Fragmentation of a Golgi-Localized Chimeric Protein Allows Detergent Solubilization and Reveals an Alternate Conformation of the Cytoplasmic Domain[†]

Carolyn S. Sevier and Carolyn E. Machamer*

Department of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received July 22, 1997; Revised Manuscript Received October 16, 1997[⊗]

ABSTRACT: Golgi resident proteins maintain their localization despite a continual protein and lipid flux through the organelle. To study Golgi retention mechanisms, we have focused upon the chimeric protein Gm1. This protein contains the Golgi transmembrane domain targeting signal from the infectious bronchitis virus M protein and the lumenal and cytoplasmic domain of the vesicular stomatitis virus glycoprotein (VSV G). The Gm1 protein is targeted to the Golgi where it forms an unusually stable detergent-resistant oligomer. The formation of oligomeric structures may aid retention of Golgi resident proteins. Thus, determining the stabilization mechanism may shed light on Golgi protein retention. Previous work determined that the transmembrane domain is required for the targeting and oligomerization of Gm1, but it is the cytoplasmic tail that stabilizes the complexes [Weisz, O. A., Swift, A. M., and Machamer, C. E. (1993) *J. Cell Biol. 122*, 1185−1196]. However, further study of the oligomer has been difficult due to its insolubility. Here we report that fragmenting the Gm1 protein into several pieces facilitates solubilization by sodium dodecyl sulfate (SDS). By analyzing the fragments produced after cleavage, we determined that the stability of the oligomer is not caused by covalent linkage of Gm1 to itself or other proteins. The fragment corresponding to the transmembrane domain and tail of Gm1 had an enhanced mobility in SDS gels relative to the same fragment of the parent VSV G protein. The enhanced migration of the tail fragment does not reflect sequence differences or post-translational modification, but correlates with Golgi localization and oligomerization. We suggest that the enhanced mobility of the Gm1 tail fragment reflects an altered conformation which serves to stabilize the detergent-resistant oligomers.

Proteins destined for secretion or delivery to the plasma membrane follow a common pathway through the cell. Newly synthesized proteins move from the lumen and membrane of the endoplasmic reticulum (ER)[1] through the cisternae of the Golgi complex to the cell surface. Originally it was proposed that arrival of proteins at the cell surface occurs by default (*1*). In the absence of a signal which identifies the protein as a resident of an organelle in the exocytic pathway, a protein will move with the "bulk" flow of lipids and proteins to the cell surface. This view is presently undergoing modification as groups have found that several proteins are selectively sorted and concentrated prior to export from the ER (*2*, *3*). Recently, rapid progress has been made toward understanding the steps of protein movement along the secretory pathway (reviewed in ref *4*). However, although significant advances have been made in the understanding of protein transport and the signals involved, few details are known about the mechanisms used to target individual proteins to specific destinations along the exocytic pathway (such as the ER and the Golgi complex).

Our interests center on dissecting the process of protein targeting to the Golgi apparatus. This organelle consists of a polarized stack of flattened, disk-shaped cisternae arranged near the microtubule organizing center. As proteins and lipids pass through the Golgi apparatus, they undergo post-translational modification and are subsequently sorted and packaged into vesicles destined for secretory granules, lysosomes, or the plasma membrane (reviewed in refs *5*−*7*). We would like to understand the signals that target proteins to the Golgi apparatus, and the mechanism by which Golgi-localized proteins maintain their steady-state distribution despite the constant protein and lipid traffic through this organelle.

In general, the strategies for protein localization within the exocytic pathway can be described in terms of a mechanism of retrieval or retention. These mechanisms are not mutually exclusive: a protein can contain both retrieval and retention information. Retrieval refers to the process in which a protein that is transported beyond its steady-state compartment is recognized by some component later in the secretory pathway and returned to its proper place. The best example of retrieval is the return of proteins containing the

[†] This work was supported by National Institutes of Health Grant GM42522.

* Author to whom correspondence should be addressed. Postal address: Department of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, 725 North Wolfe St., WBSB G-8, Baltimore, MD 21205. Fax: 410-955-4129. E-mail: carolyn_machamer@qmail.bs.jhu.edu.

[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1997.

[1] Abbreviations: DME, Dulbecco's modified Eagle's medium; dMM, deoxymannojirimycin; endo H, endoglycosidase H; ER, endoplasmic reticulum; IBV, infectious bronchitis virus; NCS, *N*-chlorosuccinimide; SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; VSV G, vesicular stomatitis virus glycoprotein.

S0006-2960(97)01782-0 CCC: $15.00
Published on Web 01/06/1998

KDEL (Lys-Asp-Glu-Leu) ER localization signal back to the ER if they are transported to the Golgi complex (reviewed in ref *8*). Proteins localized by a mechanism of retention maintain their steady-state distribution by interacting with neighboring proteins and/or lipids that prevent the protein access to transport vesicles of the exocytic pathway. An example of this occurs in the Golgi complex, where resident Golgi proteins interact with each other to form oligomeric structures (*9*, *10*). The interaction between Golgi proteins was originally believed to be mediated by the transmembrane domains, yet now it appears that the regions flanking the transmembrane domain promote oligomer formation (*11*, *12*).

In order to gain a detailed understanding of the signals and mechanisms involved in the targeting of Golgi proteins, we have used the infectious bronchitis virus (IBV) M protein (formerly referred to as E1) as a model. When the M protein is expressed in mammalian cells in the absence of other viral proteins it is targeted to the *cis* Golgi (*13*). Structurally, the M protein consists of a short *N*-glycosylated amino-terminal domain, three membrane spans, and a long carboxyl-terminal cytoplasmic tail. The first membrane-spanning domain (m1) of the M protein is sufficient to retain the M protein in the Golgi when tested in the context of a single transmembrane protein (*14*). In addition, the m1 domain can confer Golgi localization to a plasma membrane protein. When the membrane-spanning domain of a plasma membrane protein, the vesicular stomatitis virus glycoprotein (VSV G), is replaced with m1, the resulting chimera (Gm1) is retained in the Golgi (*15*).

Upon investigation of the mechanism used to retain Gm1, it was observed that Gm1 forms an unusually stable, heterogeneous oligomer including at least 12 Gm1 subunits (*9*). This oligomer is resistant to solubilization by non-ionic detergents (referred to as Triton X-100-resistant oligomer), while a large portion is additionally resistant to solubilization with SDS (SDS-resistant oligomer). Thus, the Gm1 protein has given us the opportunity to study the requirements for localization to the Golgi complex via a mechanism of oligomerization or retention. It appears that the transmembrane domain is critical for the targeting and oligomerization of Gm1 (forming a Triton X-100-resistant oligomer), but it is the VSV G tail that stabilizes the oligomer and is responsible for its SDS resistance (*9*).

We have been studying the role of the VSV G tail in oligomer stabilization and detergent resistance of Gm1. Using chemical fragmentation, we have determined that the extreme stability of the detergent-resistant oligomer is not mediated by a covalent interaction of the tail with itself or another protein. Instead, the conformation of the tail differs between mutant Gm1 proteins that reach the cell surface, relative to those retained in the Golgi apparatus as a large oligomeric complex. Thus the conformation of the tail correlates with localization and oligomerization of Gm1 molecules and may be the stabilizing agent of the detergent-resistant oligomers formed by Gm1.

EXPERIMENTAL PROCEDURES

Cells and Transfection. HeLa cells were maintained in Dulbecco's modified Eagle's medium (DME) with 5% fetal calf serum at 37 °C in 5% CO_2. HeLa cells plated in 35-mm dishes (60−70% confluent) were infected with the recombinant vaccinia virus vTF7-3 encoding T7 RNA polymerase (*16*) at a multiplicity of infection of 10−20. After 30 min of adsorption at 37 °C, the inoculum was replaced with 0.75 mL of serum-free medium containing 5 μg of a vector (pAR2529) encoding the appropriate gene behind the T7 promoter and 15 μL of LipofectACE (GIBCO BRL, Gaithersburg, MD). When 6-cm dishes were infected with vTF7-3, 2.25 mL of serum-free medium with 15 μg of vector and 45 μL of cationic lipid were used. Expression was analyzed 3−4 h postinfection. Proteins expressed included Gm1 and Gm1 with point mutations in the m1 domain (*9*, *15*, *17*) and VSV G CS2 (*18*), which have been described previously. Mutant Gm1 proteins were named by appending the original amino acid (in single-letter code) followed by the new amino acid.

Radiolabeling and Immunoprecipitation. HeLa cells expressing VSV G, Gm1, or mutant VSV G or Gm1 proteins were starved in serum-free, methionine-free DME for 15 min prior to labeling for the indicated time with L-[^{35}S] in vitro cell labeling mix (Amersham Corp., Arlington Heights, IL) in serum-free, methionine-free DME. Cells were chased for various times in DME containing 5% fetal calf serum and a 3-fold excess of unlabeled methionine. For treatment of cells with deoxymannojirimycin (Boehringer Mannheim, Indianapolis, IN), a 1.0 mM final concentration of drug was added to the labeling medium. Cells were lysed in detergent solution (50 mM Tris, pH 8.0, 1% NP-40, 0.4% deoxycholate, 62.5 mM EDTA) with 0.13 TIU/mL aprotinin, and proteins were immunoprecipitated with a polyclonal anti-VSV G antibody and fixed *Staphylococcus aureus* (Calbiochem-Novabiochem, La Jolla, CA). Oligosaccharide processing was determined as described previously using 0.4 mu endoglycosidase H (New England Biolabs, Beverly, MA) (*17*, *19*). After heating at 100 °C for 3 min in Laemmli sample buffer containing 5% β-mercaptoethanol, proteins were analyzed on 10% SDS−polyacrylamide gels as described (*20*). Labeled proteins were detected by fluorography (*21*).

Chemical Cleavage. HeLa cells plated in 6-cm dishes (60−70% confluent) were transiently transfected with VSV G, Gm1, and mutant constructs as described above. Cells were metabolically labeled for 90 min, and proteins were immunoprecipitated and separated on a 10% SDS−polyacrylamide gel. Proteins were located by autoradiography and excised from the gel. Chemical cleavage of asparagine−glycine bonds with hydroxylamine was carried out as described (*22*) with some modifications. Briefly, gel pieces were swelled in 5% methanol and washed at 4 °C in four changes of 5% methanol. Pieces were then incubated in 500 μL of reaction mixture (2 M hydroxylamine·HCl, 6 M guanidine·HCl, 15 mM Tris, 4.5 M LiOH) and placed at 45 °C for 3 h. After cleavage, pieces were washed in four changes of 5% methanol. Gel pieces were submerged in 2× Laemmli sample buffer containing 10% β-mercaptoethanol for 2 h at 37 or 4 °C overnight prior to electrophoresis. Cleavage on the carboxyl side of tryptophan residues was completed using *N*-chlorosuccinimide (NCS) essentially as described (*23*). Briefly, gel pieces were swelled in distilled water and washed at room temperature with two changes of distilled water followed by two washes with a urea/water/acetic acid (1 g/1 mL/1 mL) mixture. Pieces were then incubated in 0.015 M NCS in a urea/water/acetic acid (1

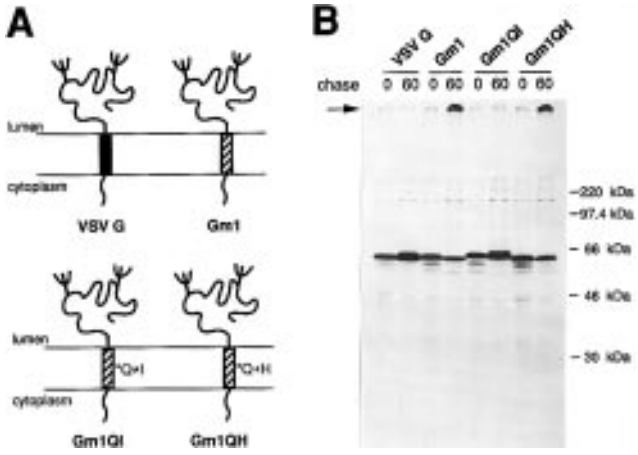

FIGURE 1: Formation of SDS-resistant oligomers by Golgi resident proteins. (A) Schematic of the topology for the VSV G, Gm1, Gm1QI, and Gm1QH proteins. The membrane bilayer is depicted as two straight lines. All four proteins contain a large lumenal domain containing two N-linked glycosylation sites and a short cytoplasmic tail of 29 amino acids. The VSV G, Gm1, and Gm1 mutant proteins differ only in the sequence of the transmembrane domains. (B) Immunoprecipitation of Gm1, VSV G, and related constructs. HeLa cells expressing these proteins were metabolically labeled for 5 min and chased for 0 or 60 min. Cells were lysed, and proteins were immunoprecipitated and separated by SDS−PAGE. Note the SDS-resistant form of the Golgi-localized proteins Gm1 and Gm1QH at the top of the stacking gel (arrow).

g/1 mL/1 mL) mixture for 30 min at room temperature. After cleavage, pieces were washed in two changes of distilled water prior to equilibration in sample buffer without β-mercaptoethanol for 90 min at room temperature. Pieces were incubated in sample buffer with 10% β-mercaptoethanol overnight at 4 °C prior to electrophoresis. Hydroxylamine- or NCS-treated gel pieces were heated at 100 °C for 3 min preceding analysis on a 12.5% polyacrylamide gel. Cleavage fragments were detected by fluorography or Western blotting.

Western Blotting. Proteins were electrophoretically transferred to nitrocellulose. After blocking in 5% nonfat dry milk in 10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween-20, the membrane was incubated with a monoclonal antibody specific for the VSV G tail (*24*). Subsequently, the membrane was treated with alkaline phosphatase-conjugated goat anti-mouse (Bio-Rad Laboratories, Hercules, CA) or horseradish peroxidase-conjugated sheep anti-mouse (Amersham Corp.) IgG antibody. Bands were visualized by either the addition of alkaline phosphate substrates or the use of enhanced chemiluminescence (Amersham Corp.).

RESULTS

Gm1 Oligomerization Correlates with Retention in Golgi Complex. Our current studies have focused on determining the catalyst that promotes the detergent resistance acquired by Gm1 upon arrival in the Golgi complex. The only difference in primary sequence between VSV G and Gm1 is the transmembrane domain (Figure 1A). The SDS-insoluble form of the Gm1 oligomer is readily apparent when immunoprecipitates are analyzed by SDS−polyacrylamide gel electrophoresis (Figure 1B, arrow). A portion of the Gm1 protein runs into the gel and migrates as a 70 kDa monomer. However, about 50% remains in the stacking gel. The Gm1 SDS-resistant oligomer is extraordinarily stable and is resistant to solubilization by a variety of conditions, including various reducing agents, nonreducing conditions, detergents, chaotropic agents, salt concentrations, temperatures of solubilization, or sample elution (*9*). When analyzed by sucrose gradient centrifugation, the Gm1 protein migrates as a large, heterogeneous Triton X-100-resistant oligomer (approximately 23S), a portion of which is resistant to solubilization by SDS (*9*). SDS resistance is not a characteristic of either parent protein.

The formation of the SDS-resistant pool of Gm1 was time dependent as demonstrated by pulse-chase labeling where no SDS-resistant Gm1 was observed after a 5 min label (Figure 1B). In previous experiments, an approximately 10 min lag was required before SDS-resistant oligomers could be detected which correlates with the time required for Gm1 to arrive at the Golgi complex (*9*). The transmembrane domain is responsible for the Golgi localization and the formation of detergent-resistant oligomers of Gm1. When a glutamine in the transmembrane span of Gm1 is changed to isoleucine (Gm1QI), the mutant protein is transported to the cell surface (*15*, *17*) and no SDS-resistant oligomers are detected (Figure 1B). However, Gm1 with a tolerated substitution in the transmembrane domain (Gm1QH) is retained in the Golgi apparatus (*17*) and forms SDS-resistant oligomers (Figure 1B).

Although the transmembrane domain is required for the formation of Gm1 oligomers, the tail is responsible for its extreme stability. It was originally hypothesized that the tail mediates the stability of the oligomer via interaction with other cytoplasmic proteins (*9*). An attempt was made to identify components of the SDS-resistant oligomer. Several different approaches were used to isolate putative components, including affinity chromatography, radiolabeling of endogenous cellular proteins prior to Gm1 transfection, and cross-linking of Gm1 within microsomes (data not shown). However, no direct evidence of proteins interacting with the SDS-resistant oligomer has been obtained. In addition, we have previously been unable to rule out a covalent interaction of the tail with itself or another protein. Because of the extraordinary stability of the oligomer and its resistance to solubilization by a variety of conditions, it is difficult to determine whether other proteins or covalent links are present in the SDS-resistant oligomer. Thus, we asked if fragmentation of the Gm1 protein into several large pieces would facilitate solubilization by SDS and thus allow examination of the Gm1 tail.

Chemical Cleavage Allows Solubilization of SDS-Resistant Oligomer. To break the Gm1 protein into several large fragments, the Gm1 SDS-resistant oligomer was treated with hydroxylamine under conditions which result in the selective cleavage between asparagine and glycine residues (*22*). Two such cleavage sites are present in the lumenal VSV G head, resulting in three complete cleavage fragments: a C-terminal fragment containing the cytoplasmic and transmembrane domain plus 75 amino acids of the lumenal domain (15.9 kDa), an N-terminal piece (19.7 kDa), and the central fragment containing the portion of the lumenal domain that joins the N-terminal and C-terminal fragments (34.7 kDa). To carry out the hydroxylamine cleavage procedure, Gm1 and VSV G were expressed in HeLa cells using a vaccinia virus-mediated expression system. Cells were radiolabeled for 90 min and lysed, and proteins were immunoprecipitated and separated by SDS−PAGE (Figure 2A). The VSV G,

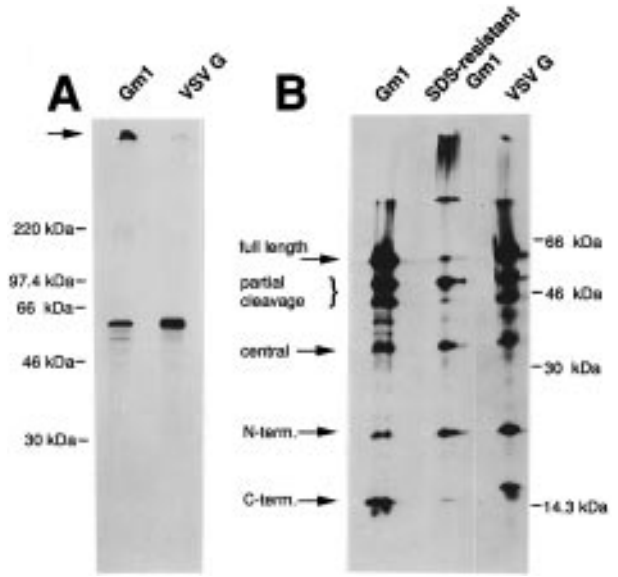

FIGURE 2: Fragmentation of the lumenal domain of Gm1 allows solubilization with SDS. (A) Gm1 and VSV G were expressed in HeLa cells. After a 90 min radiolabel, cells were lysed and proteins were immunoprecipitated and separated by SDS−PAGE. The arrow denotes the SDS-resistant form of Gm1 present at the top of the stacking gel. (B) VSV G, Gm1, and the Gm1 SDS-resistant species were excised from a gel comparable to that shown in A and treated with hydroxylamine as described in Experimental Procedures. Under these conditions, hydroxylamine cleaves selectively between adjacent asparagine−glycine residues in the VSV G and Gm1 protein sequences to generate three complete cleavage fragments. Cleavage products were separated on a 12.5% SDS−polyacrylamide gel. The SDS-resistant Gm1 is cleaved by this method. The C-terminal fragment of Gm1 runs slightly faster than the corresponding fragment of VSV G, suggesting that the Gm1 tail is not covalently linked to itself or another protein.

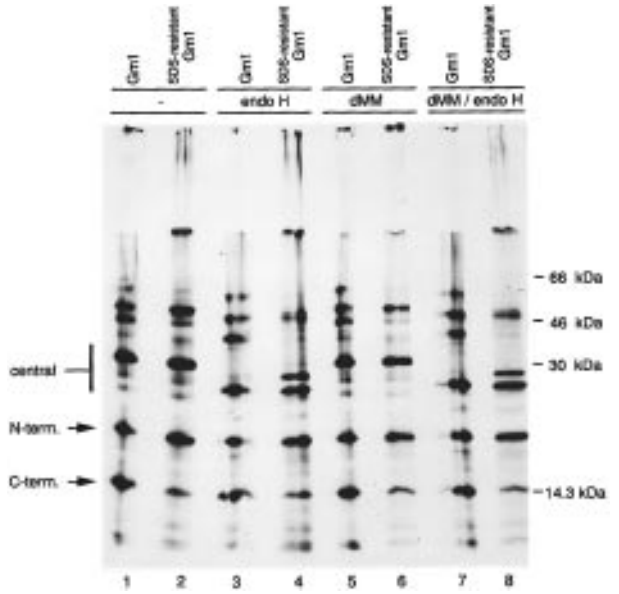

FIGURE 3: One oligosaccharide of the SDS-resistant form of Gm1 cannot be accessed by endoglycosidase H. Gm1 was transiently expressed in HeLa cells and metabolically labeled in the presence (lanes 5−8) or absence (lanes 1−4) of the mannosidase I inhibitor deoxymannojirimycin (dMM). Cells were lysed, and Gm1 was immunoprecipitated. Immunoprecipitates shown in lanes 3, 4, 7, and 8 were treated with endoglycosidase H (endo H) prior to electrophoresis. Proteins were separated on a 10% SDS−polyacrylamide gel. SDS-sensitive and -resistant Gm1 were excised from the gel and treated with hydroxylamine as described in Experimental Procedures. Fragments were separated on a 12.5% SDS−polyacrylamide gel. Gm1 has two N-linked glycosylation sites which are both found in the central hydroxylamine fragment. One oligosaccharide in the SDS-resistant form of Gm1 is inaccessible to endo H since it is uncleaved in dMM-treated cells, where all *N*-glycans are unprocessed and therefore sensitive to cleavage by endo H.

Gm1, and the Gm1 SDS-resistant species (which remains at the top of the stacking gel) were excised from a gel comparable to that seen in Figure 2A and treated with hydroxylamine. The cleavage products were separated on a second SDS−polyacrylamide gel (Figure 2B).

Hydroxylamine yielded the expected fragments: three complete cleavage fragments, two partial cleavage products, and the full-length protein. However, most significant was that the hydroxylamine-mediated fragmentation allowed SDS solubilization of the Gm1 SDS-resistant oligomer. After hydroxylamine cleavage there was a notable decrease in the signal of Gm1 in the stacker as compared with the first gel. Solubilization of the fragmented SDS-resistant oligomer was more complete in some experiments (Figure 3), which suggested that all of the oligomer could be solubilized after fragmentation. It should be noted that the recovery of the SDS-resistant Gm1 protein after cleavage was lower than the recovery of the SDS-sensitive Gm1 protein. Efficient recovery of protein from the stacking gel, where SDS-resistant Gm1 migrates, is difficult. In addition, the relative amounts of N-terminal, central, and C-terminal cleavage products of the solubilized SDS-resistant Gm1 were somewhat different than those of the SDS-sensitive Gm1 or VSV G proteins (Figure 2B). However, there were no apparent differences in migration of the fragments from SDS-sensitive and SDS-resistant Gm1.

The hydroxylamine cleavage data suggested that fragmentation of Gm1 into several large pieces allowed solubilization by SDS. The conditions of cleavage are extremely harsh (6 M guanidine, 2 M hydroxylamine, 15 mM Tris in 4.5 M LiOH, pH 9.3). However, solubilization of the oligomer was a direct result of fragmentation. Guanidine alone did not allow solubilization of the oligomer (data not shown). Likewise, when gel pieces were treated with 15 mM Tris in water, pH 9.3, the Gm1 SDS-resistant oligomer remained at the top of the gel (data not shown). We have been unable to rule out a role for LiOH in the dissociation of the oligomer. When pieces were treated with LiOH in the absence of guanidine and hydroxylamine, none of the labeled proteins (VSV G or Gm1) were recovered in the second gel (data not shown). However, solubilization by LiOH seems unlikely since other basic treatments did not solubilize the oligomer (data not shown).

Covalent Linkage with Itself or Another Protein Is Not Responsible for SDS Resistance of Gm1. By facilitating the solubilization of the oligomer, the hydroxylamine cleavage of Gm1 allowed us to determine if Gm1 was covalently linked to itself or other proteins. The hydroxylamine cleavage data indicated that SDS resistance does not involve a covalent modification of Gm1 with itself or another protein. After hydroxylamine cleavage, the C-terminal products of the SDS-resistant and SDS-sensitive Gm1 ran slightly faster than the corresponding fragment of VSV G (Figure 2B). If the cytoplasmic tail was covalently bonded with itself or another protein, a slower migration of the C-terminal Gm1 piece relative to that of VSV G would have been apparent. The difference in mobility between the C-terminal fragments

of VSV G and Gm1 is further supported by subsequent experiments discussed below.

One Oligosaccharide of SDS-Resistant Gm1 Protein Is Inaccessible to Endoglycosidase H. Processing of asparagine-linked oligosaccharides to a form resistant to cleavage by endoglycosidase H (endo H) occurs in the *medial* Golgi and serves as a convenient measure of how far a given protein has moved in this organelle. Because of the extreme stability of the SDS-resistant oligomer, the oligosaccharides on this portion of Gm1 have not previously been analyzed. The hydroxylamine procedure allowed us to assess the processing of the oligosaccharides of the Gm1 SDS-resistant oligomer for the first time. Treatment of the SDS-resistant oligomer with hydroxylamine after digestion with endo H resulted in two distinct species corresponding to the central fragment (Figure 3, lane 4). The sizes of these fragments correlate with a completely endo H-sensitive form (where both oligosaccharides present in the VSV G head were cleaved, as seen for SDS-sensitive Gm1 treated with endo H, Figure 3, lane 3) and a protein with partial sensitivity to endo H (where selective cleavage of one of the two oligosaccharides occurred). This raised the possibility that the SDS-resistant portion of Gm1 moved further into the Golgi than the SDS-sensitive portion, resulting in partial oligosaccharide processing. However, this was not the explanation. When the Gm1 oligomer was isolated from cells treated with deoxymannojirimycin (dMM) (which blocks processing by preventing mannose trimming, leaving all oligosaccharides in the endo H-sensitive form) and subjected to cleavage with endo H and hydroxylamine, a cleavage pattern the same as that for the oligomer from untreated cells was seen (compare lanes 4 and 8, Figure 3). This indicates that one oligosaccharide of the SDS-resistant Gm1 is inaccessible to endo H. To control for dMM effectiveness, VSV G, which becomes endo H resistant under normal conditions, was expressed in cells treated with dMM. Treatment with dMM blocked the processing of VSV G, maintaining both of its *N*-glycans in an endo H-sensitive form (data not shown).

C-Terminal Fragments of Gm1 and VSV G Have Different Mobilities on SDS−Polyacrylamide Gels. The observation that the Gm1 SDS-resistant oligomer does not involve a detectable modification of the cytoplasmic tail was verified by a second method. This was accomplished by cleaving the Gm1 or VSV G protein on the carboxyl side of tryptophan residues with *N*-chlorosuccinimide (NCS) and examining the relative mobilities of the tail fragments. This procedure is similar to the hydroxylamine cleavage procedure. Cleavage of the VSV G or Gm1 proteins was performed in polyacrylamide gel pieces, and the pieces were subsequently re-electrophoresed (*23*). The VSV G protein contains 15 tryptophan residues all present within the lumenal domain. Thus, one of the cleavage products contains the transmembrane domain and the cytoplasmic tail.

When VSV G and Gm1 proteins were treated with NCS a number of cleavage products were detected by autoradiography (Figure 4A). Note that NCS cleavage, like hydroxylamine cleavage, allowed solubilization of the SDS-resistant oligomer. Incubation of the oligomer in the reaction mixture in the absence of NCS did not allow solubilization (data not shown). To determine which of the NCS cleavage products contained the cytoplasmic tail, the NCS fragments

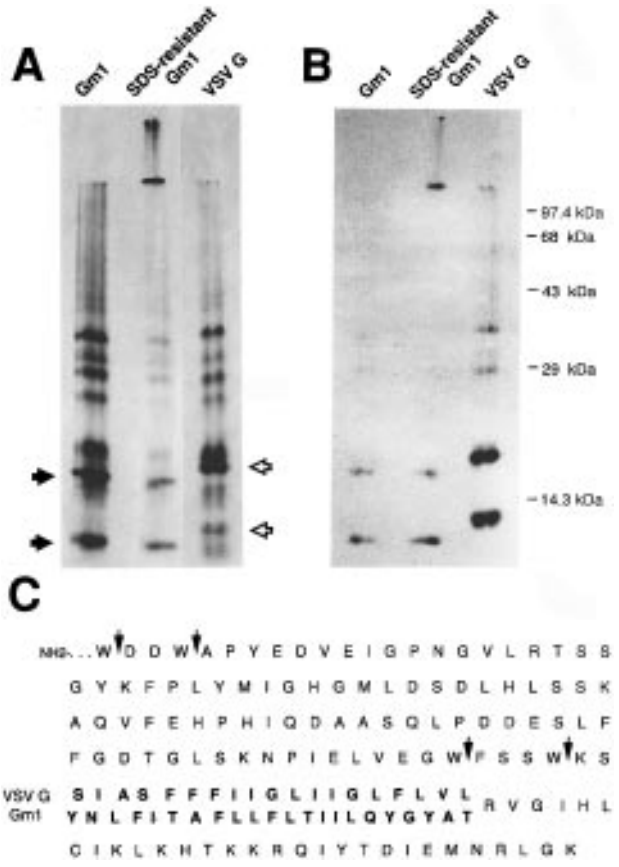

FIGURE 4: C-Terminal fragments of Gm1 and VSV G migrate differently on SDS−polyacrylamide gels. (A) HeLa cells expressing Gm1 or VSV G were labeled for 90 min, and proteins were immunoprecipitated and separated by SDS−PAGE. VSV G, Gm1, and the Gm1 SDS-resistant species were excised from the gel and treated with 0.015 M *N*-chlorosuccinimide (NCS) as described in Experimental Procedures. NCS cleaves after tryptophan residues, and there are 15 potential sites in VSV G and Gm1. Cleavage products were separated on a 12.5% SDS−polyacrylamide gel. Open and filled arrows denote the C-terminal fragments of VSV G and Gm1, respectively, as determined by immunoblotting. (B) A gel comparable to that shown in part A was transferred to nitrocellulose and probed with an antibody against the VSV G tail (*24*). The antibody recognizes the fragments that are different in size between VSV G and Gm1. The size difference between the fragments is approximately 1−2 kDa. (C) Amino acid sequence of the C-terminal one-third of VSV G and Gm1 proteins. Arrows denote the postulated cleavage sites that generate the C-terminal fragments detected in the blot (panel B). The transmembrane domains of VSV G and Gm1 are shown in bold type.

were transferred to nitrocellulose and probed with a monoclonal antibody specific for the last 11 residues of the G tail (*24*). Two fragments containing the tail sequence were generated (Figure 4B). On the basis of the mobilities of the tail fragments of SDS-sensitive Gm1, SDS-resistant Gm1, and VSV G, it is clear that the Gm1 tail is not covalently linked to itself or another protein. Even more pronounced than seen with hydroxylamine cleavage (Figure 2B), the SDS-sensitive Gm1 and SDS-resistant Gm1 tail pieces migrated faster than the G tail pieces.

Based on the migration of the fragments compared with molecular mass standards, the two tail fragments are 7−9 and 16−17 kDa. These sizes correlate with the sizes expected if NCS cleaves at tryptophan residues proximal to the transmembrane domain. For each fragment, there are two potential tryptophan residues separated by three or four

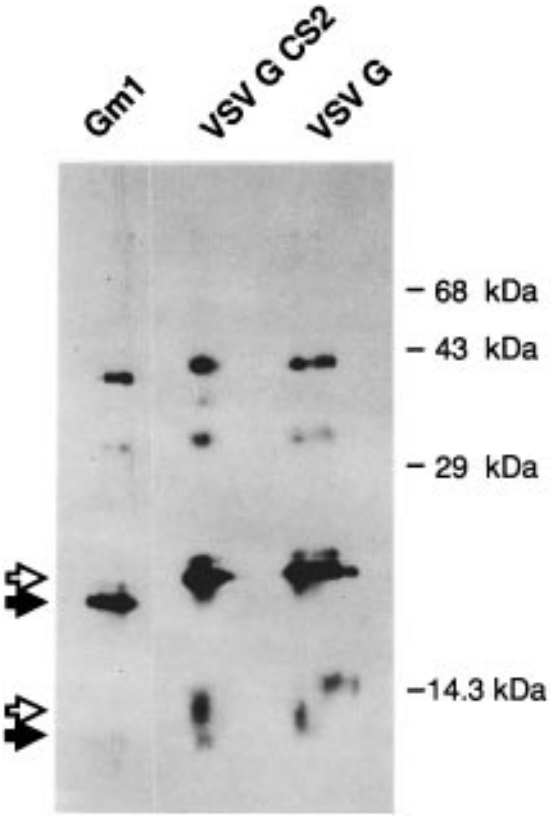

FIGURE 5: Palmitoylation of VSV G tail does not alter migration of C-terminal fragment. VSV G, VSV G CS2, and Gm1 were expressed in HeLa cells; cells were lysed, and proteins were immunoprecipitated and separated by SDS−PAGE. VSV G, VSV G CS2, and Gm1 were excised from the gel and treated with NCS as described in Experimental Procedures. Cleavage products were separated on a 12.5% SDS−polyacrylamide gel, transferred to nitrocellulose, and probed with an antibody against the VSV G tail (*24*). The C-terminal fragments of palmitoylated VSV G (open arrows) co-migrated with the C-terminal fragments of the non-palmitoylated VSV G CS2. The migration of these fragments was slowed compared to that of the C-terminal fragments of Gm1 (filled arrows), which are inefficiently palmitoylated.

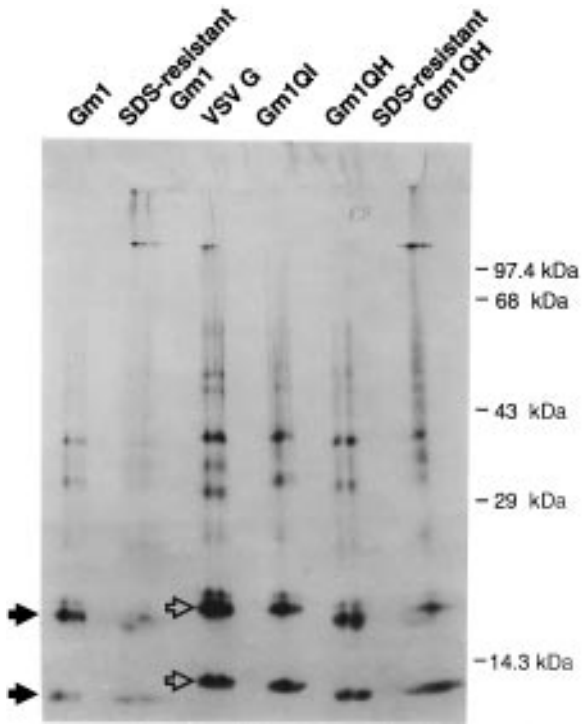

FIGURE 6: Mobility of C-terminal fragments correlates with Golgi localization. VSV G, Gm1, and related constructs were expressed in HeLa cells. Cells were lysed, and proteins were immunoprecipitated and separated by SDS−PAGE. VSV G, Gm1, and the SDS-resistant Gm1 were excised from the gel and treated with NCS as described in Experimental Procedures. Cleavage products were separated on a 12.5% SDS−polyacrylamide gel and transferred to nitrocellulose. Proteins were probed with an antibody against the VSV G tail (*24*). Note that the C-terminal fragments from the plasma membrane proteins VSV G and Gm1QI (open arrows) co-migrate. Likewise, the Golgi-localized Gm1 and Gm1QH C-terminal fragments (filled arrows) co-migrate. This suggests that the different compositions of the transmembrane domains of VSV G vs Gm1 do not account for differences in mobilities of the tail fragments. The differences in migration between the various C-terminal fragments correlate with the localization of the protein within the cell and its oligomerization.

amino acids that could be cleaved (Figure 4C). However, preferential cleavage of the more N-terminal tryptophans in VSV G vs the more C-terminal tryptophans in Gm1 cannot account for the size differences of the tail pieces. The three or four amino acid differences between the tryptophans is compensated for by the longer transmembrane domain of Gm1 as compared to VSV G.

Differences in Mobilities of C-Terminal Fragments Correlate with Golgi Localization. It is unlikely that a post-translational modification of the tail could explain the mobility differences between the VSV G and Gm1 proteins. The VSV G tail is palmitoylated subsequent to exit from the ER (*25*). Gm1 is not palmitoylated efficiently (*26*), even though it reaches the compartment where this modification occurs. However, palmitate addition to the VSV G tail does not explain the slower mobility of the VSV G pieces relative to the Gm1 fragments. When a nonpalmitoylated VSV G protein containing a cysteine-to-serine mutation in the tail (*18*) was cleaved with NCS, the nonpalmitoylated VSV G (VSV G CS2) tail fragments migrated like the wild-type VSV G pieces (Figure 5). Other post-translational modifications are unlikely to explain the differences in mobility of the tail fragments. For example, neither the VSV G or Gm1 tail is phosphorylated (data not shown). In addition, the different compositions of the transmembrane domains of VSV G vs Gm1 do not account for the differences in mobilities of the tail fragments. When Gm1 with a single point mutation in the transmembrane domain (Gm1QI) was cleaved with NCS the tail fragments co-migrated with the VSV G tail fragments (Figure 6).

The identical mobilites of the Gm1QI and VSV G pieces are additionally intriguing considering Gm1QI is transported efficiently to the plasma membrane. The tail fragments of Gm1QH, which is Golgi-localized and forms detergent-resistant oligomers, migrated like Gm1 (Figure 6). Thus, the differences in migration between the various C-terminal fragments correlated with the localization of the protein within the cell and its oligomerization.

DISCUSSION

SDS-Resistant Gm1 Oligomer Is Solubilized by Fragmentation of Lumenal Domain. We are interested in the correlation between oligomerization and Golgi localization. In addition, we would like to understand what accounts for the extreme stability of the detergent-resistant oligomers

formed by Gm1 in the Golgi apparatus. Previous work determined that the Gm1 transmembrane domain is required for Golgi localization and oligomerization (*15*). The cytoplasmic tail stabilizes the oligomers and is responsible for the SDS resistance (*9*). However, study of the role of the tail in stabilizing the SDS-resistant Gm1 oligomer has been difficult in light of the extraordinary stability of the oligomer and its resistance to solubilization by a variety of conditions. Here we report a method for dissociating the detergent-resistant complex. Chemical fragmentation of the lumenal domain of Gm1 with hydroxylamine or NCS allowed solubilization. The ability to solubilize Gm1 facilitated the analysis of the oligomers formed by Gm1 and allowed a closer examination of the features of these SDS-resistant oligomers.

It is intriguing that cleavage at just two sites in the lumenal domain of Gm1 allows solubilization by SDS. Previous data argued against a role for the lumenal domain in the stabilization of the SDS-resistant oligomer. When the lumenal portion of Gm1 was replaced with a smaller head (human alpha chorionic gonadotropin), oligomers were still able to form (*9*). The inability of endo H to access one of the oligosaccharides in the SDS-resistant oligomer (Figure 3) suggests a different arrangement of the lumenal domain in the SDS-resistant oligomer compared to the SDS-sensitive portion of Gm1. However, this may reflect a conformational change of the lumenal domain of the SDS-resistant form after exposure to SDS.

Covalent Linkage Is Not Responsible for SDS Resistance of Gm1. The hydroxylamine and NCS cleavage methods provided a way to dissociate the SDS-resistant oligomer and examine if the unusual stability of the SDS-resistant oligomer is mediated by a covalent modification of the tail. On the basis of the mobility of tail fragments produced by chemical cleavage of Gm1, it is clear that Gm1 does not interact covalently with itself or another protein (Figures 2B and 4B). In fact, the mobility of the Gm1 tail fragment was enhanced relative to the VSV G tail piece.

Solubilization of the SDS-resistant form of Gm1 permitted us to assess if any differences existed between the SDS-resistant and SDS-sensitive forms of Gm1. The hydroxylamine fragments of the solubilized SDS-resistant Gm1 co-migrated with the fragments of the SDS-sensitive form of Gm1 (Figure 2B), suggesting that the SDS-resistant and SDS-sensitive populations of Gm1 are not distinct. However, after hydroxylamine cleavage, a difference between the intensities of fragments produced by cleavage of the SDS-resistant Gm1 protein compared to SDS-sensitive Gm1 and VSV G was apparent (Figure 2B). The level of C-terminal fragment was reduced compared to the N-terminal and central fragments. Conversely, the partial cleavage product corresponding to the N-terminal and central fragment was more intense than the partial product containing the central and C-terminal regions. We do not attribute these differences to two distinct populations of Gm1. Rather, we suggest the material found at the stacking and separating gel interface contains the "missing" central and C-terminal partial fragment as well as the C-terminal fragment, emphasizing the role of the tail in conferring SDS insolubility.

The ability to solubilize the oligomer also allowed us to determine if other protein components were present in the oligomeric complex. Initial analysis of the oligomer suggested that the cytoplasmic tail stabilized the oligomer by interacting with other cellular proteins such as cytoskeletal elements (*9*). However, after solubilization of the oligomer, no labeled fragments corresponding to proteins other than VSV G or Gm1 were detected (Figure 2B). In addition, several antibodies raised against putative Golgi matrix proteins or cytoskeletal elements were tested for reactivity with the fragmented Gm1 oligomer by blotting. No direct evidence of proteins interacting with the SDS-resistant oligomer was obtained (data not shown).

Mobility of C-Terminal Fragments of VSV G and Gm1 May Reflect Conformational Differences. The C-terminal fragments resulting from chemical cleavage of VSV G and Gm1 have different electrophoretic mobilities. All cleavage sites are present in the lumenal domain. Thus, the C-terminal fragments contain the transmembrane domain sequence as well as the cytoplasmic tail. The mobility differences between the VSV G and Gm1 carboxyl-terminal fragments produced by chemical cleavage cannot be ascribed to the transmembrane domain composition. The tail fragment of a Gm1 protein with a single amino acid change from glutamine to isoleucine (Gm1QI) co-migrated with the VSV G tail fragment (Figure 6). In addition, a post-translational modification of the tail does not appear to account for the slower mobility of the carboxyl fragment of the VSV G protein. The VSV G tail is palmitoylated efficiently, unlike the Gm1 tail (*26*). However, when the cysteine in the VSV G protein is mutated to a serine residue, which is unable to support the addition of palmitate (*18*), the nonpalmitoylated carboxyl-terminal fragment co-migrated with the palmitoylated form of VSV G (Figure 5). The tail does not appear to undergo any other post-translational modifications (such as phosphorylation) that would explain the differences in mobilities.

The differences in migration of the cytoplasmic fragments produced by chemical cleavage correlated with the localization of the protein within the cell and with detergent-resistant oligomerization. The fragments of the plasma membrane proteins VSV G and Gm1QI ran differently than the Golgi-localized Gm1 and Gm1QH protein fragments (Figure 6). We suggest that the different mobilities of the VSV G and Gm1 tail fragments result from conformational differences in the cytoplasmic domains. The conformation of the tail could influence the amount of SDS binding which, in turn, is reflected as an electrophoretic mobility shift. Previous observations support this interpretation. The cytoplasmic tails of VSV G and Gm1 are differentially sensitive to trypsin when microsomal membranes containing these proteins are digested (*9*). Likewise, the trypsin treatment of the plasma membrane proteins VSV G and Gm1QI produce the same pattern of bands, which differ from the tryptic fragments generated after digestion of the Golgi-localized proteins Gm1 and Gm1QH (O. A. Weisz and C. E. Machamer, unpublished observation). In addition, since Gm1 reaches the compartment where palmitate is added (*27*), the inefficient addition of palmitate to the Gm1 tail suggests the cysteine residue is inaccessible to the palmitoyl transferase. Thus, although the sequence is identical, the structure assumed by the cytoplasmic tail appears to differ.

The conformational differences between the cytoplasmic domains of the cell surface and Golgi-localized proteins may be a result of a structural change during transit through the

exocytic pathway. In fact, during intracellular transport, the VSV G tail increases in accessibility to proteases, suggesting a modification of the arrangement of the tail (*28*). A conformational change in the tail could be prompted by an association with cytosolic transport machinery. Or, the varying membrane composition, both lipid and protein, through the exocytic pathway could affect the orientation of the protein within the lipid bilayer. The differences in conformation could also reflect the cellular recognition of Golgi targeting information within the membrane span of Gm1 or Gm1QH which could alter the structure of the cytoplasmic tail.

Nature of SDS-Resistant Oligomer. The cytoplasmic domain of a protein that forms oligomeric structures in the Golgi appears to be structurally distinct from the tail of a protein that resides at the plasma membrane. In light of this and other data, a role for the tail in promoting and structurally maintaining the oligomeric complex is suggested. However, it is still not clear what accounts for the extreme stability of the oligomer. Thus, a main question remains to be answered. If nothing is covalently attached to the tail and no other protein components are in the oligomeric structure, what interaction is causing this unusually stable oligomer? It is conceivable that the removal of certain lipids during the solubilization process results in a collapsed structure that resists SDS solubilization. We are currently exploring the role of Gm1-associated lipids, as well as assessing the contribution of the tail, in the formation and stabilization of the detergent-resistant oligomers formed by Gm1.

ACKNOWLEDGMENT

We thank the Machamer lab and Dr. Ora Weisz for helpful advice and discussions. We also thank Dr. Riad Qanbar for critical reading of the manuscript.

REFERENCES

1. Pfeffer, S. R., and Rothman, J. E. (1987) *Annu. Rev. Biochem. 56*, 829−852.
2. Mizuno, M., and Singer, S. J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5732−5736.
3. Balch, W. E., McCaffery, J. M., Plutner, H., and Farquhar, M. G. (1994) *Cell 76*, 841-852.
4. Rothman, J. E., and Wieland, F. T. (1996) *Science 272*, 227−234.
5. Farquhar, M. G. (1985) *Annu. Rev. Cell Biol. 1*, 447−486.
6. Mellman, I., and Simons, K. (1992) *Cell 68*, 829−840.
7. Gonatas, N. K. (1994) *Am. J. Pathol. 145*, 751−761.
8. Pelham, H. R. B. (1990) *Trends Biochem. Sci. 15*, 483−486.
9. Weisz, O. A., Swift, A. M., and Machamer, C. E. (1993) *J. Cell Biol. 122*, 1185−1196.
10. Nilsson, T., Hoe, M. H., Slusarewicz, P., Rabouille, C., Watson, R., Hunter, F., Watzele, G., Berger, E. G., and Warren, G. (1994) *EMBO J. 13*, 562−574.
11. Munro, S. (1995) *EMBO J. 14*, 4695−4704.
12. Nilsson, T., Rabouille, C., Hui, N., Watson, R., and Warren, G. (1996) *J. Cell Sci. 109*, 1975−1989.
13. Machamer, C. E., Mentone, S. A., Rose, J. K., and Farquhar, M. G. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6944−6948.
14. Machamer, C. E., and Rose, J. K. (1987) *J. Cell Biol. 105*, 1205−1214.
15. Swift, A. M., and Machamer, C. E. (1991) *J. Cell Biol. 115*, 19−30.
16. Fuerst, T. R., Niles, E. G., Studier, F. W., and Moss, B. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8122−8126.
17. Machamer, C. E., Grim, M. G., Esquela, A., Chung, S. W., Rolls, M., Ryan, K., and Swift, A. M. (1993) *Mol. Biol. Cell 4*, 695−704.
18. Rose, J. K., Adams, G. A., and Gallione, C. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2050−2054.
19. Machamer, C. E., Florkiewicz, R. Z., and Rose, J. K. (1985) *Mol. Cell Biol. 5*, 3074-3083.
20. Laemmli, U. K. (1970) *Nature 227*, 680−685.
21. Bonner, W. M., and Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83−88.
22. Saris, C. J. M., van Eenbergen, J., Jenks, B. G., and Bloemers, H. P. J. (1983) *Anal. Biochem. 132*, 54−67.
23. Lischwe, M. A., and Ochs, D. (1982) *Anal. Biochem. 127*, 453−457.
24. Kreis, T. E. (1986) *EMBO J. 5*, 931−941.
25. Schmidt, M. F. G., and Schlesinger, M. J. (1979) *Cell 17*, 813−819.
26. Mook, A. M. (1996) Doctoral dissertation, The Johns Hopkins University School of Medicine, Baltimore, MD.
27. Bonatti, S., Migliaccio, G., and Simons, K. (1989) *J. Biol. Chem. 264*, 12590−12595.
28. Mack, D., Kluxen, B., and Kruppa, J. (1989) *J. Cell Biol. 109*, 2057−2065.

BI971782K