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Dissociation and Reassociation of Oligomeric Viral Glycoprotein Subunits in the Endoplasmic Reticulum

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The vesicular stomatitis virus (VSV) glycoprotein (G) forms noncovalently linked trimers in the endoplasmic reticulum (ER) prior to transport to the cell surface. Here we examined the formation of heterotrimers between wild-type and mutant subunits that were retained in the ER by C-terminal retention signals. When G protein was coexpressed with mutant subunits that formed trimers at the wild-type rate and were transported from the ER at the wild-type rate, heterotrimers were readily detected. In contrast, when G protein was coexpressed with mutant subunits that formed trimers at the wild-type rate, but were retained in the ER, heterotrimers were not detected unless transport of the wild-type molecules from the ER was blocked. After removal of transport block, the heterotrimers then dissociated and reassorted to homotrimers of the mutant protein that were retained in the ER and wild-type trimers that were transported to the cell surface. These and other results presented here indicate that there is an equilibrium between G protein trimers and monomers in vivo, at least in the ER. This equilibrium may function to allow escape of wild-type subunits from aberrant retained subunits.

The intracellular transport of secretory and cell surface proteins begins with translocation of the polypeptide chains into the lumen of the endoplasmic reticulum (ER). Folding of the polypeptide chain can begin immediately after translocation and often requires a substantial amount of time after synthesis to be completed. In many cases the folding of monomeric subunits is followed by oligomerization, and oligomerization is often a prerequisite for further transport (for reviews, see references 20, 21, and 39). Protein transport to the cell surface continues in vesicles budding from the ER which fuse with the *cis*-Golgi cisternae. The protein transport continues through the Golgi cisternae, and eventually vesicles that budded from the *trans*-Golgi network reach the plasma membrane and fuse with it (36).

Viral membrane proteins have been used for many years as probes for the study of various steps along the exocytic pathway. The vesicular stomatitis virus (VSV) glycoprotein (G) has been one of the major model proteins for such studies, and much has been learned about its folding and transport properties. The VSV G protein is synthesized as a precursor of 511 amino acids, and its N-terminal signal sequence (16 amino acids) is cleaved during insertion into the ER (25, 41). Following insertion, the N terminus and the majority of the extracellular domain containing two N-linked oligosaccharides are in the lumen of the ER. The protein is anchored by a typical membrane span of 20 hydrophobic amino acids, and a highly charged C-terminal cytoplasmic domain of 29 amino acids remains on the cytoplasmic side (40, 41).

In the ER, newly synthesized, incompletely disulfide-bonded G protein is associated with a protein called GRP78 or BiP (29) which is thought to assist in folding of G protein as well as many other membrane proteins (35). Once the correct disulfide bonds are formed in G protein, BiP is released ($t_{1/2}$ = 4 min). Following release of BiP, the monomeric subunits of G protein assemble to form noncovalently linked trimers (24) with a half time of about 7 to 10 min (8, 10). G protein trimers are then transported to the Golgi apparatus, a process that requires 15 to 20 min following synthesis (1, 38). An additional 10 to 15 min are required before the protein reaches the cell surface (22).

There are two major classes of proteins that are known to be retained in the ER. First there are proteins that contain specific signals for retention (reviewed in reference 35) that are recognized by receptors (44, 49). Second, many misfolded proteins are known to be retained in the ER. In some but not all cases, these proteins are associated with BiP (5, 7, 15, 29; reviewed in reference 20).

Mutations in the extracellular and transmembrane domains of the VSV G protein often cause misfolding and retention of aggregated G protein complexes in the ER. In the case of extracellular domain mutants, these complexes contain BiP protein also (7, 29). Deletions within the cytoplasmic domain of the VSV G protein generally slow down export of the protein from the ER, yet the extracellular domains of these proteins appear to fold correctly, be released from BiP, and oligomerize at the wild-type rate (10, 29). The slow transport of these mutants has suggested a role for the cytoplasmic domain in promoting G protein export from the ER (38). In addition, one mutant G protein bearing a new cytoplasmic domain sequence of 12 amino acids (designated 1473) is completely blocked in transport from the ER (38), yet its extracellular domain appears to fold, be released from BiP, and trimerize with wild-type kinetics (10, 29). Because this cytoplasmic domain sequence can be transferred to other proteins and cause their retention in the ER (19), we suggested that it contains a specific retention signal for the ER. A related cytoplasmic domain sequence from the adenovirus E19 protein also causes protein retention in the ER (32, 33).

The purpose of the studies reported here was to determine first whether the monomeric subunits of VSV G proteins synthesized on separate polysomes are free to diffuse in the ER and assemble from a common pool to form heterotrimers with G proteins synthesized on other polysomes. This has been shown to occur with glycoproteins from influenza virus

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variants (3) or closely related subtypes (45). The second goal was to determine whether heterotrimers would form between wild-type G protein subunits and G protein mutants that are able to trimerize but are blocked in transport by retention signals. If the signals interfering with transport were dominant in heterotrimers, then this might provide a strategy for interfering specifically with viral glycoprotein transport. Our studies suggest that these signals may be dominant in heterotrimers but also reveal a novel property of G protein trimers. G protein trimers are in equilibrium with G protein monomers and can therefore reassort to form retained mutant homotrimers and transported wild-type homotrimers. Although an equilibrium between a membrane protein and a soluble subunit (major histocompatibility complex class I and β_2-microglobulin) has been described (20a), we are not aware of viral membrane proteins that have been shown to exist in equilibrium with monomeric subunits in vivo.

MATERIALS AND METHODS

Reagents. Brefeldin A (BFA) was a gift from Jennifer Lippincott-Schwartz. Monensin and morpholineethanosulfonic acid (MES) were purchased from Sigma; endoglycosidase H (endo H) was from Boehringer Mannheim. Antipeptide sera recognizing the cytoplasmic domains of G and GE1 proteins have been described previously (17, 30).

Plasmid construction mutagenesis. Plasmids encoding VSV G and GE1 proteins (pARG, pARGE1) under control of the T7 promoter were as described previously (51) and employed the pAR2529 vector (13, 42). pAR1473 was generated by excising the sequence encoding the 1473 G protein from pSVG1473 DNA (38) with *Bam*HI and *Hpa*I. The DNA was made blunt ended by filling it in with the Klenow fragment of DNA polymerase I, and *Bam*HI linkers were added to both ends. The fragment was then cloned into the *Bam*HI site of pAR2529. pG_s-KDEL was generated by site-directed mutagenesis (54). DNA encoding G protein was cloned into the *Xho*I site of M13mp8X (18), and single-stranded DNA was prepared (18). Oligonucleotide-directed mutagenesis with 5'AGTTGGAAAAGCGAAAAGGATGAGCTCTAATTT ATCATAGGGTT3' was then used to replace the sequence encoding the first seven amino acids of the transmembrane domain of G protein (SSIASFF) with DNA encoding SEKDEL followed by a stop codon. For expression in the vaccinia virus T7 system, the mutated gene was cloned into the *Xho*I site of pAR2529X (51). The sequence changes were verified by using the dideoxy sequencing method (43).

Expression, radiolabeling, and coprecipitation. Protein expression was carried out in BHK-21 cells which were about 75% confluent on 35-mm-diameter dishes. Cells were infected with recombinant vaccinia virus encoding the T7 RNA polymerase (vTF7-3) (13) at a multiplicity of 10 to 20 in 400 μl of Dulbecco's modified Eagle medium (DME) for 30 min at 37°C. The inoculum was removed, and the cells were transfected with plasmid DNA(s) in 1 ml of DME, using the lipofection technique (12) but employing dimethyldioctadecyl ammonium bromide (Sigma) as the cationic lipid (37a). The amounts of the two DNAs added were adjusted to give equal expression of the encoded proteins. The cells were incubated for 3.5 h at 37°C, and then the medium was replaced by DME containing 5% fetal calf serum. After incubation for another hour at 37°C, the cells were labeled with the indicated amounts of [^{35}S]methionine (Trans ^{35}S-label; ICN Biomedicals Inc., Irvine, Calif.) in 400 μl of methionine-free DME.

In experiments employing chase periods the cultures were rinsed with phosphate-buffered saline (10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 150 mM NaCl [pH 7.2]) and the incubation was continued for various times in DME containing 2.5 mM unlabeled methionine and 5% fetal calf serum. At the end of the chase period, the medium was removed and the dishes were rinsed with phosphate-buffered saline. Lysis of the cells was done on ice, using 1% Triton X-100 in a buffer consisting of 20 mM MES, 30 mM Tris, 100 mM NaCl, 1 mM EDTA disodium salt, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid (EGTA) (1× MNT buffer), pH 5.8. Nuclei were removed by centrifugation at 10,000 × *g* for 1 min, and the supernatant was precleared for 6 h at 0°C by incubation with 4 μl of antiserum recognizing an unrelated protein and then additional incubation at 0°C with 30 μl of fixed *Staphylococcus aureus* for the same time. After removal of the precipitate, the supernatant was divided in two or three parts, the volume was brought to 1 ml with lysis solution, and sodium dodecyl sulfate (SDS) was added to a final concentration of 0.1%. Immunoprecipitation with the various antibodies (1 μl of anti-VSV serum, 4 μl of antipeptide sera) was at 0°C for 6 h, followed by incubation for 6 h with 30 μl of prewashed *S. aureus*. Immunoprecipitates were washed three times with cold lysis solution containing 0.1% SDS, analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 10% acrylamide gels, and visualized by fluorography (2). Endo H treatment of immunoprecipitates was done as described previously (38).

Analysis of trimer formation. Trimer formation was analyzed by sedimentation on sucrose density gradients (10). Cells grown on 35-mm-diameter dishes were labeled with 25 μCi of [^{35}S]methionine in methionine-free medium for 5 min. The labeling medium was removed, and the cells were incubated in DME containing 2.5 mM unlabeled methionine and 5% fetal calf serum for various times. Cells were lysed in 300 μl 2× MNT (pH 5.8), the nuclei were removed by centrifugation, and 200 μl of the supernatant was loaded onto 4.8 ml of a 5 to 20% (wt/wt) sucrose gradient in 1× MNT buffer (pH 5.8) containing 0.1% Triton X-100. The gradients were centrifuged in an SW50.1 rotor (Beckman Instruments, Inc.) for 16 h at 16,000 rpm at 4°C. Sixteen fractions were collected from the bottom of the tube, and the volume of each was brought to 1 ml with a solution containing 1% Nonidet P-40, 0.4% sodium deoxycholate, 0.2% SDS, 66 mM EDTA, and 10 mM Tris (pH 7.4). Immunoprecipitations prepared with anti-VSV serum were washed once with a solution of 10 mM Tris, 0.1% SDS, 1% sodium deoxycholate, 1% Nonidet P-40, 0.15 M NaCl (pH 7.4) (RIPA buffer) and then analyzed by SDS-PAGE. For coprecipitation of heterotrimers after sucrose gradient analysis, the volume of the collected fractions was brought to 1 ml with 1× MNT (pH 5.8) containing 1% Triton X-100 and the solution was cleared as described previously. Then SDS was added to a final concentration of 0.1%, and immunoprecipitation was done at 0°C, using antiserum to the cytoplasmic domain of G protein (17). The immunoprecipitates were washed twice with 1× MNT (pH 5.8) containing 1% Triton X-100 and 0.1% SDS.

RESULTS

We have previously described mutated VSV G proteins with altered cytoplasmic domains that can either block, retard, or have little effect on the transport of G protein from the ER to the Golgi apparatus (for references, see reference 10). The subset of the mutants employed in the studies

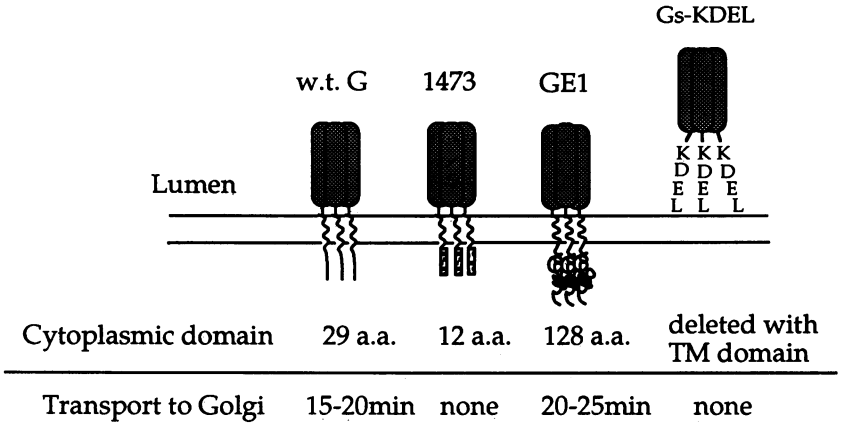

FIG. 1. Wild-type and mutant VSV glycoproteins. The two mutant proteins (1473 and GE1) with new cytoplasmic domains (37, 38) are illustrated along with the soluble protein G_s-KDEL containing the retention signal for the ER (SEKDEL; 31). The half time of transport of wild-type and GE1 proteins from the ER to Golgi was estimated from the time required for each protein to obtain endo H-resistant oligosaccharides. The 1473 and G_s-KDEL proteins are retained in the ER and do not obtain endo H-resistant oligosaccharides.

described here and one additional mutant containing the KDEL retention signal (31) are diagrammed in Fig. 1. The half time for transport of each mutant from the ER to the Golgi is given also. All of these proteins form trimers in the ER at the same rate as wild-type G protein and appear to have correctly folded extracellular domains.

Coprecipitation assay for heterotrimers. To determine whether heterotrimers would form between VSV G proteins expressed from two different plasmid DNAs in the same cell, we expressed the wild-type VSV G protein, which has a 29-amino-acid cytoplasmic domain, and a mutant designated GE1, with a much longer 128-amino-acid cytoplasmic domain derived from the cytoplasmic domain of the infectious bronchitis virus E1 glycoprotein (Fig. 1). The GE1 mutant has also been called G23 (37). These proteins were chosen because we have specific antipeptide sera recognizing the cytoplasmic domains of each (17, 30) and because G and GE1 have very different mobilities on SDS-polyacrylamide gels. The GE1 mutant is also transported at a rate similar to that of G protein.

To obtain a high level of coexpression of both proteins we used the vaccinia virus-T7 hybrid system of Fuerst et al. (13) and transfected separate plasmids encoding G and GE1 under T7 promoter control into BHK cells that had been infected with vTF7-3, a vaccinia virus recombinant encoding the bacteriophage T7 RNA polymerase. Cells were labeled with [^{35}S]methionine and then lysed in a detergent solution at pH 5.8, a pH that is known to stabilize the G trimer (8). Lysates from cells expressing G and GE1 separately or lysates of cells expressing both proteins simultaneously were precipitated with the specific antipeptide sera recognizing the cytoplasmic domains of G or GE1 proteins (designated G_T and E_T) or with a polyclonal antiserum (G) recognizing the extracellular domains of both proteins. As shown in Fig. 2, the antipeptide sera were completely specific for the G and GE1 proteins when the proteins were expressed alone (lanes 1, 2, 4, and 5), while the serum recognizing the extracellular domain precipitated both proteins (lanes 3 and 6). In contrast, when the G and GE1 proteins were coexpressed, antibody to the cytoplasmic domain of G or GE1 precipitated a significant fraction of the other protein (Fig. 2, lanes 7 and 8), indicating that a complex of G and GE1 (presumably heterotrimers) had formed. That the coprecipitating proteins had the sedimentation coefficient expected for heterotrimers was shown by sucrose velocity gradient sedimentation followed by coprecipitation (data not shown). To determine whether the complexes might form in vitro after lysis of the cells, we transfected cells separately with plasmids encoding G and GE1, mixed the cells, and lysed them together. No coprecipitation of G and GE1 was observed by using antibodies recognizing the cytoplasmic domain of either of the two proteins (data not shown). If the coexpression of G and GE1 were equal in all cells, then one would predict that 75% of all subunits should be in heterotrimers if mixing were completely random (3). We observed levels of heterotrimers that were clearly lower than 75%, but this could be due to unequal coexpression in many cells or dissociation during immunoprecipitation rather than to nonrandom mixing.

Wild-type G protein does not form heterotrimers with the nontransported mutant 1473. We next examined the efficiency of heterotrimer formation between wild-type G protein and a mutant designated 1473 which is retained in the

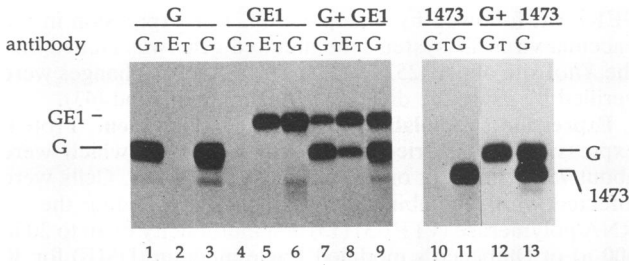

FIG. 2. Detection of heterotrimers by coprecipitation. Cells infected with vTF7-3 were transfected with pARG, pARGE1, and pAR1473 (encoding G, GE1, or 1473 proteins) or cotransfected with pARG and pARGE1 or pARG and pAR1473. Cells were labeled with [^{35}S]methionine for 30 min and then incubated in chase medium containing excess unlabeled methionine for 1 h. Lysis and immunoprecipitation of proteins from lysates is described in Materials and Methods. Immunoprecipitations were with antipeptide sera recognizing the cytoplasmic domains of G (G_T, lanes 1, 4, 7, 10, and 12) and GE1 (E_T, lanes 2, 5, and 8) or with polyclonal serum recognizing the extracellular domain of G protein (G, lanes 3, 6, 9, 11, and 13). Immunoprecipitates were analyzed by SDS-PAGE followed by fluorography.

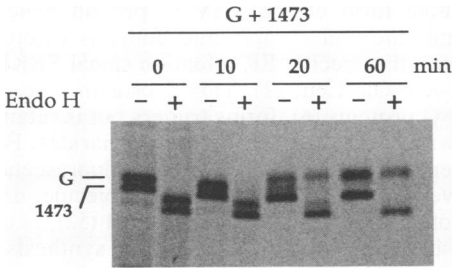

FIG. 3. Independent processing of G and 1473 proteins. Cells infected with vTF7-3 were cotransfected with pARG and pAR1473, labeled for 10 min with [^{35}S]methionine, and chased for the indicated times in excess cold methionine. The cells were lysed and immunoprecipitated by using polyclonal serum recognizing the extracellular domain of G. The immunoprecipitates were incubated with 3 mU of endo H as described in Materials and Methods and analyzed by SDS-PAGE and fluorography.

ER but is known to form trimers at the wild-type rate (Fig. 6A) (10). The 1473 mutant shares the same ectodomain and transmembrane domain with G protein but has a foreign 12-amino-acid cytoplasmic domain replacing the normal cytoplasmic domain of 29 amino acids. This protein is not recognized by the antipeptide serum against the cytoplasmic domain of G (Fig. 2, lane 10). The smaller 1473 protein migrates faster than G protein on SDS-PAGE. When G and 1473 were coexpressed and labeled with [^{35}S]methionine, antibody to the extracellular domain precipitated both proteins, but antibody to the cytoplasmic tail of G precipitated only G protein (Fig. 2, lanes 12 and 13).

In other experiments we attempted to detect any transient heterotrimers between G and 1473 after short pulse-labels with [^{35}S]methionine and chase times of from 5 to 30 min. We were able to detect heterotrimers between G and GE1 proteins after only 10 min, but no heterotrimers between G and 1473 were detected at any time (data not shown). This result indicates either that heterotrimers did not form or that they formed and then dissociated rapidly.

Independent processing of G protein and a nontransported mutant after coexpression. If G protein and the nontransported mutant 1473 were not forming stable heterotrimers, one would expect completely independent behavior of both proteins during transport. To examine the transport of the coexpressed proteins we performed a pulse-chase experiment in BHK cells. At various times after synthesis we examined the extent of oligosaccharide processing on both proteins, using endo H. This enzyme cleaves high-mannose oligosaccharides added in the ER, but it does not cleave oligosaccharides which have been modified by the Golgi enzymes *N*-acetylglucosamine transferase and mannosidase II (23, 46). Because *N*-acetylglucosamine transferase is located in the medial Golgi compartment (11), the acquisition of endo H resistance by glycoproteins marks their arrival at the medial Golgi compartment. As shown in Fig. 3, half of the expressed G protein became endo H-resistant after 20 min of chase and it was fully resistant after 1 h of chase. This is the normal rate for G protein expressed alone (38; data not shown). In contrast, the coexpressed 1473 protein remained endo H sensitive (Fig. 3). This result indicates that G protein was transported to the medial Golgi with wild-type kinetics, whereas the mutant protein was retained in the ER. The independent sorting of the mutant and wild-type proteins was consistent with our inability to detect heterotrimer formation between these two proteins.

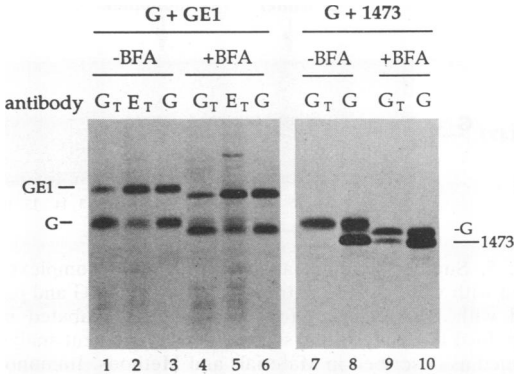

FIG. 4. Effect of BFA on heterotrimer formation. Cells infected with vTF7-3 were cotransfected with pARG and pARGE1 or with pARG and pAR1473. Cells were labeled with [^{35}S]methionine for 30 min, incubated in chase medium for 1 h, and immunoprecipitated as described in Materials and Methods. BFA was added 1 h before labeling at a concentration of 1 μg/ml, and it was present during the labeling and chase periods. Immunoprecipitates were analyzed as described in the legend to Fig. 2.

BFA allows heterotrimer formation between G and 1473. We initially considered two explanations for the inability to detect heterotrimers between G protein and the nontransported mutant. First, the mutant might fold such that it was capable of forming trimers only with other mutant molecules. Second, the wild-type molecule and mutant molecules might somehow be physically separated from each other before trimerization. For example, the wild-type molecules might move to a subcompartment of the ER before trimer formation.

To distinguish between these two possibilities, we tested the ability of G and 1473 to assemble into heterotrimers under conditions in which protein transport from the ER to the Golgi was blocked. For this experiment we treated the cells with the compound BFA, which causes fusion of the Golgi apparatus with the ER (9, 14, 27) and blocks transport of secretory and membrane proteins out of the ER. We then coexpressed G and 1473 proteins or G and GE1 proteins in BFA-treated or untreated cells. In BFA-treated cells, BFA was added 1 h before labeling and it was present during the labeling and chase periods. Cell lysates were precipitated with appropriate anti-cytoplasmic domain-specific sera or serum recognizing the ectodomain, as indicated in Fig. 4. As shown in Fig. 4, G_T and E_T cytoplasmic domain antibodies were able to coprecipitate G and GE1, respectively, both in the absence (lanes 1 and 2) or presence (lanes 4 and 5) of BFA, indicating that heterotrimers formed in both cases. This shows that the ability of G and GE1 to form heterotrimers is not affected by BFA treatment. In contrast, G-1473 complexes were not detected in the absence of BFA, but in the presence of BFA they were readily detected with the G_T serum (Fig. 4, lanes 7 and 9). This result suggested that retention of G and 1473 proteins in the same compartment allowed their monomeric subunits to mix and form hybrid trimers. In these experiments the electrophoretic mobility of the transport-competent proteins was increased in the BFA-treated samples. This results from a lack of complete oligosaccharide processing in the presence of BFA.

To determine whether the 1473 protein, which was coprecipitated with G from BFA-treated cells, was in a heterotrimeric complex with G, we examined the sedimentation profile of the G-1473 complex by sucrose velocity gradient

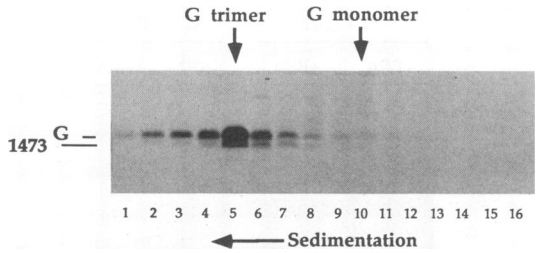

FIG. 5. Sucrose gradient analysis of G-1473 complexes. Cells infected with vTF7-3 were cotransfected with pARG and pAR1473, treated with BFA, labeled for 30 min, and incubated in chase medium for 1 h. Cell lysis and sucrose density gradient analysis were performed as described in Materials and Methods. Immunoprecipitation of the fractions was with antipeptide serum recognizing the cytoplasmic domain of G protein. Immunoprecipitates were analyzed as described in the legend to Fig. 2.

centrifugation after coexpression in the presence of BFA. Detergent lysates were centrifuged under standard conditions (10), and fractions were precipitated with the anti-G tail peptide serum. As shown in Fig. 5, the G-1473 complexes sedimented as trimers, indicating that they were assembled into heterotrimers in BFA-treated cells. To determine whether the effect of BFA on the formation of heterotrimers of G and 1473 was related to effects on the rates of trimer formation, we determined these rates in the presence or absence of BFA. The results (Fig. 6) showed that BFA did not significantly affect the rates of trimer formation.

Heterotrimers formed in the presence of BFA dissociate after removal of BFA. Because the effects of BFA on transport are reversible (9, 26, 27), it was possible to examine transport of the G-1473 heterotrimers that were formed in the presence of BFA and then released from the transport block after removal of the BFA. Because initial experiments suggested that the wild-type and mutant proteins dissociated after BFA removal (1473 remained in the ER, and G protein was transported to the cell surface), we examined the stability of the G-1473 heterotrimers after BFA removal.

To determine the stability of G-1473 complexes, heterotrimers were formed in the presence of BFA and detected by coprecipitation with the G_T antibody (Fig. 7A, lane 1). At 1 h after BFA removal, more than 95% of the prelabeled 1473 protein had dissociated from G protein, and at 2 h the heterotrimers were not detectable (Fig. 7A, lanes 3 and 5). Precipitation of proteins from each time point with antibody recognizing the ectodomain showed that the G and 1473 proteins were expressed at similar levels and were stable (Fig. 7A, lanes 2, 4, and 6). We therefore conclude that the disappearance of the G-1473 heterotrimers did not result from selective degradation. Quantitation (from this and other experiments) of the rate of heterotrimer dissociation is shown in Fig. 7B and indicates that it occurs with a half time of about 20 min after removal of BFA.

Heterotrimer formation between G and G_s-KDEL also requires a transport block. The results described above indicated that heterotrimers between wild-type G protein and a mutant that is blocked in transport from the ER could be detected but only if the proteins were both retained in the same compartment. To determine whether these results as well as the dissociation of heterotrimers were peculiar to this one membrane-anchored, retained mutant, we decided to examine another mutant G protein, G_s-KDEL. This protein is a soluble form of the VSV G protein which has the transmembrane and cytoplasmic domains deleted and replaced with the specific ER retention signal SEKDEL (Ser-Glu-Lys-Asp-Glu-Leu; 31). This G protein, like other soluble VSV G proteins (6), forms trimers but is retained within the ER with endo H-sensitive oligosaccharides. Pulse-chase experiments as well as indirect immunofluorescence microscopy have shown that the G_s-KDEL molecule, unlike some other proteins bearing the KDEL signal (52), is completely retained in the ER for up to 6 h after synthesis (data not shown).

To examine heterotrimer formation between G and G_s-KDEL we again used the coprecipitation assay, employing antibody to the cytoplasmic domain of G protein (Fig. 8A). When the G_s-KDEL protein was expressed with wild-type G protein, only about 5% of the G_s-KDEL protein was detected in heterotrimers as judged from precipitation with G_T antibody. Just as with the membrane-bound, retained mutant, blockage of transport with BFA allowed extensive heterotrimer formation. Approximately 60% of the G_s-KDEL protein was now precipitated with the G_T antibody (lanes 3 and 4). We also found that the G_s-KDEL-G heterotrimers, like the G-1473 heterotrimers, dissociated after removal of BFA (Fig. 8B).

Wild-type G protein homotrimers can dissociate and form heterotrimers with G_s-KDEL. It was clear from the previous experiments that heterotrimers formed from wild-type and mutant G protein subunits could dissociate. To determine whether this instability was peculiar to heterotrimers formed from mutant and wild-type subunits, we asked whether preformed wild-type homotrimers could subsequently dissociate and reassociate with mutant subunits to form heterotrimers.

The experiment was carried out as follows. We treated cells expressing G and G_s-KDEL proteins with the ionophore monensin to inhibit transport of G protein from the medial to the *trans*-Golgi compartment (16, 47). The proteins were then labeled with [^{35}S]methionine. Under these conditions wild-type G protein trimers should move to the medial Golgi, while the G_s-KDEL homotrimers should remain in the ER. The cells were then treated with BFA to return all wild-type G protein homotrimers from the Golgi to the ER.

Cells expressing G and G_s-KDEL that were incubated with monensin for 1 h did not show any heterotrimers between G and G_s-KDEL, as determined by lack of coprecipitation of G_s-KDEL with G when antibody to the G cytoplasmic domain was used. This is the result expected if the two proteins were segregated into the Golgi and ER compartments (Fig. 9, lane 1). However, after addition of BFA and incubation for 1 additional h, the G_s-KDEL was precipitated along with G protein when antibody to the cytoplasmic domain was used (Fig. 9, lane 5). We conclude from this experiment that the G protein homotrimers that were returned to the ER are able to dissociate and reassociate to form heterotrimers with the mutant subunits that remained in the ER.

DISCUSSION

In this report we have described experiments examining the formation of heterotrimers between wild-type and mutant VSV glycoproteins expressed from different plasmid DNAs in the same cell. When wild-type G protein was coexpressed with a mutant bearing a much larger cytoplasmic domain but transported at the same rate as G protein, heterotrimers formed. We conclude that once VSV G mono-

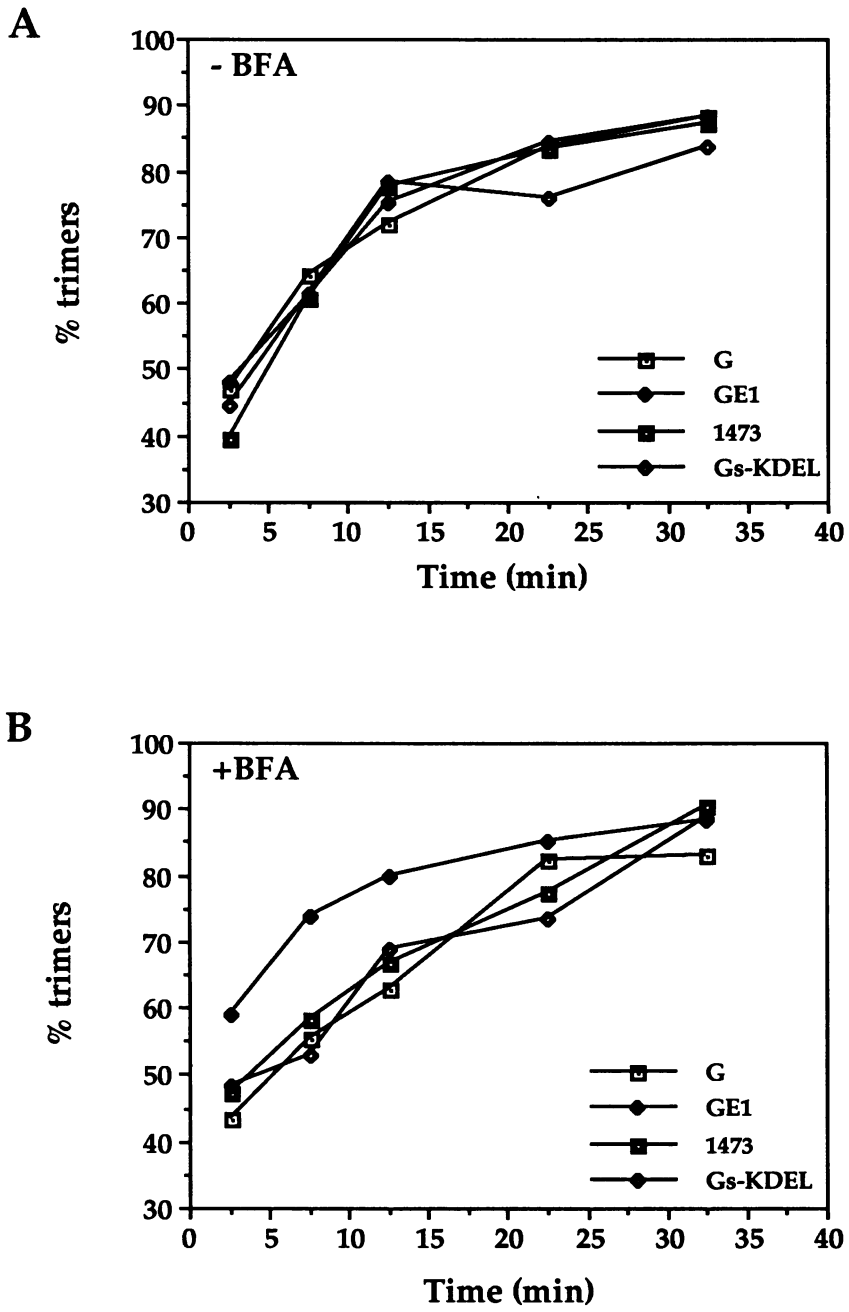

FIG. 6. Kinetics of trimer formation in the absence (A) or presence (B) of BFA. Cells infected with vTF7-3 were transfected with the appropriate plasmids, labeled with [^{35}S]methionine for 5 min, and then incubated in chase medium for the indicated times. Lysis of the cells and sucrose density gradient sedimentation were performed as described in Materials and Methods. Immunoprecipitation of the various fractions was with serum recognizing the ectodomain of G protein. The immunoprecipitates were analyzed by SDS-PAGE followed by fluorography. The intensity of the bands was quantitated by scanning densitometry of the fluorograms, and the percentage of total protein present in the fractions corresponding to the trimeric G protein was calculated for each time point.

mers are formed and correctly folded they are free to diffuse from the site of synthesis and form mixed oligomers with subunits that were translated on different polysomes. This result is similar to earlier results showing formation of heterotrimers between different influenza virus hemagglutinin proteins during coinfection with two influenza virus variants (3) or with viruses from different subtypes (45).

When wild-type G protein was coexpressed with a mutant G protein bearing a retention signal for the ER in its cytoplasmic domain or the KDEL retention signal on the C terminus of the soluble ectodomain, both wild-type and mutant proteins formed trimers, but heterotrimers were not detected unless protein transport from ER to Golgi was blocked by BFA. This compound causes fusion of *cis* and medial Golgi with the ER and blocks protein transport from ER to Golgi (9, 14, 27). Surprisingly, these heterotrimers

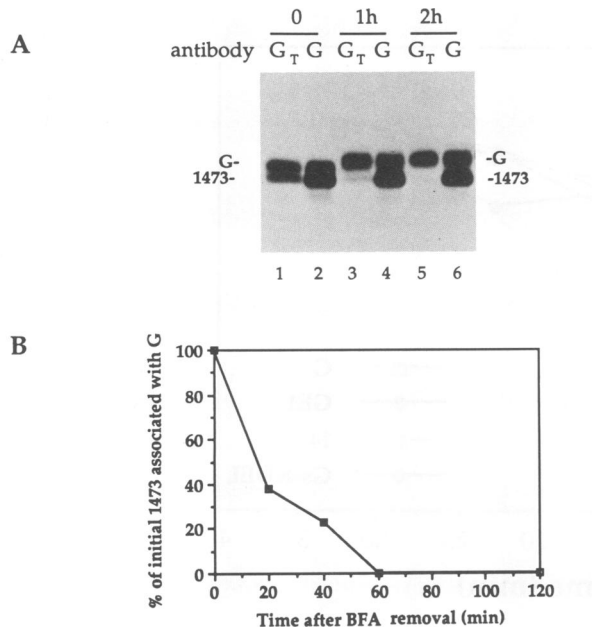

FIG. 7. Dissociation of G-1473 heterotrimers after removal of BFA. (A) Cells were infected with vTF7-3 and cotransfected with pARG and p1473. The cells were then pretreated with 1 μg of BFA per ml for 1 h, labeled with [^{35}S]methionine for 30 min in the presence of BFA, and incubated in chase medium for 1 h. BFA was removed by washing the cells twice with phosphate-buffered saline, and the incubation was continued in BFA-free medium for the indicated times. Cell lysates were immunoprecipitated with sera recognizing the cytoplasmic domain of G protein (G_T, lanes 1, 3, and 5) or the extracellular domain of G protein (G, lanes 2, 4, and 6). The immunoprecipitates were analyzed by SDS-PAGE followed by fluorography. (B) Quantitation of the data from panel A and from another experiment are shown. The percentage of 1473 protein initially in heterotrimers is plotted. This was determined by scanning densitometry.

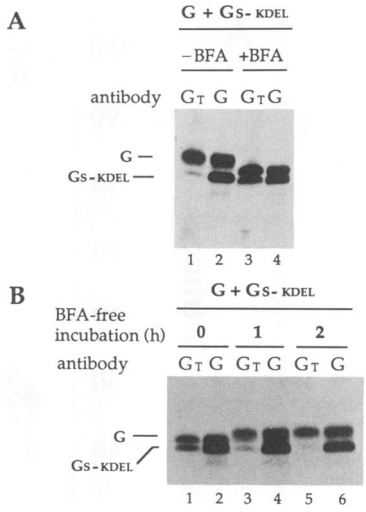

FIG. 8. Coprecipitation of G and G_s-KDEL in the presence of BFA. (A) Cells infected with vTF7-3 were cotransfected with pARG and pARG$_s$-KDEL and then treated with BFA, labeled, chased, and immunoprecipitated with the indicated antibodies as described in the legend to Fig. 4. (B) Cells infected with vTF7-3 and cotransfected with pARG and pARG$_s$-KDEL were treated with BFA, labeled, chased in the presence and then absence of BFA, lysed, and immunoprecipitated as described in the legend to Fig. 7. Immunoprecipitates were analyzed as described in the legend to Fig. 2.

were found to dissociate rapidly and then reassociate to form homotrimers after removal of the transport block. The wild-type homotrimers were then transported to the cell surface, and the mutants were retained in the ER. The dissociation of trimers was not only a property of heterotrimers formed from mutant and wild-type subunits, because wild-type trimers blocked in transport from the Golgi subsequently dissociated and formed heterotrimers with subunits of mutant trimers in the ER after BFA addition.

A major conclusion from our studies is that in the ER the VSV G trimer must be in an equilibrium with a pool of monomeric subunits. The half time for trimer dissociation is 20 min or less because this is the rate of dissociation observed after removal of BFA. The rate could in fact be much faster, with the 20-min half time simply reflecting the time required for reversal of the effects of BFA. The half time for reversal of the BFA effects is known to be about 15 min (9, 27).

It is possible that the G trimer dissociation-reassociation is a reaction specific to the ER and that it serves to ensure that wild-type subunits are not trapped by interactions with subunits that cannot be transported. After transport to the Golgi the trimeric structure might become more stable. However, our results indicate that G protein trimers that have been transported to the Golgi and are then returned to the ER do dissociate and form heterotrimers.

Results obtained in vitro with detergent-solubilized G protein have shown that G protein trimers can be unstable at neutral pH. After solubilization in the detergent Triton X-100, G protein trimers dissociate upon centrifugation in sucrose gradients unless the pH is lowered to below 6.0 (8, 10). The low pH shifts G protein into its membrane fusion conformation, and in this conformation the trimers are much more stable (8). In the detergent octyl glucoside, however, the trimers appear stable to centrifugation at neutral pH (28). Recent fluorescent energy transfer experiments on G protein solubilized with octyl glucoside show that G trimers can

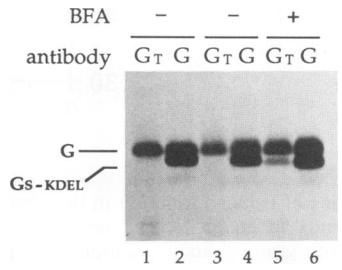

FIG. 9. Effect of BFA on monensin-treated BHK cells coexpressing G and G_s-KDEL. Cells infected with vTF7-3 were cotransfected with pARG and pARG$_s$-KDEL, treated with 7 μg of monensin per ml for 1 h, and then labeled with [^{35}S]methionine for 30 min and incubated in a medium containing excess unlabeled methionine for 1 h. Both labeling and chase were performed in the presence of monensin. The cells were either lysed immediately (lanes 1 and 2) or they were incubated for 1 more h without (lanes 3 and 4) or with 1 μg of BFA per ml (lanes 5 and 6). Immunoprecipitation was performed by using antibody to the cytoplasmic domain of G protein (G_T, lanes 1, 3, and 5) or polyclonal serum against the extracellular domain of G protein (G, lanes 2, 4, and 6). Immunoprecipitates were analyzed as described in the legend to Fig. 2.

dissociate and reassociate in solution (28). The half time for dissociation of trimers was determined to be 3 min at pH 7.5.

Another interesting aspect of our results was that under conditions in which wild-type protein was being exported from the ER, we were unable to detect even transient formation of heterotrimers between wild-type G protein and the mutant G protein (1473) retained in the ER by the foreign cytoplasmic domain. What is the mechanism of this apparent segregation of mutant and wild-type proteins? It could imply physical separation of the two types of subunits within the ER before trimer formation. In this case segregation might involve binding of the retention signal in the cytoplasmic domain to a protein localized to a subregion on the cytoplasmic side of the ER or segregation of the wild-type molecule through cytoplasmic interactions. Addition of BFA might disrupt this segregation. There are reports that proteins are segregated within the ER (39, 48, 50).

There is an alternative explanation for the segregation which we cannot rule out at present. In this model, the heterotrimers would actually form but be so transient that they were not detected in the pulse-chase experiments used in this study. The apparent segregation might result from a very rapid reassortment between hetero- and homotrimers combined with rapid selection of only the wild-type homotrimers into the vesicles budding from the ER. In this model the retention signal in the cytoplasmic tail or the KDEL retention signal would be dominant in heterotrimers. Because we cannot determine the precise half times for trimer dissociation in the ER or the half time for export of wild-type homotrimers from the ER, it is not possible to decide whether this model is reasonable.

Although the results on heterotrimer formation between G and the 1473 mutant were very similar to those obtained with G and G_s-KDEL, we did find a low but detectable level (approximately 5%) of heterotrimers forming between G and G_s-KDEL even in the absence of BFA. This might occur because a fraction of the G_s-KDEL molecules is being recycled between the ER and a post-ER compartment after binding to a receptor (31, 34). Those G_s-KDEL molecules being cotransported to the post-ER compartment with wild-type G protein might be free to form heterotrimers. Other studies (53) on the formation of heterotrimers between G protein and slowly transported mutant forms with cytoplasmic domain deletions have shown that heterotrimers form, but only with the small fraction of the mutant that leaves the ER with wild-type G protein. This result also suggests that heterotrimer formation requires cotransport.

One possible practical application for mutant subunits of oligomeric proteins would be in blocking exocytosis of wild-type subunits and subsequent virus assembly. Our work shows clearly that this approach is not feasible with a virus such as VSV whose envelope glycoprotein forms trimers that are unstable and exist in equilibrium with monomeric forms. Even when heterotrimers are formed with mutant subunits, the heterotrimers are then free to dissociate and reassort. However, when an interaction of sufficiently high affinity occurs between two proteins in the ER, such as that occurring between the human immunodeficiency virus envelope glycoprotein (gp160) and its CD4 receptor, it is possible to block transport of the viral glycoprotein by using a retention signal attached to CD4 (4). With other viruses such as influenza virus which have more stable oligomeric glycoproteins, the approach of using retention signals on individual subunits may indeed be practical if the subunits mix and form heterotrimers.

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