.** 1988. Vesicular stomatitis virus G proteins with altered glycosylation sites display temperature-sensitive intracellular transport and are subject to aberrant intermolecular disulfide bonding. J. Biol. Chem. **263**:5955–5960.
20. **Moore, H. P., and R. B. Kelly.** 1985. Secretory protein targeting in a pituitary cell line: differential transport of foreign secretory proteins to distinct secretory pathways. J. Cell Biol. **101**: 1773–1781.
21. **Puddington, L., C. E. Machamer, and J. K. Rose.** 1984. Cytoplasmic domains of cellular and viral integral membrane proteins substitute for the cytoplasmic domain of the vesicular stomatitis virus glycoprotein in transport to the plasma membrane. J. Cell Biol. **102**:2147–2157.
22. **Riedel, H., C. Kondor-Koch, and H. Garoff.** 1984. Cell surface expression of fusogenic vesicular stomatitis virus glycoprotein from cloned cDNA. EMBO J. **3**:1477–1483.
23. **Rose, J. K., and J. E. Bergmann.** 1982. Expression from cloned cDNA of cell-surface and secreted forms of the glycoprotein of vesicular stomatitis virus in eukaryotic cells. Cell **30**:753–762.
24. **Rose, J. K., and J. E. Bergmann.** 1983. Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis virus glycoprotein. Cell **34**:513–524.
25. **Rose, J. K., and R. W. Doms.** 1988. Regulation of protein export from the endoplasmic reticulum. Annu. Rev. Cell Biol. **4**: 257–288.
26. **Rose, J. K., and C. J. Gallione.** 1981. Nucleotide sequences of the mRNAs encoding the vesicular stomatitis virus G and M proteins determined from cDNA clones containing the complete coding regions. J. Virol. **39**:519–528.
27. **Rothman, J. E., and H. F. Lodish.** 1977. Synchronized transmembrane insertion and glycosylation of a nascent membrane protein. Nature (London) **269**:775–780.
28. **Tarentino, A. L., and F. Maley.** 1974. Purification and properties of an endo-B-acetylglucosaminidase from Streptomyces griseus. J. Biol. Chem. **249**:811–817.
29. **Tse, A. G., A. N. Barclay, A. Watts, and A. F. Williams.** 1985. A glycophospholipid tail at the carboxyl terminus of the Thy-1 glycoprotein of neurons and thymocytes. Science **230**:1003–1008.
30. **Tykocinski, M. L., H. K. Shu, D. J. Ayers, E. I. Walter, R. T. Getty, R. K. Groger, A. Hauer, and M. E. Medof.** 1988. Glycolipid reanchoring of T-lymphocyte surface CD8 using the 3′ end sequence of decay-accelerating factor's mRNA. Proc. Natl. Acad. Sci. USA **85**:3555–3559.
31. **Whitt, M. A., L. Chong, and J. K. Rose.** 1989. Glycoprotein cytoplasmic domain sequences required for rescue of a vesicular stomatitis virus glycoprotein mutant. J. Virol. **63**:3569–3578.
32. **Wieland, F. T., M. L. Gleason, T. A. Serafini, and J. E. Rothman.** 1987. The rate of bulk flow from the endoplasmic reticulum to the cell surface. Cell **50**:298–300.
33. **Wilson, I., J. Skehel, and D. Wiley.** 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3Å resolution. Nature (London) **292**:366–373.