

Multiple GTP-Binding Proteins Regulate Vesicular Transport from the ER to Golgi Membranes

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Abstract. Using indirect immunofluorescence we have examined the effects of reagents which inhibit the function of ras-related rab small GTP-binding proteins and heterotrimeric $G_{\alpha\beta\gamma}$ proteins in ER to Golgi transport. Export from the ER was inhibited by an antibody towards rab1B and an NH_2 -terminal peptide which inhibits ARF function (Balch, W. E., R. A. Kahn, and R. Schwaninger. 1992. *J. Biol. Chem.* 267:13053–13061), suggesting that both of these small GTP-binding proteins are essential for the transport vesicle formation. Export from the ER was also potently inhibited by mastoparan, a peptide which mimics G protein binding regions of seven transmembrane spanning receptors activating and uncoupling heterotrimeric G proteins from their cognate receptors. Consistent with this result, purified $\beta\gamma$ subunits inhibited the export of VSV-G from the ER suggesting an initial event in transport vesicle assembly was regulated by a heterotrimeric G protein. In contrast, incubation in the presence of

GTP γ S or AIF₍₃₋₅₎ resulted in the accumulation of transported protein in different populations of punctate pre-Golgi intermediates distributed throughout the cytoplasm of the cell. Finally, a peptide which is believed to antagonize the interaction of rab proteins with putative downstream effector molecules inhibited transport at a later step preceding delivery to the cis Golgi compartment, similar to the site of accumulation of transported protein in the absence of NSF or calcium (Plutner, H., H. W. Davidson, J. Saraste, and W. E. Balch. 1992. *J. Cell Biol.* 119:1097–1116). These results are consistent with the hypothesis that multiple GTP-binding proteins including a heterotrimeric G protein(s), ARF and rab1 differentially regulate steps in the transport of protein between early compartments of the secretory pathway. The concept that G protein-coupled receptors gate the export of protein from the ER is discussed.

GTTP-binding proteins are key components of biochemical complexes which regulate vesicular transport through the exocytic pathway of eukaryotic cells. Transport of protein from the ER to the Golgi complex requires members of both the ras-related *rab* and *ARF* families of small GTP-binding proteins in yeast and mammalian cells (reviewed in Balch, 1990; Goud and McCaffrey, 1991; Pryer et al., 1992). ARF is a component of non-clathrin-coated vesicles and believed to be involved in vesicle formation and coat assembly (Serafini et al., 1991; Kahn et al., 1992; Balch et al., 1992; Taylor et al., 1992) (reviewed in Duden et al., 1991; Waters et al., 1991). Although the function(s) of rab proteins are unknown, the rab-related protein Ypt1p in yeast (Haubruck et al., 1989), and at least three rab proteins (rab1A, rab1B, and rab2) in mammalian cells are required for ER to Golgi transport (Plutner et al., 1990, 1991; Tisdale et al., 1992). In addition, the small GTP-binding protein Sar1p, which requires both Sec12p and Sec23p for function (Kaiser and Schekman, 1990), is essential for vesicle formation in

yeast (Nakano and Muramatsu, 1989; d'Enfert et al., 1991; Nishikawa and Nakano, 1991; Oka et al., 1991). A homologue of SAR1 is also likely to be required for ER export in higher eukaryotes given the recent discovery of a protein which cross-reacts with an anti-SEC23 antibody in the pre-Golgi region of mammalian cells (Orci et al., 1991). Consistent with the proposed role of multiple small GTP-binding proteins in ER to Golgi transport, both GTP γ S, a non-hydrolyzable analog of GTP, and reagents which inhibit either rab or ARF function are potent inhibitors of ER to Golgi transport in vitro (Beckers and Balch, 1989; Plutner et al., 1990, 1991; Balch et al., 1992).

The potential role of another major class of GTP-binding proteins, the signal transducing heterotrimeric G proteins (composed of α , β , and γ subunits), in vesicular transport has received less attention. This class of G proteins is coupled to a diverse range of ligand-activated transmembrane receptors present on the cell surface. In this way they serve to transduce extracellular signals to a more limited range of intracellular proteins including ion channels and those catalyzing the formation of intracellular second messengers (Ross, 1989; Bourne et al., 1990, 1991; Simon et al., 1991).

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Evidence for their potential involvement in vesicular transport stems from the early observations that regulated secretion in (neuro)endocrine and exocrine cells may proceed via signal transducing G proteins (reviewed in Burgoyne, 1987). Only recently has indirect evidence suggested that heterotrimeric G proteins may also function in vesicular trafficking of protein through the constitutive exocytic pathway (reviewed in Balch, 1992; Barr et al., 1992). AIF₍₃₋₅₎, a reagent which activates heterotrimeric G proteins but not small GTP-binding proteins (Kahn et al., 1991), inhibits ER to Golgi transport (Beckers and Balch, 1989) and leads to accumulation of non-clathrin-coated vesicles during intra-Golgi transport (Melancon et al., 1987). In support of the hypothesis that G proteins may be required for vesicle fission or fusion in the secretory pathway, both pertussis toxin and $\beta\gamma$ subunits perturb vesicular export from the trans Golgi compartment (Barr et al., 1991). In addition, recruitment of coat proteins found on clathrin-coated vesicles budding from the *trans*-Golgi compartment is sensitive to the G protein activator AIF₍₃₋₅₎ (Robinson and Kreis, 1992; Wong and Brodsky, 1992). In the early secretory pathway, it has been proposed that both AIF₍₃₋₅₎ and purified $\beta\gamma$ subunits modulate the cyclical membrane association of β -COPS and ARF (putative components of non-clathrin coats) (Orci et al., 1991; Serafini et al., 1991b; Waters et al., 1991), through a G protein-mediated event (Donaldson et al., 1990, 1991; Ktistakis et al., 1992). Finally, one of the members of the G protein family ($G_{\alpha 13}$) is found in the Golgi complex (Stow et al., 1991). Overexpression of $G_{\alpha 13}$ results in the pertussis toxin-sensitive retardation of the flow of glycosaminoglycans through the Golgi stack (Stow et al., 1991).

The cumulative results suggest that a biochemical cascade involving multiple GTP-binding proteins may regulate different aspects of vesicle budding, targeting and/or fusion between compartments of the exocytic pathway. To begin to address the role of GTP-binding proteins in regulating ER to Golgi transport in mammalian cells, we have explored the biochemical and morphological consequences of incubating cells *in vitro* in the presence of reagents which antagonize the function of both small GTP-binding proteins and heterotrimeric G proteins. These reagents give rise to distinct morphological phenotypes resulting in either inhibition of export from the ER or accumulation in pre-Golgi intermediates. These results provide new evidence to support the hypothesis that at least five GTP-binding proteins ($G_{\alpha\beta\gamma}$, rab1A, rab1B, rab2, ARF) and possibly SAR1 participate in the regulation of transport at the first stage of the secretory pathway in mammalian cells. The potential role for a heterotrimeric G protein poised at the base of this cascade to "gate" the exit of protein from the ER through agonist coupled G protein receptors is discussed.

Materials and Methods

Materials

G_{α} and $G_{\alpha\beta\gamma}$ were prepared from bovine brain as described previously (Sternweis and Robishaw, 1984). These preparations were typically enriched in G_{i1-2} and G_o , but also contained a spectrum of other uncharacterized G proteins. $\beta\gamma$ subunits were prepared from bovine brain (Bokoch, 1987). Transducin $\beta\gamma$ was kindly provided by Y.-K. Ho (University of Chicago, Chicago, IL). Pertussis and cholera toxins were obtained from

Sigma Chemical Co. (St. Louis, MO). ECG 163 and ECG 125 were provided by R. Ghadira (The Scripps Research Institute, La Jolla, CA). Mastoparan 11 and 17 were provided by T. Higashima (University of Texas Southwestern Medical School, Dallas, TX). GTP γ S was obtained from Calbiochem-Behring Corp. (La Jolla, CA). Antibodies towards rab1B were as described previously (Plutner et al., 1991).

Incubation Conditions and Analysis of Transport

The ER to Golgi transport assays using clone 15B semi-intact cells infected with tsO45 VSV were performed as described previously (Beckers et al., 1987; Beckers and Balch, 1989). Briefly, transport incubations contained in a final total volume of 40 μ l (final concentration): 25 mM Hepes-KOH (pH 7.2), 75 mM KOAc, 2.5 mM MgOAc, 5 mM EGTA, 1.8 mM CaCl₂, 1 mM ATP, 5 mM creatine phosphate, 0.2 IU of rabbit muscle creatine phosphokinase, 25–75 μ g cytosol, and 5 μ l (25–30 μ g of protein; $1-2 \times 10^5$ cells) of semi-intact cells. Transport was initiated by transfer to 32°C. After termination of transport by transfer to ice, the membranes were pelleted by a brief (15 s) centrifugation in a microfuge at top speed. For analysis of processing of VSV-G protein to the Man₅ form, the pellet was subsequently solubilized in an endoglycosidase D (endo D)¹ digestion buffer and digested with endo D as described previously (Beckers et al., 1987). Endo D digestions were terminated by adding a 5 \times concentrated gel sample buffer (Laemmli, 1970) and boiling for 5 min. The samples were analyzed by SDS-PAGE, autoradiographed, and the fraction of VSV-G protein processed to the endo D-sensitive form determined by densitometry (Beckers and Balch, 1989; Beckers et al., 1987).

Experimental procedures for digitonin permeabilization, incubation *in vitro*, indirect immunofluorescence, and analysis of processing of VSV-G protein to endo H-resistant forms using NRK cells were as described previously (Plutner et al., 1992). In all experiments, NRK cells were permeabilized with 40 μ g/ml digitonin.

Pertussis and Cholera Toxin Treatment of Cells

To determine the effect of pertussis or cholera toxins on transport, clone 15B, or NRK cells were pretreated with pertussis (1 μ g/ml) or cholera (1 μ g/ml) toxin from 4 to up to 16 h. After infection with tsO45 VSV, cells were labeled with [³⁵S]-met at 39.5° for 5 min. Aliquots were then directly incubated in labeling medium, or permeabilized and incubated in the transport cocktail described above. Transport was terminated by transfer to ice and the fraction of VSV-G protein processed to the endo D- or endo H-sensitive forms using 15B or NRK cells, respectively, as described previously (Plutner et al., 1992). The effectiveness of the toxin treatment was evaluated by labeling *in vitro* post toxin incubation in the presence of [³²P]-NAD as described (Bokoch and Gilman, 1984).

Results

VSV-G Protein Accumulates in Distinct Transport Intermediates in the Presence of GTP γ S

Processing of VSV-G protein from the high mannose (Man₆) form to the Man₅ form by the *cis/medial*-Golgi enzyme α 1,2 mannosidase I in semi-intact clone 15B CHO cells is inhibited by the non-hydrolyzable analog of GTP, GTP γ S (Beckers and Balch, 1989). To define the morphological site of inhibition (either transport vesicle formation from the ER or vesicle targeting/fusion to Golgi compartments) digitonin permeabilized NRK cells infected with tsO45 VSV-G protein were prepared as described previously (Plutner et al., 1992) and incubated in the presence of increasing concentrations of GTP γ S. As shown in Fig. 1 (lanes c–e), incubation in the presence of as little as 1 μ M GTP γ S completely inhibited transport and processing of VSV-G protein from the endo

1. *Abbreviations used in this paper:* BFA, brefeldin A; CGN, *cis*-Golgi network; CtX, cholera toxin; endo D, endoglycosidase; endo H, endoglycosidase; GTP γ S, guanosine-5'-O-(3-thiophosphate); Man II, α -1,2-mannosidase; MC-LR, microcystin; OKA, okadaic acid; PtX, pertussis toxin; t β γ , transducin $\beta\gamma$; VSV-G, vesicular stomatitis virus glycoprotein.

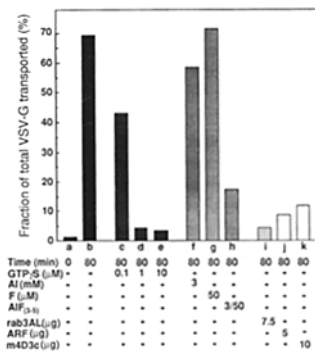


Figure 1. Effect of inhibitors of GTP-binding proteins on processing of VSV-G protein to the endo H_R form. NRK cells were permeabilized and incubated in suspension for 80 min in vitro as described previously (Plutner et al., 1992). The fraction of VSV-G protein processed to the endo H_R form containing terminal GlcNAc was determined as described (Plutner et al., 1992).

The concentrations of reagents

used in each assay are as indicated (40 μ l final volume). Rab3AL is a 16-residue peptide which is homologous to the rab1 effector domain (Plutner et al., 1990). ARF is a 16-residue peptide identical to the NH₂ terminus of hARF1 (Balch et al., 1992). m4D3c is a mAb which is specific for rab1B and inhibits rab1 function in vitro (Plutner et al., 1991).

H-sensitive (endo H_S) ER form to endo H-resistant (endo H_R) Golgi forms in NRK cells. Consistent with this result, incubation in the presence of excess (25 μ M) GTP γ S (to insure a strong phenotype) had a striking effect on the morphological transport of VSV-G. After 45 min of incubation in vitro, conditions which normally result in the efficient delivery of VSV-G to the Golgi compartments enriched in α -1,2-mannosidase II (Man II) (Plutner et al., 1992), VSV-G protein was concentrated in numerous small, punctate structures scattered throughout the cytoplasm (Fig. 2 A, *small arrowheads*). This distribution was similar to the distribution of VSV-G after ~5–10 min of incubation in vitro in the absence of GTP γ S (Plutner et al., 1992). These punctate structures for the most part did not colocalize with Golgi compartments containing Man II (Fig. 2, compare A, VSV-G, and B, Man II, *open arrows*), although in some cases VSV-G protein could also be detected in a more diffuse compartment in the vicinity of the Man II containing Golgi compartments (Fig. 2, A and B, *large solid arrow*), representing VSV-G protein retained in the ER network found in the perinuclear region.

Since VSV-G is vectorially transported from the ER to the Man II containing Golgi compartments via peripheral and central p58 containing compartments (Plutner et al., 1992), we examined if VSV-G matured to these structures in the presence of GTP γ S. Incubation for 45 min in the presence of GTP γ S resulted in the accumulation of VSV-G protein in numerous small, punctate structures which showed only limited colocalization with p58 containing compartments in both the peripheral and central regions of the cell (Fig. 2, compare C, VSV-G, and D, p58, *open arrows*). Typically, <10–20% of the VSV-G protein colocalized with p58 after incubation for 45 min in the presence of GTP γ S (Fig. 2, C and D, *solid arrows* and *boxed region*). In contrast, in the absence of GTP γ S >75% of the VSV-G protein was found in p58-containing compartments after a similar time (Plutner et al., 1992). We observed an identical level of inhibition with other GTP analogs such as GMPPCP and GMPPNP, but have not observed inhibition either biochemically (based on VSV-G protein oligosaccharide processing) or morphologically in the presence of up to 100 μ M GDP β S (not shown). These results suggest that reagents which prevent GTP hydrolysis inhibit an early step in export of protein from the ER and maturation of the p58-containing compartment.

Rab1 Is Required for Export from the ER

To define the role of GTP-binding proteins in ER to Golgi transport, we first focused on the role of the rab1 protein. We have previously demonstrated that a mAb specific for rab1B (m4D3c) inhibits an early step in processing of VSV-G protein from the Man₉ to the Man₅ form in perforated clone 15B CHO cells (Plutner et al., 1991). As shown in Fig. 1 (lane k), processing of VSV-G protein to the endo H_R form in digitonin permeabilized NRK cells was also strongly inhibited by m4D3c. However, and in contrast to the effects of GTP γ S which allowed partial mobilization of VSV-G protein into punctate transport intermediates, incubation in the presence of the anti-rab1B antibody completely blocked exit of VSV-G protein from the ER (Fig. 3). VSV-G protein was not detected in small punctate intermediates nor downstream compartments containing either p58 or Man II (data not shown). The morphological phenotype observed in the presence of m4D3c was identical to that observed when permeabilized cells were incubated in the absence of either ATP or cytosol (Plutner et al., 1992). Inhibition by the m4D3c antibody suggests that rab1B or a protein complex containing rab1B is essential for an initial step in export of VSV-G protein from the ER.

To explore the basis for inhibition by m4D3c, we examined the morphological distribution of rab1B after different times of incubation in vitro. As shown in Fig. 4, before incubation in vitro rab1B was present on Golgi compartments (Fig. 4 A, *large arrows*) and on numerous small punctate structures, consistent with its distribution in intact cells (Plutner et al., 1991). After 15 and 30 min of incubation we have consistently detected an increase in the intensity of immunostaining of rab1B in Golgi membranes (Fig. 4, B and C, *large arrows*) and its appearance on small punctate structures distributed throughout the peripheral and central regions of the cell (Fig. 4, B and C, *small arrows*). This distribution was similar to the pattern observed when VSV-G protein was transported to the p58-containing intermediates during incubation in vitro for a similar time period (Plutner et al., 1992). After 60 min of incubation rab1B attained the steady state distribution shown in Fig. 4 (D, *small and large arrows*).

To determine if the punctate compartments containing rab1B were part of the early secretory pathway we compared the distribution of rab1B with that of p58 before and after incubation in vitro. As shown in Fig. 5, before incubation both rab1B and p58 were predominately colocalized in the Golgi region (Fig. 5, A, p58, and B, rab1B, *large arrowheads*), although weakly staining punctate structures which contained p58 or rab1B could also be detected in the peripheral cytoplasm (Fig. 5, A, p58, and B, rab1B, *small arrowheads*). When cells were incubated for 45 min in vitro, typically between 40–70% of the p58-containing punctate compartments were found to colocalize with rab1B (Fig. 5, C, p58, and D, rab1B, *small arrows*), although colocalization was not always complete (Fig. 5, C, p58, and D, VSV-G, *large arrows*). A similar result was observed after only 15–20 min of incubation (not shown), suggesting that in addition to the central Golgi stack, many of the peripheral p58-containing compartments rapidly acquired a pool of membrane-associated rab1B during incubation of NRK cells in vitro.

What is the extent of colocalization between rab1B and VSV-G during transport? As shown in Fig. 6, VSV-G was ex-

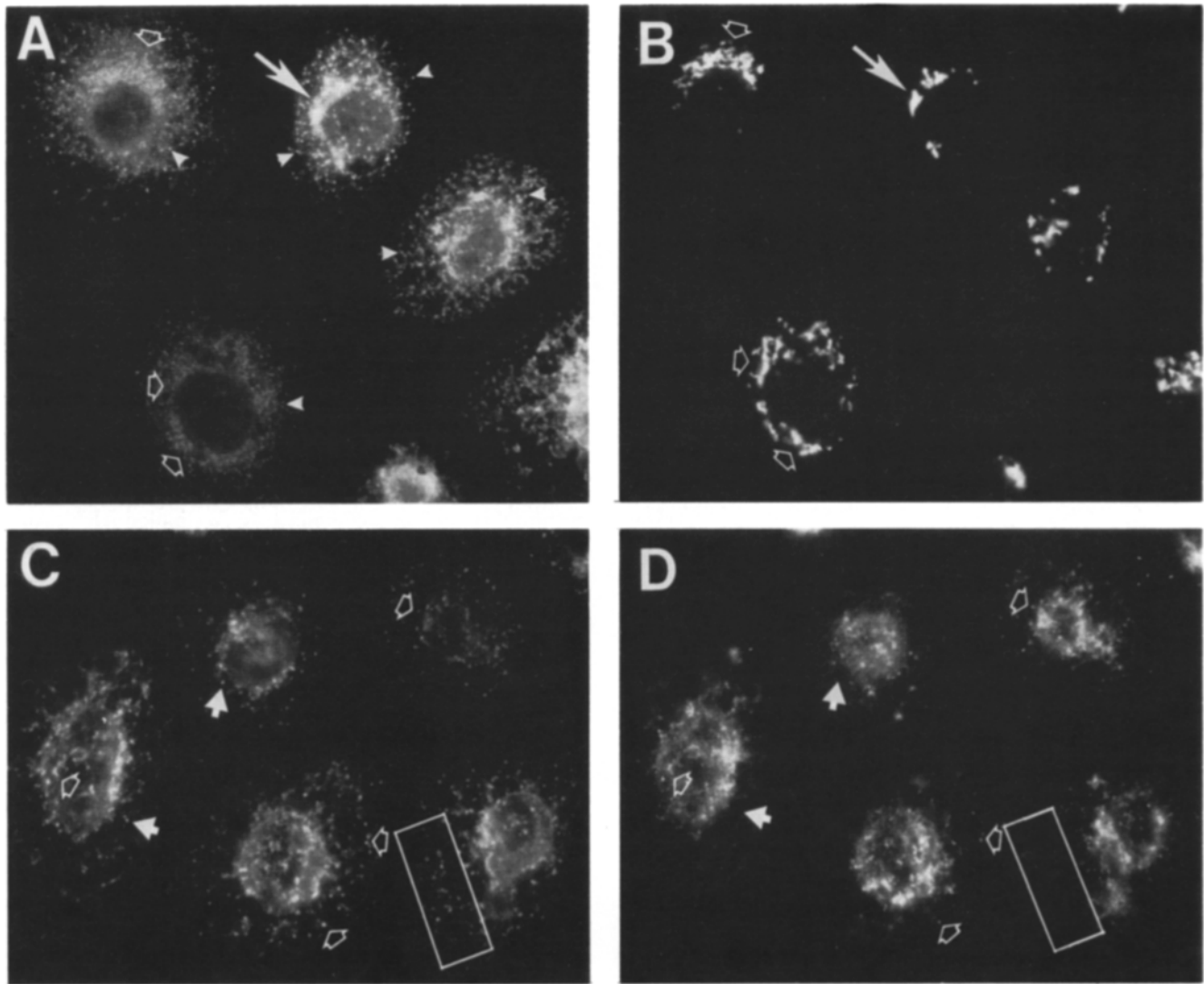


Figure 2. GTP γ S inhibits transport to Golgi compartments containing Man II and p58. (A and B) NRK cells were permeabilized, incubated for 45 min in a complete cocktail containing cytosol and ATP in the presence of 25 μ M GTP γ S, and the distribution of VSV-G (A) and Golgi compartments containing Man II determined using indirect immunofluorescence as described previously (Plutner et al., 1992). Arrowheads indicate numerous small punctate structures containing VSV-G which accumulate in the presence of GTP γ S; open arrows indicate regions which lack colocalization of VSV-G with Man II; the solid arrow indicates a region which shows partial colocalization of VSV-G and Man II. (C and D) NRK cells were permeabilized, incubated for 45 min in a complete cocktail in the presence of 25 μ M GTP γ S, and the distribution of VSV-G (C) and pre-Golgi compartments containing p58 (D) determined using indirect immunofluorescence as described previously (Plutner et al., 1992). Open arrows indicate punctate structures containing VSV-G which colocalize with p58; solid arrows indicate punctate structures containing VSV-G which do not colocalize with p58; the boxed region indicates numerous small punctate structures accumulating in the presence of GTP γ S which contain VSV-G but not p58.

clusively found in the ER before incubation in vitro (Fig. 6 B), whereas rab1B was largely confined to the perinuclear Golgi region (Fig. 6 A, large arrows) and in small punctate structures. After incubation for only 15 min in vitro, a time point which precedes processing of VSV-G to the endo H_R form, but results in the strong colocalization of VSV-G with the p58-containing compartments (Plutner et al., 1992), typically 50–75% of the punctate structures containing VSV-G also colocalized with rab1B (Fig. 6, C, rab1B, and D, VSV-G). An identical result was observed after 45-min incubation (Fig. 6, E, E' [rab1B]), and F, F' [VSV-G]). At earlier timepoints (between 5 and 10 min) when VSV-G was being

recruited from its diffuse reticular distribution to the first detectable punctate structures which lacked p58, a weak but reproducible colocalization of VSV-G protein with rab1B with these punctate structures could be detected (not shown). In contrast, when permeabilized cells were incubated in the presence of GTP γ S for 45 min, VSV-G protein containing punctate intermediates for the most part (typically <10%) did not contain rab1B (Fig. 7, compare A, VSV-G, with B, rab1B, arrowheads), although rab1B could still be detected on other punctate structures and in the more central Golgi region (Fig. 7 B). Thus, GTP γ S blocks rab1B association with early transport intermediates.

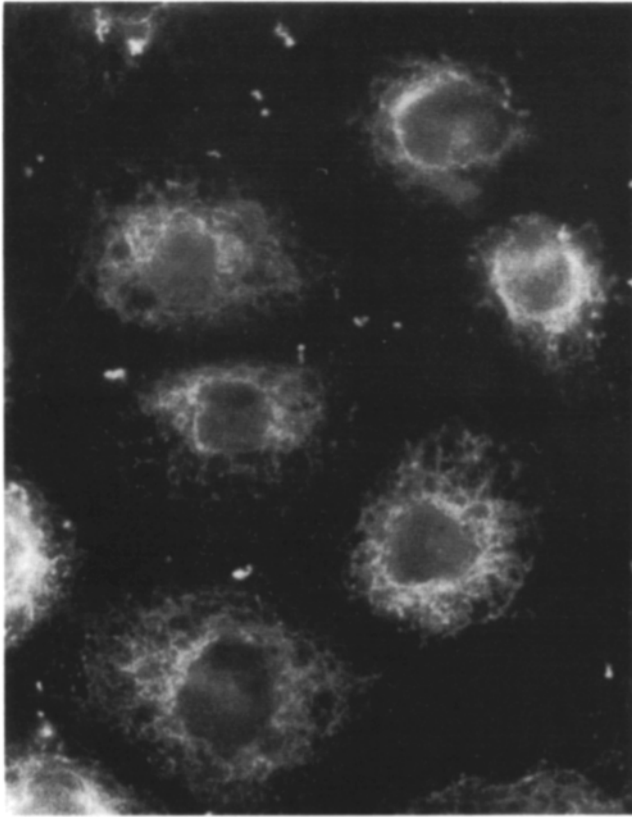


Figure 3. An antibody which inhibits rab1 function prevents export of VSV-G protein from the ER. NRK cells were permeabilized and preincubated for 60 min on ice in the presence of cytosol and 5 μ g of a monoclonal reagent (m4D3c) which is specific for rab1B (Plutner et al., 1991). Cells were supplemented with ATP and incubated for 45 min at 32°C as described previously (Plutner et al., 1992). The distribution of VSV-G protein was determined as described (Plutner et al., 1992). The punctate fluorescent structures observed outside the cell boundaries are an artifact associated with the detection of small aggregates of m4D3c by the Texas red-conjugated second reagent used to localize VSV-G protein.

Rab3AL and ARF Peptides Block Different Steps in Transport between the ER and the Golgi

We have previously demonstrated that a peptide (rab3AL) homologous to the putative effector domain of rab1 inhibits a late Ca^{2+} -dependent fusion step in ER to Golgi transport (Plutner et al., 1990). More recently, a peptide identical to the NH₂ terminus of human ADP-ribosylation factor 1 (hARF1) which prevents hARF1 from serving as a cofactor in cholera-toxin-catalyzed ADP-ribosylation of G_s (Kahn et al., 1992), was found to inhibit the transport and processing of VSV-G protein from the Man₉ oligosaccharide ER form to the Man₅ Golgi-associated form in perforated clone 15B CHO cells (Balch et al., 1992). Consistent with this result, the addition of excess recombinant hARF1 protein which was posttranslationally myristylated (myr-hARF1) inhibited transport of VSV-G (Balch et al., 1992) suggesting that hARF1 and possibly other transport components involved in vesicular trafficking may function efficiently only when present at optimal ratios (Balch et al., 1992).

To assess the morphological consequences of these re-

agents on VSV-G protein transport, digitonin permeabilized cells were incubated in vitro for 45 min in the presence of either the hARF1 or rab3AL peptides at concentrations which inhibit >95% processing of VSV-G to the endo H_R form (Fig. 1, lanes *i* and *j*). In the presence of the rab3AL peptide VSV-G protein was exported from the ER and accumulated in punctate, pre-Golgi structures (Fig. 8 *A*). These structures did not colocalize with Man II-containing Golgi compartments (not shown), but did show partial (typically 25–50%) colocalization with p58 (not shown). In contrast, incubation in the presence of the hARF1 peptide resulted in the complete inhibition of export of VSV-G protein from the ER. No detectable maturation to either early or late punctate intermediates was observed (Fig. 8 *B*). A similar result was observed in the presence of excess recombinant myr-hARF1 protein (Fig. 8 *C*). In this case, export from the ER reticulum was largely inhibited, although a limited number of small, uniform punctate structures distributed throughout the cytoplasm could be detected (Fig. 8 *C*, *arrows*). These results suggest that ARF or a component(s) facilitating ARF function are critical for initiating transport vesicle formation from the ER while a downstream effector molecule may be required for rab1 to function in targeting or fusion of transport vesicles to acceptor compartments.

AIF₍₃₋₅₎ Permits Transport to the p58-containing Compartment but not the cis-Golgi Reticulum

In contrast to GTP γ S, which is likely to inhibit the function of most GTP-binding proteins, AIF₍₃₋₅₎ binds to heterotrimeric G proteins, but not members of the *rab* or *ARF* gene families (Kahn, 1991). The AIF₍₃₋₅₎ complex is believed to interact with the GDP-bound form of G _{α} , the fluoride group serving as a mnemonic for the γ -phosphate, directly activating the protein. As shown in Fig. 1, incubation of digitonin permeabilized cells in the presence of 50 μ M AlCl₃ or 3 mM KF separately had no effect on processing of VSV-G protein to the endo H_R form (Fig. 1, lanes *f* and *g*). However, incubation in the combined presence of AlCl₃ (50 μ M) + KF (3 mM) inhibited processing of VSV-G protein to the endo H-resistant form by ~75% (Fig. 1, lane *h*), the maximum level of inhibition obtainable with this reagent. Morphological analyses were consistent with this result. Incubation in the presence of either AlCl₃ (Fig. 9, *A* and *D*) or KF (Fig. 9, *B* and *E*) alone did not inhibit the morphological movement of VSV-G protein into the punctate transport intermediates or to Golgi compartments containing Man II (Fig. 9, *A* and *B* [VSV-G], *D* and *E* [Man II], *solid arrows*). In contrast, while incubation in the presence of the AIF₍₃₋₅₎ complex allowed transport of VSV-G to the punctate p58-containing compartments, it largely prevented transport to Man II-containing Golgi compartments (Fig. 9, *C*, VSV-G, and *F*, Man II), suggesting a considerably reduced level of vesicular traffic to the more central Golgi region.

Mastoparan Inhibits Export from the ER

To further analyze the possibility that a heterotrimeric G protein(s) regulates ER to Golgi transport, we examined the effects of the tetradecapeptide mastoparan and related compounds. Mastoparan has been extensively studied in its capacity to activate G proteins (Mousli et al., 1990). It is a cat-

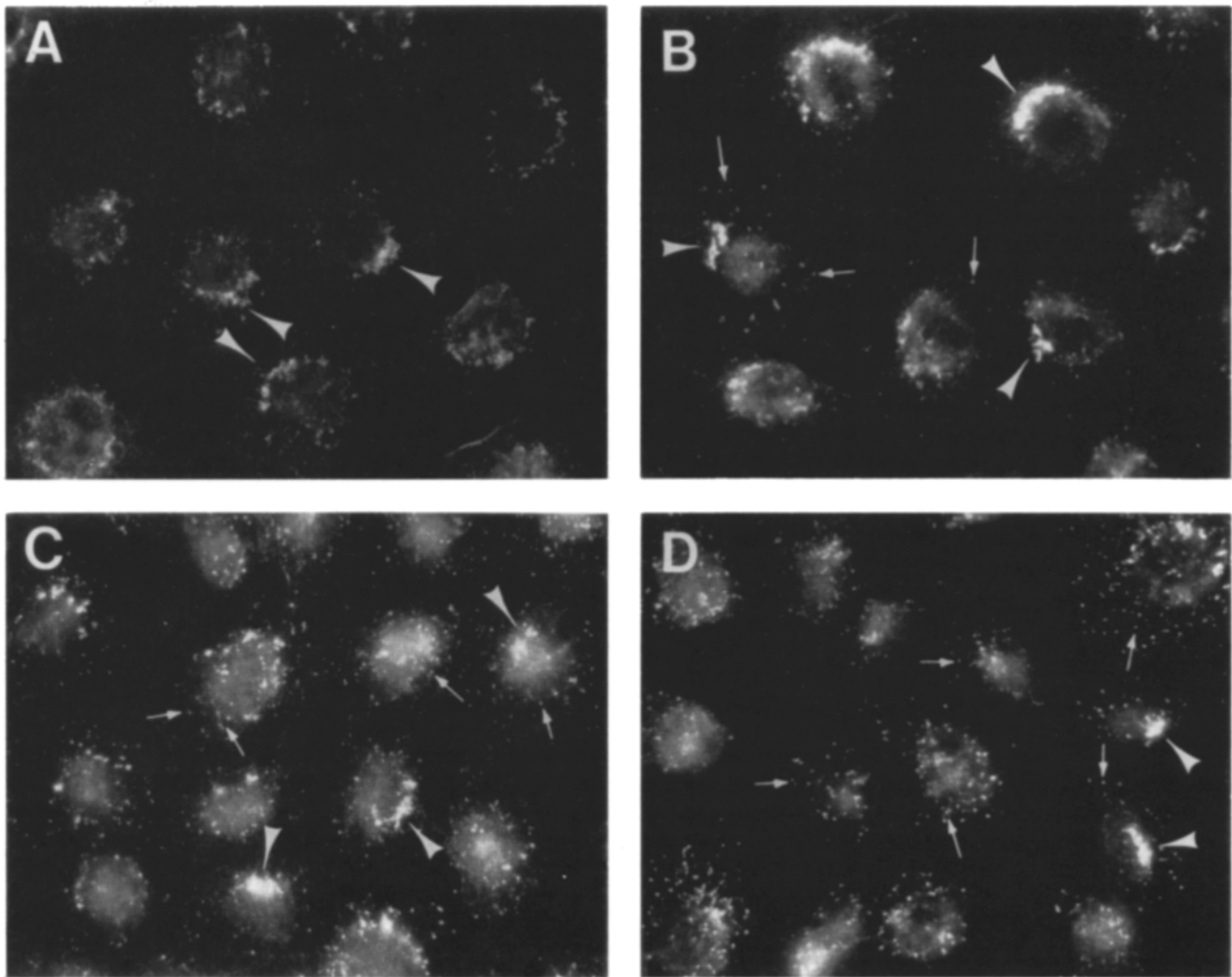


Figure 4. Redistribution of rab1B during incubation *in vitro*. NRK cells were permeabilized and incubated in the presence of a complete cocktail on ice for 60 min (A) or at 32°C for 10 min (B), 30 min (C), or 60 min (D). The cells were fixed and the distribution of rab1B determined by indirect immunofluorescence using the monoclonal antibody (m5C6b) (Plutner et al., 1992). Large arrowheads indicate rab1B associated with the larger perinuclear Golgi compartments; the small arrows indicate the association with small, punctate structures distributed throughout the peripheral and central cytoplasm.

ionic amphiphilic peptide which binds to the carboxyl terminus of G_{α} subunits blocking receptor interaction (Ross, 1989; Okamoto et al., 1991; G. M. Balch, unpublished data) and triggers GDP/GTP exchange leading to G protein activation (Weingarten et al., 1990; Higashijima and Ross, 1991). If a heterotrimeric G protein were involved in ER to Golgi transport, uncoupling it from its cognate receptors might inhibit transport.

As shown in Fig. 10 (A), addition of increasing concentrations of mastoparan resulted in potent inhibition of the transport of VSV-G protein from the ER to the *cis*-Golgi compartment *in vitro* (based on processing of VSV-G from the Man_9 to the Man_5 oligosaccharide form). Partial inhibition by mastoparan was observed at concentrations of $<0.2 \mu\text{g}$ ($2.5 \mu\text{M}$), with an EC_{50} of $\sim 7.5 \mu\text{M}$. Greater than 80–90% inhibition was observed in the presence of $1 \mu\text{g}$ ($17 \mu\text{M}$) mastoparan. Mastoparan related peptides (mast-X and polistes mast, Table I) were also found to inhibit transport with

polistes-mastoparan being the most potent, inhibiting transport at an $EC_{50} < 2.5 \mu\text{M}$ (Table I). These results are consistent with the reported EC_{50} concentrations of mastoparan required to activate G-protein-coupled effectors (Higashijima et al., 1990; Mousli et al., 1990). Addition of a functionally related basic secretagogue, compound 48/80, which also stimulates mast cell degranulation (Mousli et al., 1990), was also found to be a potent inhibitor of ER to Golgi transport *in vitro* (Fig. 10 A). Identical results were obtained with processing of VSV-G protein to the endo H_R form in digitonin permeabilized cells (see Fig. 13).

The ability of mastoparan to activate G proteins is dependent on both its amphiphilicity and hydrophobicity. Amino acid substitutions which alter either the charge distribution or the hydrophobic moment alter its capacity to activate G proteins (Higashijima et al., 1990). In addition, unrelated peptides which form cationic, amphiphilic structures will also substitute for mastoparan and activate G proteins, albeit

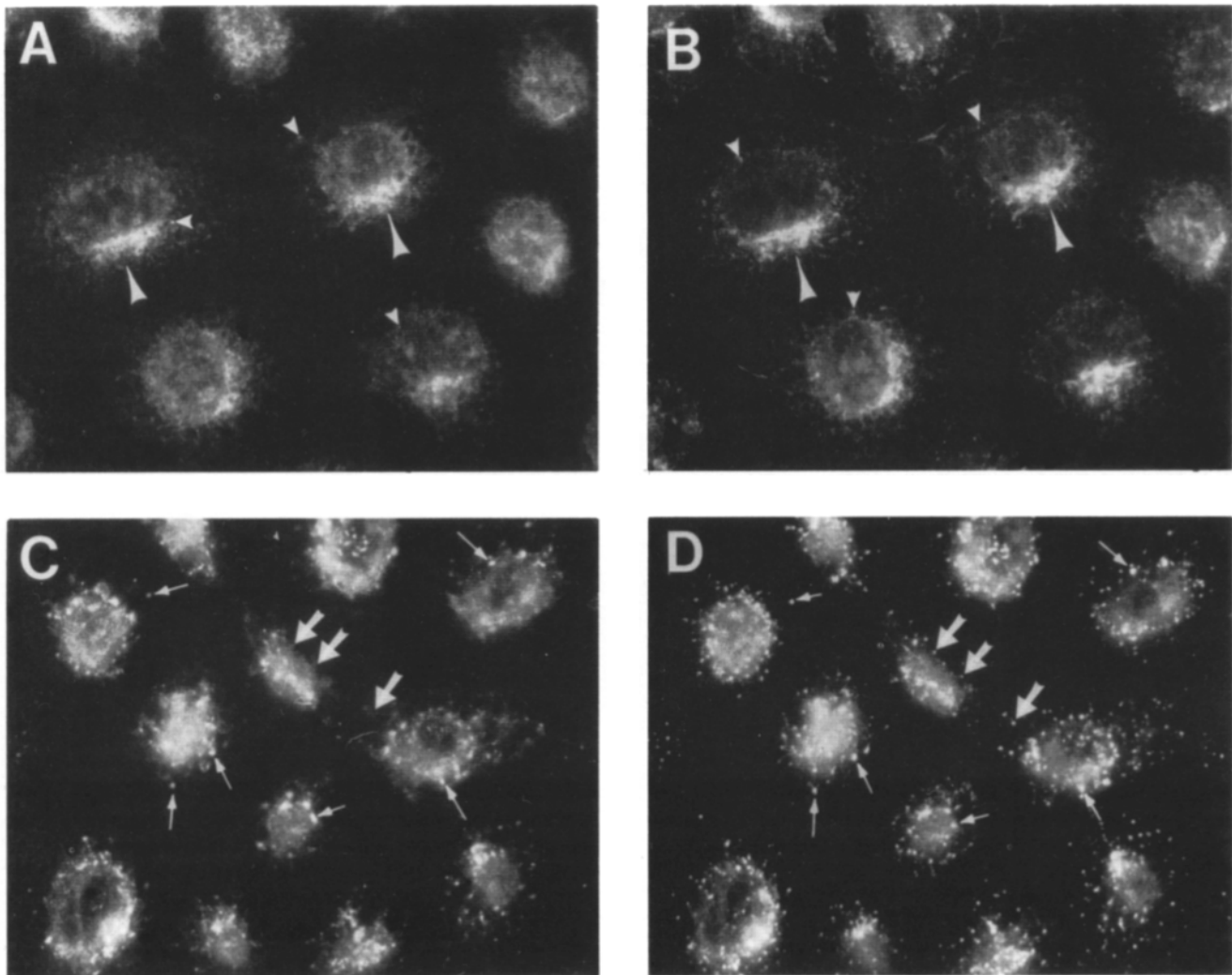


Figure 5. Rab1B colocalizes with p58 containing compartments in the central and peripheral regions of the cytoplasm. NRK cells were permeabilized and incubated on ice (*A* and *B*) or for 45 min at 32°C in the presence of a complete cocktail, and the distribution of p58 (*A* and *C*) or rab1B (*B* and *D*) determined using indirect immunofluorescence (using 5C6b) as described (Plutner et al., 1992). (*A* and *B*) The large arrowheads indicate the distribution of rab1B containing Golgi stacks; the small arrowheads indicate rab1B and p58 associated with small punctate structures before incubation. (*C* and *D*) The large arrows indicate vesicles which contain rab1B but not p58; the small arrows indicate vesicles in which rab1B and p58 are colocalized.

to a lesser efficiency (Higashijima et al., 1990; Mousli et al., 1990). To assess the specificity of mastoparan and its relationship to peptides with similar amphipathic structure and charge distribution, several additional mastoparan-related peptides were tested. Removal of the two terminal amino acids (mast-3) or single amino acid substitutions which increase the potency of mastoparan (mast-7) were also found to inhibit ER to Golgi transport (Fig. 10 *B*; Table I). In contrast, substitutions which inhibit the formation of the amphipathic α -helix or the ability to bind to membranes (mast-11 and mast-17) eliminated the capacity of mastoparan to efficiently inhibit transport (Fig. 10 *B*; Table I). These results are consistent with the observation that mastoparan partitions between the aqueous phase and the surface of membranes. It has been demonstrated that the lateral surface concentration, not the total concentration in solution, is likely to determine the extent of G protein activation (Higashijima

et al., 1990). We have observed a similar effect in our assays (not shown).

In addition to mastoparan, several other peptides which form amphiphilic cationic structures were tested (Table I). When compared with mastoparan, the hARF1 peptide which has similar structural properties (Kahn et al., 1992) had a nearly identical EC_{50} of inhibition (Fig. 10 *B*; Table I). In contrast, the ECG 125 peptide of unrelated sequence to mastoparan or the hARF1 peptide (Table I), but which forms α -helical structures with similar amphiphilic and hydrophobic properties had no effect on transport (Fig. 10 *B*; Table I).

Mastoparan Inhibits an Early Step in Transport

Transport of protein between the ER and the Golgi compartment in vivo proceeds via distinct punctate pre-Golgi intermediates (Saraste and Kuismanen, 1984; Saraste and Svens-

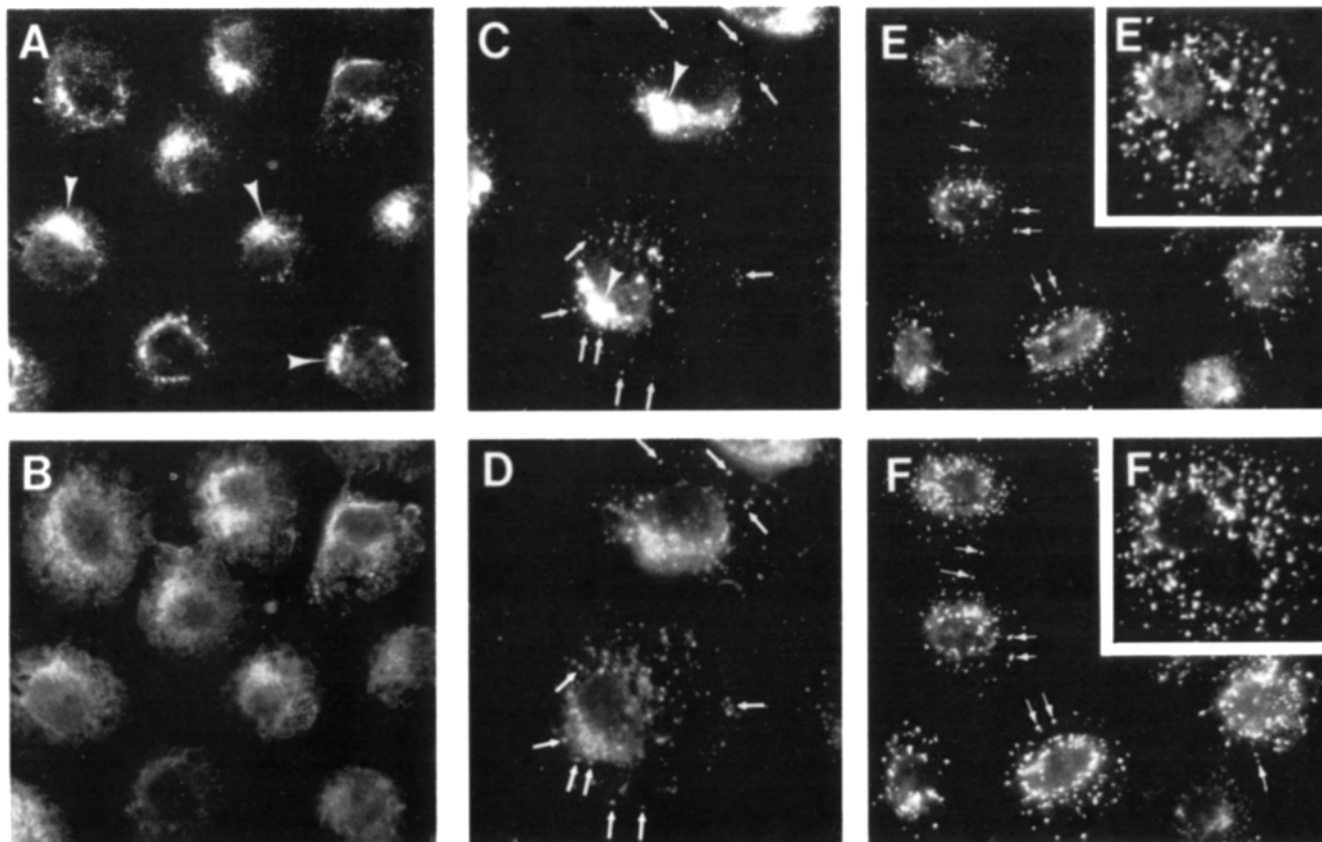


Figure 6. Rab1B and VSV-G occupy the same punctate structures after incubation in vitro. NRK cells were permeabilized and incubated on ice (A and B), 15 min at 32°C (C and D) or 45 min at 32°C (E, E', F, and F'). The distribution of VSV-G (B, D, F, and F') or rab1 (A, C, E, and E') was determined by indirect immunofluorescence (Plutner et al., 1992). (A) The large arrowheads indicate the distribution of rab1 containing Golgi stacks. (C, D, E, and F) The small arrows indicate vesicles in which rab1B and VSV-G are colocalized. (E', F') Inset is an enlarged magnification of a cell illustrating the striking colocalization after a 45-min incubation in vitro.

son, 1991; Schweizer et al., 1990; Schweizer et al., 1991). Similarly, transport of VSV-G protein in vitro in semi-intact cells proceeds through early pre-Golgi intermediates during the first 20 min of incubation (the "lag" period) before processing of oligosaccharides by the *cis/medial*-Golgi enzyme α -1,2 mannosidase I (Beckers et al., 1990; Schwaninger et al., 1991; Plutner et al., 1992). Export of VSV-G protein from the ER in vitro is largely complete during this first 20 min of incubation (Beckers and Balch, 1989; Beckers et al., 1990; Schwaninger et al., 1991; Plutner et al., 1992).

To determine the step in transport sensitive to mastoparan, semi-intact cells were incubated for increasing time at 32°C (to promote the exit of VSV-G from the ER into transport intermediates) before the addition of mastoparan. After the addition of mastoparan, cells were either transferred to ice or the incubation continued to chase any VSV-G protein which was transported beyond a mastoparan-sensitive step to the Golgi compartment. Since processing is not rate limiting (Beckers et al., 1987), if mastoparan inhibited a late vesicle fusion step then addition of the reagent at any time point should immediately terminate processing, phenotypically similar to transferring cells to ice (shown in Fig. 10 C, *closed circles*). Alternatively, if mastoparan were only to inhibit an early step involved in exit from the ER or targeting to the Golgi compartment, then addition of mastoparan after 20 min would not be expected to inhibit any further fusion,

resulting in extensive processing of VSV-G protein during the chase period in the presence of peptide.

As shown in Fig. 10 C, using a concentration of mastoparan sufficient to inhibit \sim 70–80% of VSV-G protein transport when added prior to the incubation, VSV-G rapidly entered ($t_{1/2} = \sim$ 5 min) a mastoparan-resistant intermediate during the lag period. Addition of peptide after 15 min of incubation, a time point at which an undetectable amount of the VSV-G protein has been delivered to the Golgi (Fig. 10, *closed circles*, $t = 15$ min), resulted in >70–80% of the total VSV-G protein transported in the control (Fig. 10 C, *closed circles*, $t = 90$) being processed during the subsequent chase period in the presence of peptide (Fig. 10 C, *open circles*, $t = 15$ min). Given the rapid and irreversible inhibition of transport by mastoparan ($t_{1/2}$ of \sim 30 s at 32°C) (data not shown), these results suggest that an early biochemical step is sensitive to a G protein-coupled receptor.

Mastoparan Inhibits the Exit of VSV-G Protein from the ER

Although our biochemical results suggested that an early step occurring during the lag period was inhibited by mastoparan, two morphological consequences were possible. One possibility consistent with the above results was that mastoparan blocked export from the ER per se, resulting in the

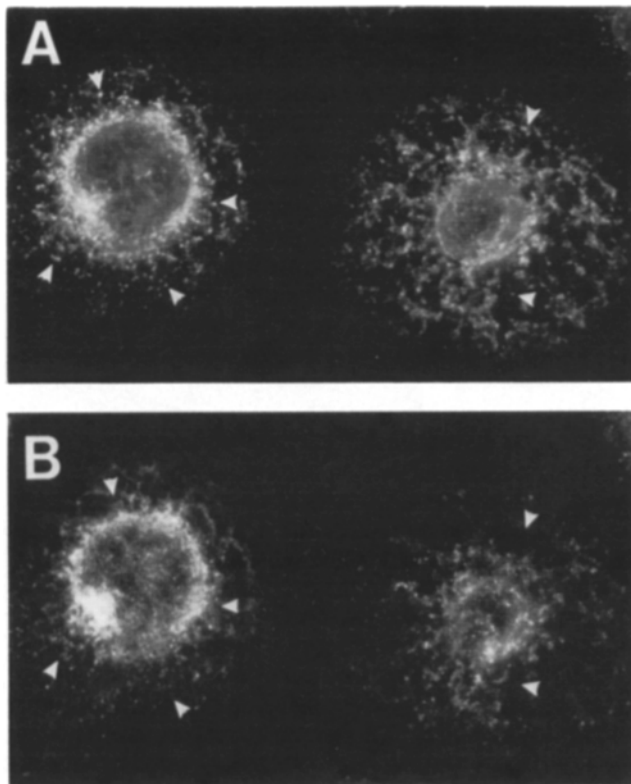
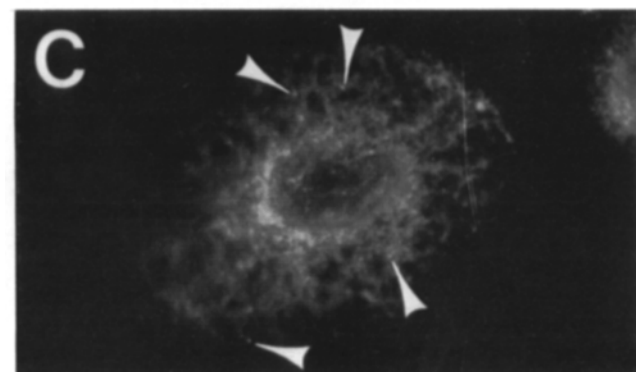
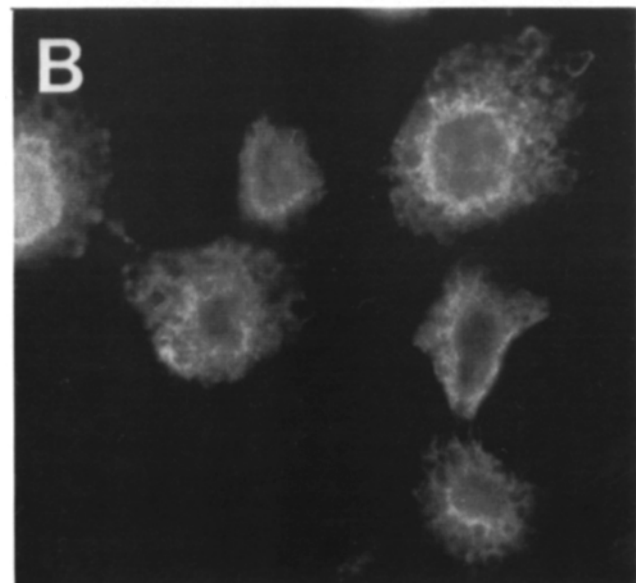
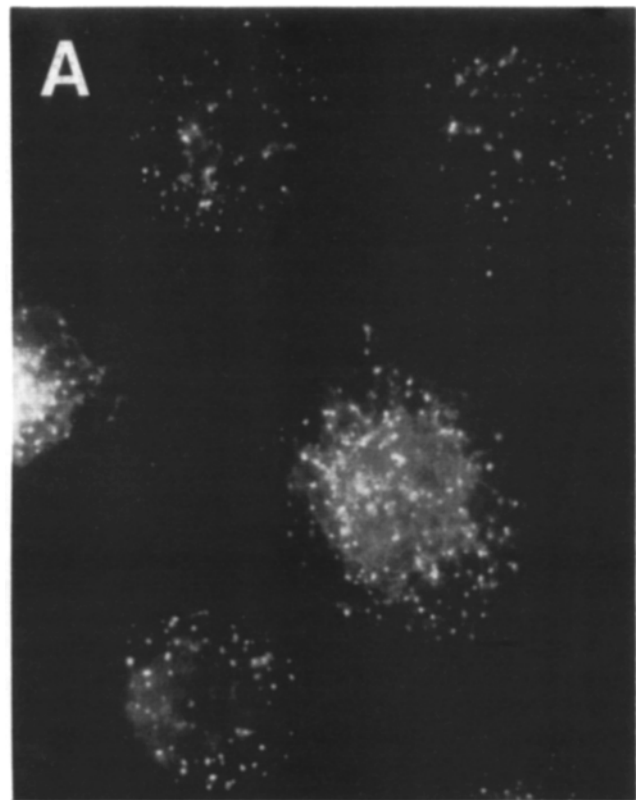


Figure 7. GTP γ S inhibits colocalization of VSV-G and rab1B. NRK cells were permeabilized, incubated for 45 min in a complete cocktail in the presence of 25 μ M GTP γ S, and the distribution of VSV-G (A) and rab1B (B) determined using indirect immunofluorescence as described for Fig. 6. Solid arrows indicate punctate structures containing VSV-G which do not colocalize with rab1B.

retention of VSV-G in the morphologically distinct ER network. Alternatively, export of VSV-G protein into punctate pre-Golgi transport intermediates (Saraste and Kuismanen, 1984; Saraste and Svensson, 1991; Schweizer et al., 1990, 1991; Plutner et al., 1992) may occur in the presence of mastoparan, but these intermediates may not fuse with the *cis*-Golgi compartment as a consequence of inhibition of some early priming step sensitive to mastoparan.

To determine the morphological effects of mastoparan on VSV-G protein transport, permeabilized cells were incubated in the presence of peptide for 80 min. As shown in Fig. 11, addition of mastoparan had a striking effect on export from the ER. In this case, no movement of VSV-G protein into the punctate pre-Golgi vesicular intermediates (Fig. 11 B, VSV-G), or to the Golgi compartment was observed (Fig. 11 C, VSV-G, and C', Man II), VSV-G protein being retained in a typical ER observed prior to incubation *in vitro* (Plutner et al., 1992). Export from the ER was also strongly inhibited

Figure 8. Rab3AL and ARF peptides inhibit transport at different steps during ER to Golgi transport. NRK cells were permeabilized, incubated for 45 min in a complete cocktail in the presence of 37.5 μ g of the rab3AL peptide (per 200 μ l reaction volume) (A), 25 μ g of the ARF peptide (per 200 μ l reaction volume) (B) or 5 μ g of posttranslationally myristylated hARF1 (Balch et al., 1992) (C) and the distribution of VSV-G protein determined using indirect immunofluorescence as described previously (Plutner et al., 1992).



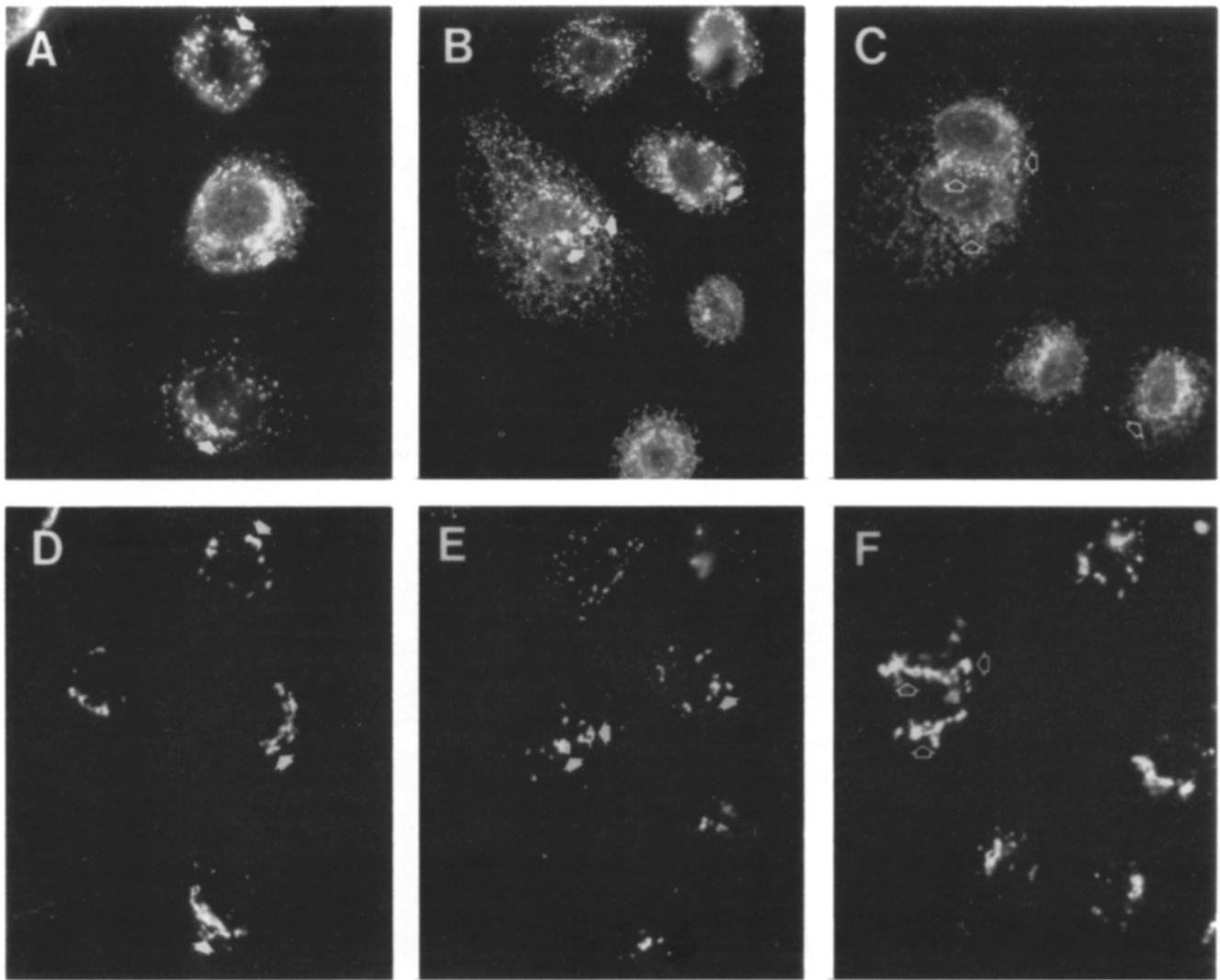


Figure 9. Effects of AIF₍₃₋₅₎ on VSV-G protein transport in vitro. NRK cells were permeabilized and incubated for 45 min in a complete cocktail containing 5 mM KF (*A* and *D*), 50 μ M AlCl₃ (*B* and *E*) or the AIF₍₃₋₅₎ complex prepared in assay buffer 5 min before the addition of cells and cytosol (*C* and *F*). The distribution of VSV-G (*A*, *B*, and *C*) or Man II (*D*, *E*, and *F*) was determined as described previously (Plutner et al., 1992).

by mastoparan 7 (mast-7), a more potent analog of the native peptide (Fig. 11 *D*). In contrast, incubation in the presence of 10-fold excess of mastoparan 11 (mast-11) or mastoparan 17 (mast-17) did not significantly inhibit morphological transport to punctate pre-Golgi intermediates or to the Golgi complex (Fig. 11, compare *E*, mast-11, and *F*, mast-17, to *A*, control). Under all conditions tested there was no significant difference in the distribution of the Golgi compartments compared to control incubations lacking peptides after similar periods of incubation (not shown).

We have scored the fraction of cells on coverslips which failed to export VSV-G protein from the ER based on three criteria: (*a*) colocalization with an ER marker protein; (*b*) lack of formation of the punctate intermediates; and (*c*) lack of colocalization with the Golgi compartment using indirect immunofluorescence. Compared to the control, <5% of cells on coverslips incubated in the presence of mastoparan and mast-7 exported VSV-G protein from the ER. In contrast, using the inactive mastoparan analogs mast-11 and -17 we ob-

served transport to punctate pre-Golgi intermediates and Golgi complexes in >95% of the cells.

$\beta\gamma$ Subunits of Heterotrimeric G Proteins Inhibit VSV-G Protein Exit from the ER

To provide a third and more direct line of evidence that heterotrimeric G proteins participate in the export of VSV-G protein from the ER, we examined the effect of G _{$\alpha\beta\gamma$} , G _{α_s} , and $\beta\gamma$ subunits purified from bovine brain on ER to Golgi transport in permeabilized NRK cells. $\beta\gamma$ subunits bind members of the G _{α} class of GTP-binding proteins, but do not bind *ras*-related small GTP-binding proteins (Pang and Sternweis, 1989, 1990; Bokoch and Quilliam, 1990). Our experiments were based on the observation that binding of agonist by receptor results in G _{α} subunit dissociation from the G _{$\alpha\beta\gamma$} complex and GDP/GTP exchange leading to activation of G _{α} (Ross, 1989; Simon et al., 1991). Previous studies have suggested that the presence of excess $\beta\gamma$

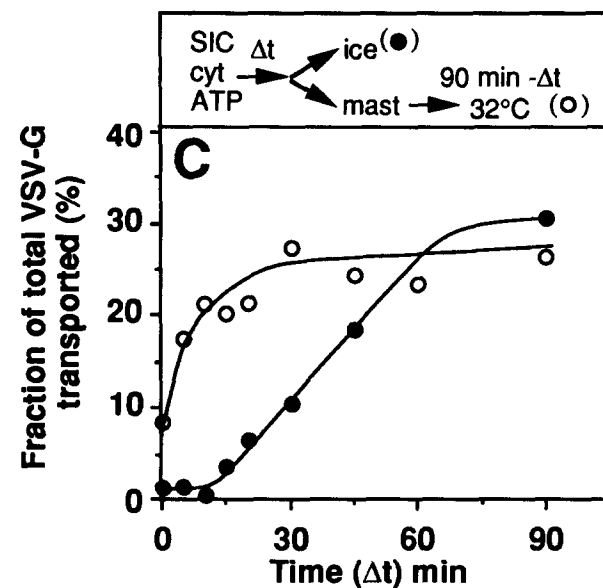
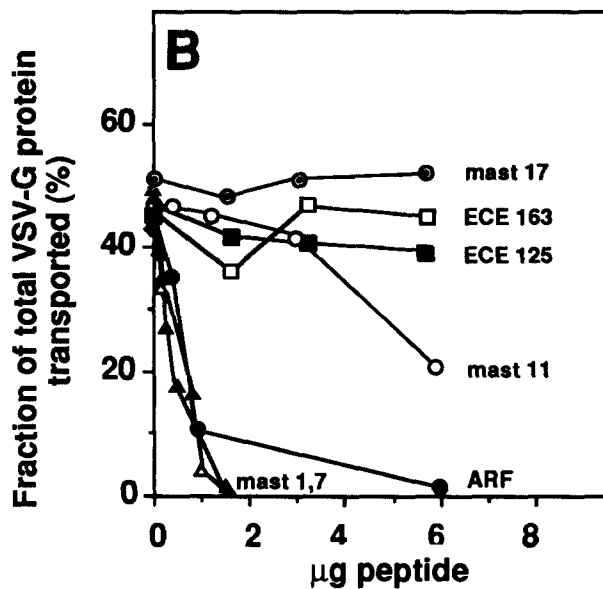
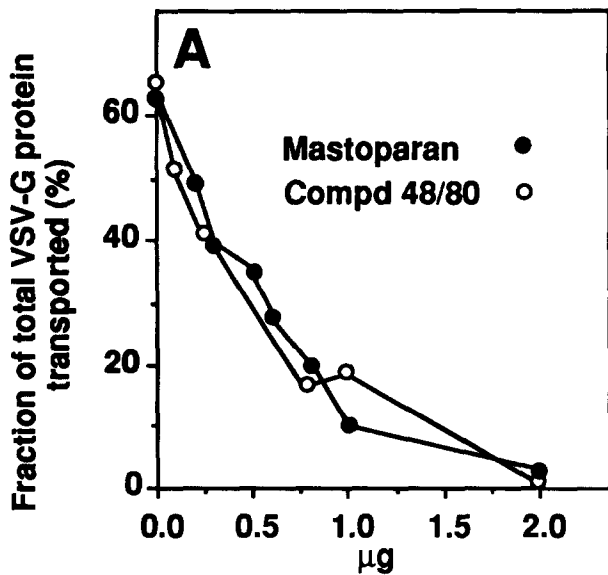


Table I. Sequences of Mastoparan and Related Peptides Used in Transport Assays

| Peptide | Sequence | EC ₅₀ * |
|---------------|-------------------------|--------------------|
| Mastoparan | INLKALAALAKKIL | 7.5 |
| Mast-3 | —LKALAALAKKIL | 5.0 |
| Mast-7 | INLKALAALAKA <u>I</u> L | 5.0 |
| Mast-11 | INLKALAAL <u>K</u> KKIL | >75 |
| Mast-17 | INLKA <u>K</u> AALAKKLL | >100 |
| Mast-X | INWKGIAAMAKKLL | 15 |
| Polistis mast | VDWKKIGQHILSVL | 2.5 |
| ARF | GNIFANLFKGLFGKKE | 7.5 |
| ECG125 | GELAQKLEQALQKLA | >100 |
| ECG163 | GEHAQKHEQALQKLA | >100 |

* Concentration (µM) of peptide required to inhibit transport by 50%. Bold letters emphasize distribution of Ile or Leu residues. Italicized letters emphasize distribution of positively charged lysine residues.

subunits can inhibit G_α activation of target effector proteins such as the adenylyl cyclase (Casey and Gilman, 1988; Katada et al., 1984; Neer and Clapham, 1988), muscarinic K⁺ channels (Okabe et al., 1990), Ca²⁺ channels (Lotersztajn et al., 1992), and phospholipase C (Boyer et al., 1989) through quenching of the free G_α pool (Okabe et al., 1990; Ross, 1989; reviewed in Brown and Birnbaumer, 1990). Thus, addition of βγ subunits to the assay would provide a more specific test for the participation of G_α in vesicular transport should an activated G_α subunit(s) be required.

Since brain G proteins, and their α or βγ subunits are prepared in the presence of detergent we first established conditions in which the buffer used for purification did not inhibit transport. Detergent concentrations of <0.005% only slightly inhibited the transport of VSV-G protein to the punctate pre-Golgi intermediates or to the Golgi complex using both biochemical and morphological assays (not shown). When a standard in vitro cocktail was supplemented with either G_α subunits (Fig. 12 C, Man II, and D, VSV-G) or G_{αβγ} (Fig. 12 E, Man II, and F, VSV-G) at concentrations up to 400 nM (in the presence of 0.005% detergent), no effect was observed on transport of VSV-G protein to the punctate intermediates or to the Golgi. In each case, >95% of the cells on coverslips responded with strong transport as shown in Fig. 12. These results suggest that neither the detergent buffer per se nor the presence of excess βγ subunits when complexed to the α subunit (G_{αβγ}) inhibit transport. In contrast, addition of purified βγ subunits at a final concentration of 200 nM inhibited export of VSV-G protein from the ER, resulting in its retention in the ER (Fig. 12 A, Man II, and B, VSV-G).

Figure 10. Mastoparan and related compounds inhibit ER to Golgi transport in vitro. (A and B) In vitro incubation conditions and analysis of transport were conducted as described previously (Beckers et al., 1990) in the presence of the indicated concentrations of peptides. Transport was detected processing of VSV-G to the endoglycosidase D (endo D) sensitive form (Beckers et al., 1987). See Table I for description of the peptides. (C) Semi-intact cells (SIC), cytosol (cyt), and ATP were incubated for increasing time (Δt) before transfer to ice (●), or addition of 15 µM mastoparan and incubation for a total time of 90 min at 32°C (○).

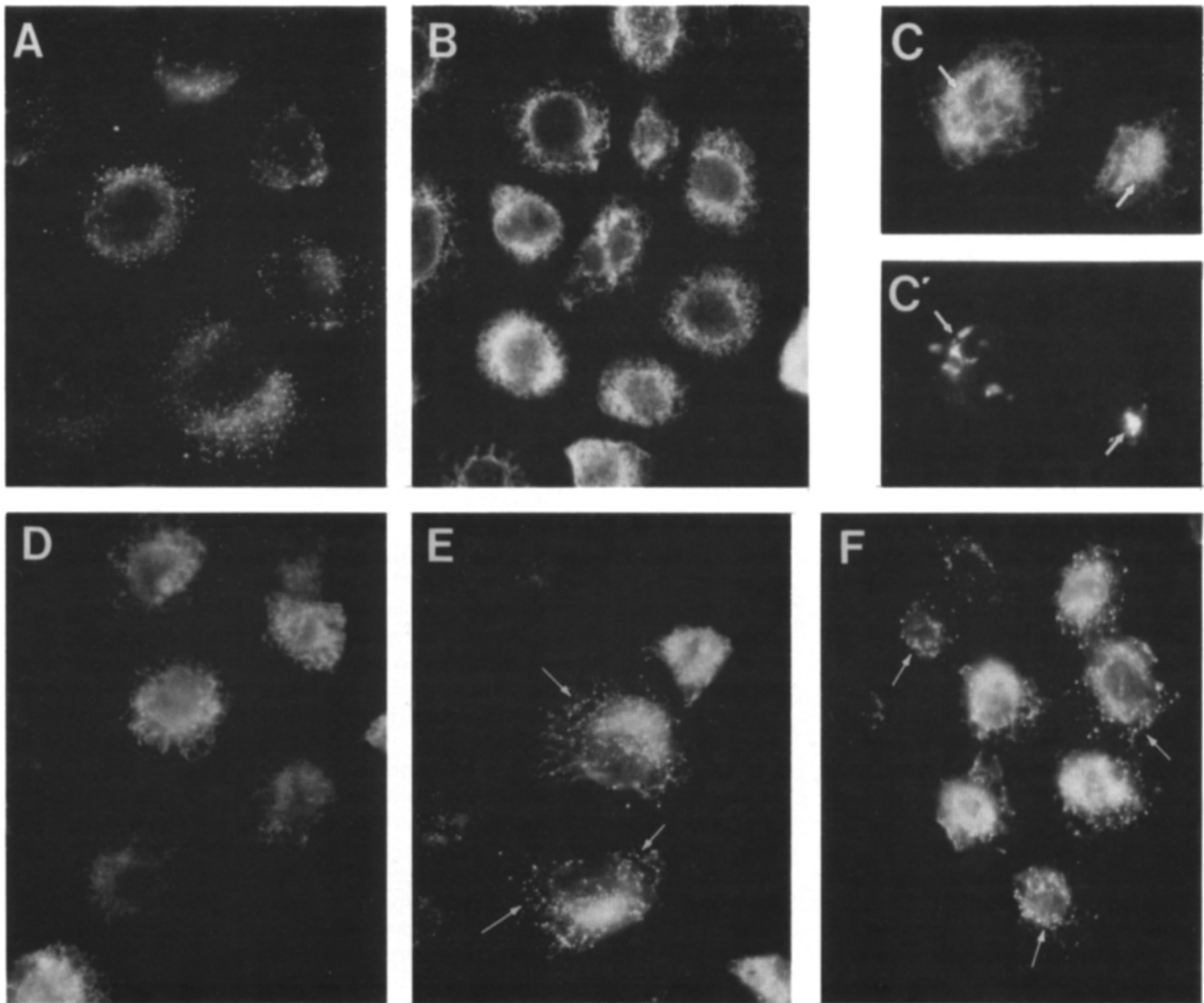


Figure 11. Mastoparan inhibits the exit of VSV-G protein from the ER. *In vitro* incubation conditions and indirect immunofluorescence were as described previously (Plutner et al., 1992). (A) The distribution of VSV-G protein after 80 min in the absence of peptide (control). (B, C, and C') Distribution of VSV-G protein (B and C) and Man II (C') after incubation for 80 min in the presence of mastoparan. (D) Distribution of VSV-G protein after incubation in the presence of 5 μ M mastoparan 7 (mast-7). (E) Transport of VSV-G protein to pre-Golgi intermediates and Golgi compartments (arrows) in the presence of 100 μ M mastoparan 11 (mast-11). (F) Transport of VSV-G protein to pre-Golgi intermediates and Golgi compartments (arrows) in the presence of 100 μ M mastoparan 17 (mast-17).

In this case <5% of the cells on coverslips showed detectable movement of VSV-G into the punctate pre-Golgi intermediates or Golgi compartments. Significant inhibition of the movement of VSV-G protein into punctate pre-Golgi intermediates could be detected at concentrations as low as 50–100 nM $\beta\gamma$ subunits (data not shown). Importantly, no inhibition was observed when $\beta\gamma$ subunits were heat-treated for 5 min at 95°C (Fig. 12 B, *inset*), confirming that the detergent associated with these subunits as not responsible for the inhibition observed. Similar results were observed when transport was followed by measuring the processing of VSV-G to the endo H_R form. The addition of 200 nM $\beta\gamma$ subunits inhibited transport to the *cis*-Golgi compartment by ~80–90% of the control which contained heat-inactivated $\beta\gamma$ subunits (Fig. 13, compare lanes *f* and *i*).

Since $\beta\gamma$ subunits form micelles which may concentrate

detergent up to 100-fold (Okabe et al., 1990), as a further control we tested the effect of transducin $\beta\gamma$ ($t\beta\gamma$) subunits which can be prepared in the absence of detergent. Transducin $\beta\gamma$ subunits inhibit both the agonist dependent and agonist independent activation of muscarinic K^+ channels, Ca^{2+} channels, and adenylate cyclase, and phospholipase C activation (Casey et al., 1989; Lotersztajn et al., 1992; Okabe et al., 1990; Yang et al., 1991; reviewed in Brown and Birnbaumer, 1990). As shown in Fig. 14 (A), addition of $t\beta\gamma$ inhibited exit from the ER network. Heat-inactivated $t\beta\gamma$ showed no inhibition (Fig. 14 B). In this case, significant retention of VSV-G protein in the ER was observed at a final concentration of ~2 μ M $t\beta\gamma$ in the assay, ~10-fold the concentration required for $\beta\gamma$ subunits prepared from bovine brain. This result is consistent with the observation that $t\beta\gamma$ subunits are less efficient in binding G_α subunits, generally

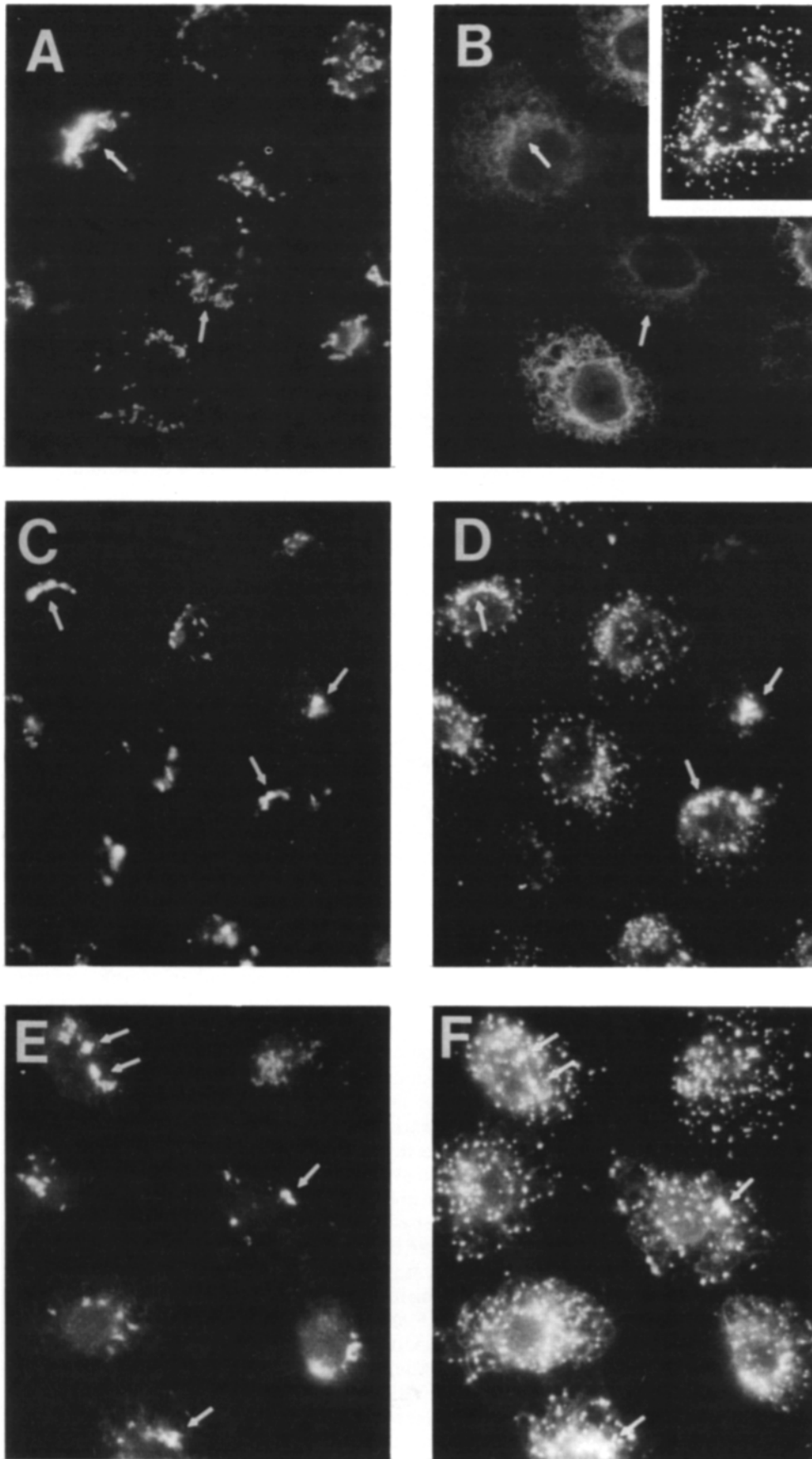


Figure 12. $\beta\gamma$ subunits prevent exit of VSV-G protein from the ER. In vitro incubation conditions and analysis of the distribution of VSV-G protein by indirect immunofluorescence were as described previously (Plutner et al., 1992). (B, D, and F) Distribution of VSV-G protein; (A, C, and E) distribution of Man II. (A and B) Incubation in the presence of 200 nM $\beta\gamma$ subunits. (B, inset) Incubation in the presence of 200 nM heat-inactivated (5 min 95°C) $\beta\gamma$ subunits. (C and D) Incubation in the presence of 200 nM G_α subunits. (E and F) Incubation in the presence of 400 nM $G_{\alpha\beta\gamma}$ subunits.

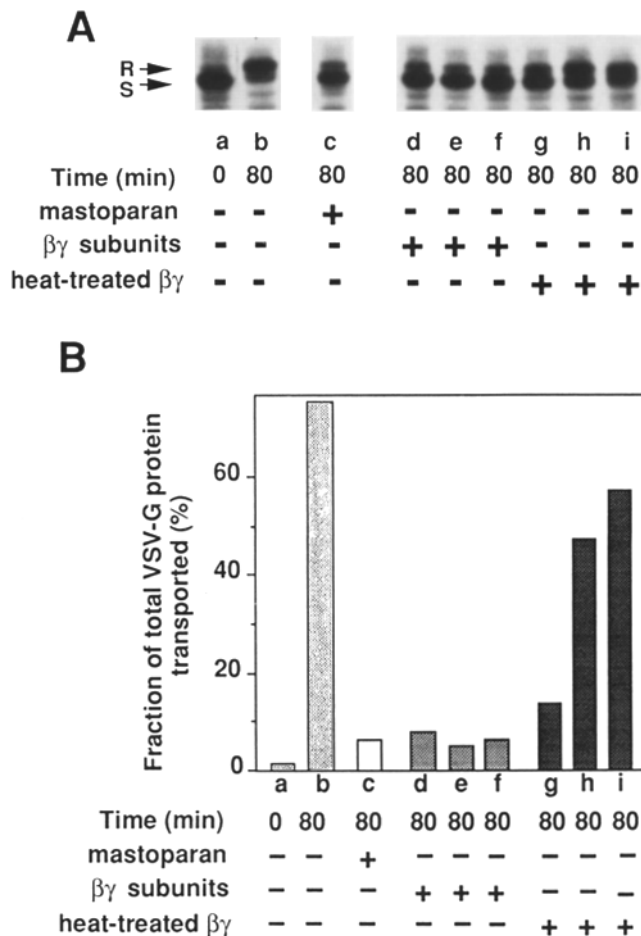


Figure 13. Biochemical analysis of inhibition of transport by $\beta\gamma$ subunits. In vitro incubation conditions and analysis of processing of VSV-G from the endoglycosidase H sensitive (ER) form (S in A) to the endoglycosidase H resistant (Golgi) form (R in A) were as described previously (Plutner et al. 1992). (A) Effect of mastoparan, brain $\beta\gamma$ subunits or heat-inactivated (5 min, 95°C) $\beta\gamma$ subunits on VSV-G transport (80 min, 32°C) to the cis/medial Golgi compartments: lanes a and b, controls; lane c, 10 μ M mastoparan; lanes d-f, $\beta\gamma$ subunits at 600 nM, 400 nM, and 200 nM $\beta\gamma$, respectively; lanes g-i, heat-inactivated $\beta\gamma$ subunits at 600, 400, and 200 nM final concentrations, respectively. (B) Quantitative analysis of the distribution of VSV-G in the endo H sensitive and endo H resistant forms for each condition shown in A.

requiring a 10-fold higher concentration to efficiently block, for example, the activation of K^+ channels (Okabe et al., 1990), or adenylyl cyclase (Casey et al., 1989).

Pertussis toxin (PtX) uncouples the G_i family of heterotrimeric proteins from activation by catalyzing the ADP-ribosylation of $G_{i\alpha}$ (reviewed in Ross, 1989; Simon et al., 1991). Since PtX ADP-ribosylates the carboxyl terminus involved in receptor recognition, it also antagonizes the effect of mastoparan on PtX-sensitive G proteins (Mousli et al., 1990; Weingarten et al., 1990). In contrast, cholera toxin (CtX) ADP-ribosylates the G_s family of G proteins leading to constitutive activation (Kahn and Gilman, 1986). We examined whether PtX- or CtX-treated cells have impaired or accelerated transport of VSV-G protein. Under conditions in which ADP-ribosylation (either PtX or CtX modification) of endogenous G_s subunits was 80–90% (data not shown), we have detected neither inhibition nor stimulation of transport

of VSV-G protein to the trans Golgi network and processing to the mature sialylated form in vivo or in vitro (data not shown). In addition, cells pretreated with PtX before permeabilization remained sensitive to inhibition by mastoparan in vitro (not shown). These results suggest that either toxin catalyzed ADP-ribosylation per se does significantly affect the action of a member of the G_i or G_o family of G proteins involved in transport between the ER and Golgi compartments, or that a PtX or CtX-insensitive G protein may regulate an early step in the secretory pathway in NRK cells.

Discussion

Inhibition of Transport by $GTP\gamma S$

The focus of the present study was to define at the morphological level the potential role of GTP-binding proteins in regulating different steps in the vesicular transport of protein between the ER and the Golgi complex. Our results are summarized in the diagram in Fig. 15.

Incubation in the presence of excess $GTP\gamma S$ (concentrations $>1 \mu$ M) lead to the accumulation of VSV-G in small, punctate structures scattered throughout the cytoplasm. These structures for the most part did not colocalize with p58, suggesting that they are very early intermediates in transport, possibly transport vesicles. This result is consistent with our previous observation that $GTP\gamma S$ inhibits an early step in transport (Beckers and Balch, 1989). A similar result has been observed for ER to Golgi transport in yeast (Rexach and Schekman, 1991). However, the precise morphological step in transport sensitive to GTP hydrolysis is still unclear. In the case of $GTP\gamma S$, the rapidity of inhibition may be reflected in the exchange rate(s) of this reagent for bound guanine nucleotide. Since the $t_{1/2}$ of inhibition for $GTP\gamma S$ is 2–3 min (Beckers and Balch, 1989), accumulation of VSV-G in punctate transport intermediates may simply reflect the inability of $GTP\gamma S$ to be exchanged sufficiently rapidly to inhibit vesicle budding, a step which may also require GTP-hydrolysis. Alternatively, GTP hydrolysis may regulate a later step and/or recycling of transport components. In the latter case, other (non-GTP binding) transport components could rapidly become rate-limiting leading to accumulation of VSV-G in early intermediates.

Previous studies on intra-Golgi transport in vitro suggested that $GTP\gamma S$ inhibits vesicular trafficking between Golgi compartments by accumulation of non-clathrin-coated transport vesicles (Malhotra et al., 1989; Melancon et al., 1987; Orci et al., 1989). Recently, a soluble factor conferring $GTP\gamma S$ sensitivity to intra-Golgi transport was identified as ARF (Taylor et al., 1992). Consistent with this result, the binding of ARF to Golgi membranes is sensitive to $GTP\gamma S$ (Serafini et al., 1991; Donaldson et al., 1991) and vesicles accumulating in the presence of $GTP\gamma S$ are highly enriched in both ARF and other coat proteins (Waters et al., 1991). While transport between the ER and the Golgi may also require ARF (Balch et al., 1992), we have previously shown that components rendering ER to Golgi transport sensitive to $GTP\gamma S$ are not exclusively present in the soluble fraction, but are retained by semi-intact cells (Beckers and Balch, 1989). Either a membrane-bound form of ARF or a different GTP-binding protein sensitive to $GTP\gamma S$ inhibits an early step leading to truncation of transport.

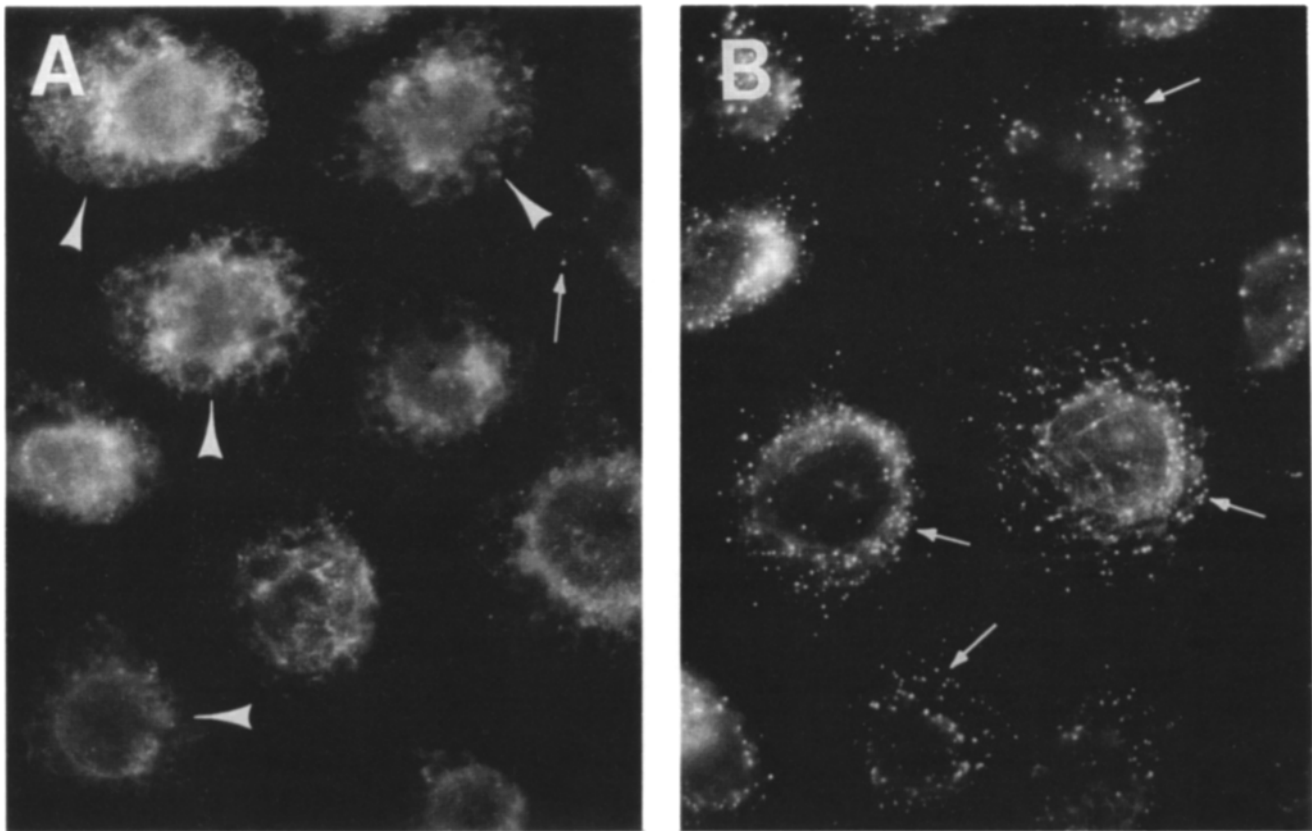


Figure 14. Transducin $\beta\gamma$ subunits inhibit VSV-G protein export from the ER. In vitro incubation conditions and use of indirect immunofluorescence for detecting VSV-G were as described (Plutner et al., 1992). (A) Retention of VSV-G protein in the ER after incubation for 80 min at 32°C in the presence of 2 μ M transducin $\beta\gamma$ subunits (large arrows). (B) Incubation as in A in the presence of transducin $\beta\gamma$ subunits inactivated by heat treatment for 5 min at 95°C. Small arrows denote the colocalization of VSV-G protein with pre-Golgi intermediates.

Role of rab1 in Transport of Protein from the ER to Golgi Membranes

Several lines of evidence now strongly suggest that rab1 is essential for ER to Golgi transport in mammalian cells. A mAb specific for rab1B prevents delivery to the Golgi based on processing of VSV by the cis Golgi enzyme α -1,2-mannosidase I (Plutner et al., 1991) or appearance in endo H-resistant forms (this study). In addition, *trans*-dominant negative mutants of rab1 defective in GTP-binding and/or hydrolysis are potent inhibitors of VSV-G protein transport in vivo (Tisdale et al., 1992). In the present study the antibody specific for rab1B inhibited export of VSV-G from the ER, suggesting that either rab1B or proteins associated with rab1B are essential for vesicle formation. Consistent with this result, VSV-G protein became rapidly associated with rab1B containing vesicles after initiation of transport in vitro. After 10–15 min of incubation, a time point at which <5% of the VSV-G protein has been processed to the endo H_R forms, >50% of the punctate compartments containing VSV-G also contained rab1B. At earlier time points rab1B could also be detected on the small punctate vesicles which contained VSV-G but did not contain p58. Interestingly, in the presence of GTP γ S, rab1B could not be detected on aborted punctate transport intermediates even after 45 min of incubation. Whether this reflects inhibition of rab1B GTP-hydrolysis which may be essential for recycling, or inhibition of a different GTP-binding protein functioning before, and perhaps necessary for the recruitment of rab1B remains to be deter-

mined. However, the striking increase in the association of rab1B with p58 and Man II containing compartments during incubation in vitro suggest that some step in the recycling of rab1B may be rate limiting. Using a different antibody, we have previously demonstrated that the smooth ER contains a resident pool of rab1B not detected by the antibodies used in the present study (Plutner et al., 1991). This pool persists throughout the duration of incubation in vitro (Balch, W. E., and H. Plutner, unpublished results) and may provide a reservoir of rab1B for vesicle budding.

The essential role of rab1 in ER to Golgi transport is consistent with the proposed role for the rab1-related YPT1 protein in vesicular transport in yeast. Ypt1p is 72–76% homologous to the mammalian rab1 proteins. Mouse rab1A will substitute for YPT1 in yeast, albeit at high levels of expression (Haubruck et al., 1989). Both biochemical and genetic evidence suggests that YPT1 functions in a vesicle targeting or fusion step (Segev et al., 1988; Bacon et al., 1989; Baker et al., 1990; Kaiser and Schekman, 1990; Becker et al., 1991; Rexach and Schekman, 1991; Segev, 1991). A similar phenotype has been observed for the related small GTP-binding protein SEC4 involved in vesicular transport from the *trans*-Golgi compartment to the cell surface (Goud et al., 1988; Walworth et al., 1989). Rab1 in mammalian cells also functions at a late step in ER to Golgi transport. We have previously demonstrated that a peptide homologous to the rab1 effector domain acts as a potent antagonist for interaction of rab proteins with putative downstream effectors (Plutner et al., 1990). In the case of ras, a similar effector domain

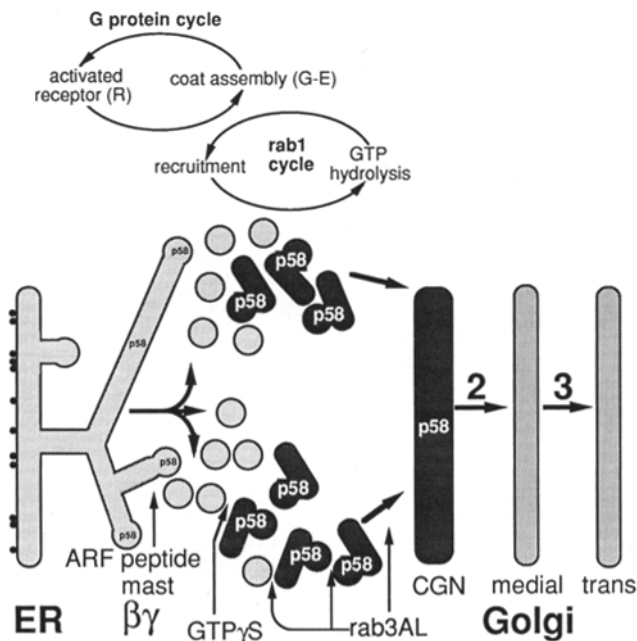


Figure 15. A diagram summarizing the role of GTP-binding proteins in regulating vesicular trafficking between the ER and the Golgi complex. The compartments labeled "p58" are post-ER compartments found in the peripheral and central regions enriched in p58 at steady-state (see Plutner et al., 1992). The thick lines denote the vectorial flow of VSV-G protein from the ER to the Golgi through p58 containing compartments to the central Golgi stack. The site of inhibition of the reagents used in this study are indicated by the arrows: (1) ARF peptide, mastoparan (*mast*) and $\beta\gamma$ subunits inhibit export from the ER; (2) GTP γ S results in the presumptive accumulation of VSV-G in transport vesicles preceding delivery to the p58 compartment; (3) the rab3AL peptide inhibits multiple fusion events leading to maturation to the CGN (see Plutner et al., 1992). The bold numbers 1 and 2 indicate that at least two transport steps are required for VSV-G protein to be transported from the ER to Golgi compartments enriched in Man II. (Top) Postulated roles for a heterotrimeric G protein(s) and rab1 in transport. Heterotrimeric G proteins coupled to receptors (*R*) participate in an initial step triggering coat assembly (*E*). Rab1 (and/or rab1-associated proteins) are recruited during vesicle formation, but function at a later step related to vesicle targeting/fusion and/or recycling of coat components.

peptide inhibits interaction with GTPase activating protein (GAP) (Marshall et al., 1989; McCormick, 1989; Hall, 1990). Given the rapid and irreversible inhibition by the rab3AL reagent ($t_{1/2} = \sim 15\text{--}30$ s) (Plutner et al., 1990), we conclude that rab1 (or some component regulated by rab1) may function in the context of a cycle in which the protein is recruited during vesicle formation but an unknown effector domain function is required for a later targeting or fusion step before delivery to the *cis*-Golgi compartment (recently referred to as the *cis*-Golgi network [CGN] [Mellman and Simons, 1992]) (Fig. 15). This may also hold true for Ypt1p and SEC4p in yeast (Goud et al., 1988; Walworth et al., 1989).

Role of ARF in Transport of Protein from the ER to Golgi Membranes

In contrast to the rab3AL peptide, an amphiphilic cationic

peptide identical to the NH₂-terminal domain of hARF1p which inhibits ARF function as a cofactor in cholera toxin-catalyzed ADP ribosylation of G, (Kahn et al., 1992), inhibited exit from the ER in NRK cells based on indirect immunofluorescence. These results suggest that ARF may be required for vesicle formation and are consistent with the effects of the peptide on processing of VSV-G during ER to Golgi transport in CHO cells (Balch et al., 1992) and on intra-Golgi transport in vitro (Kahn et al., 1992). In the latter case, Golgi stacks incubated in the presence of the ARF peptide neither formed transport vesicles nor extended tubular processes characteristically associated with these compartments when incubated in vitro (Orci et al., 1989). Addition of excess recombinant myr-hARF1 protein yielded a similar morphological phenotype to that of the peptide, preventing export from the ER. Since ARF is likely to participate in the structural scaffolds involved in vesicle assembly (Serafini et al., 1991), the stoichiometry between ARF and other transport components may be critical for their activity. Interestingly, and in contrast to the apparent role of ARF in vesicle formation shown in this study, we have previously provided evidence that the ARF peptide can also inhibit a late step in ER to Golgi transport (Balch et al., 1992). Addition of the peptide to the transport assay even after 45–60 min of incubation abruptly terminated VSV-G processing (Balch et al., 1992). Given the striking ability of the peptide to biochemically and morphologically immobilize the transport activities of both the ER and Golgi membranes, these results suggest that ARF may directly or indirectly play a role in the capacity of the Golgi to serve as both a donor and acceptor for vesicular traffic.

Role of Heterotrimeric G Proteins in Transport: Effect of Mastoparan

Mastoparan has been principally studied for its ability to bind to the carboxyl terminus of G α subunits and to serve as a mimetic for domains found in classical G-protein coupled receptors (Higashijima and Ross, 1991; Weingarten et al., 1990). Mastoparan has also been reported to permeabilize cell membranes, activate various kinases, and phosphatases (Mousli et al., 1990), and to weakly stimulate the intrinsic rate of hydrolysis and nucleotide exchange rate of the small GTP-binding proteins rho and rac (Koch et al., 1991).

For the following four reasons we believe this reagent to reflect the activity of a heterotrimeric G protein(s) in ER to Golgi transport. Firstly, the concentrations of mastoparan reported to elicit even partial membrane permeabilization are nearly 5–10-fold higher than those required to inhibit transport. The concentrations required to inhibit our assay are similar to those used to study the effect of mastoparan on purified G proteins (Higashijima and Ross, 1991). Secondly, we provided direct evidence that the effects observed here are specific. Inactive analogs of mastoparan failed to elicit inhibition (Higashijima et al., 1990; Mousli et al., 1990) nor was a synthetic peptide similar in amphiphilicity or charge able to inhibit transport. However, mastoparan does have structural homology to the ARF peptide, both being amphiphilic cationic peptides. Since the ARF peptide inhibits transport with a similar EC₅₀ and at the same morphological site as mastoparan, it will be interesting to determine whether the two peptides inhibit the function of the

same or different G protein(s). Thirdly, since mastoparan has been demonstrated to have a weak effect on guanine nucleotide exchange of the ras-related rho and rac small GTP-binding proteins (Koch et al., 1991), we tested and found no significant effect of mastoparan on rab 1B guanine nucleotide exchange (Balch, W. E., and G. M. Bokoch, unpublished results). Fourthly, we were concerned about the possibility that mastoparan inhibited a phosphatase activity. We have recently shown that both okadaic acid (OKA) and microcystin-LR (MC-LR), inhibitors of phosphatase 2A, potently inhibit VSV-G protein exit from the ER (Davidson et al., 1992). Inhibition by OKA or MC-LR can be efficiently antagonized by the protein kinase inhibitor staurosporine (Davidson et al., 1992). However, we found that staurosporine does not antagonize the inhibitory effect of mastoparan (Balch, W. E., unpublished results). This result makes it very unlikely that inhibition of export from the ER by mastoparan is related to direct or indirect effects on the phosphatase/kinase cascade regulating ER export during mitosis (Davidson et al., 1992). In addition, given the potential for mastoparan to activate G protein-regulated calcium channels (Lotersztajn et al., 1992), we have examined the effect of thapsigargin (Thastrop, 1990; Lytton et al., 1991; Ghosh et al., 1991; Kijima et al., 1991), a potent inhibitor of ER calcium channels leading to a rapid elevation of intracellular calcium, on VSV-G export. We found no effect of the drug at concentrations as high as 2.5 μ M in vitro (Balch, W. E., and H. Plutner, unpublished observations). We conclude that the effects of mastoparan are unlikely to reflect a calcium related event controlling VSV-G folding or oligomerization recently reported to influence the export of the asialoglycoprotein receptor (Lodish et al., 1992).

The effects of the G protein-activating reagent mastoparan are not inconsistent with the results obtained for the G protein-activating reagent AIF₍₃₋₅₎ given the technical limitations of the latter. In contrast to inhibition of export from the ER by mastoparan, in the presence of AIF₍₃₋₅₎ VSV-G was efficiently transported to the p58 containing compartments. Although inhibition by AIF₍₃₋₅₎ is not limited by guanine nucleotide exchange (as is the case for GTP γ S) and therefore should rapidly bind target G protein subunits, it is a reagent which is readily reversible and failed to elicit complete inhibition of processing of VSV-G protein to the endo H-resistant form (~75%). A "leaky" phenotype at the biochemical level would result in a significant accumulation of VSV-G in downstream compartments (a result which we observed), rendering a direct interpretation of the initial target of AIF₍₃₋₅₎ difficult. Thus, while the inhibition observed with AIF₍₃₋₅₎ is consistent with a role for a heterotrimeric G protein(s) in regulating ER to Golgi transport, it is unlikely to represent a useful reagent to define the morphological consequences of G protein activation in VSV-G transport.

Effect of $\beta\gamma$ Subunits

A more direct line of evidence that a heterotrimeric G protein(s) is required for ER to Golgi transport came from analysis of the effect of $\beta\gamma$ subunits on transport in vitro. Given the unique and high affinity of $\beta\gamma$ for G_{α} subunits (and not small GTP-binding proteins) (Pang and Sternweis, 1989; Brokoch and Quilliam, 1990) we tested whether the presence of excess $\beta\gamma$ subunits would perturb export from the ER. While $\beta\gamma$ subunits inhibited transport at a concentration of 100–200 nM and inhibition could be inactivated by boil-

ing, neither excess G_{α} nor excess $G_{\alpha\beta\gamma}$, prepared in the identical detergent buffer inhibited transport. The latter reagent also served as a control for the contribution of detergent associated with purified $\beta\gamma$ subunits. Soluble retinal $\beta\gamma$ ($t\beta\gamma$) subunits prepared in the absence of detergent also inhibited transport, albeit at a 10-fold higher concentration consistent with the elevated concentrations of $t\beta\gamma$ subunits necessary to perturb, for example, the activation of atrial muscarinic K⁺ channels (Okabe et al., 1990). Based on studies examining the effect of $\beta\gamma$ subunits on G_{α} activation of a number of different G protein-coupled receptors (Katada et al., 1984; Neer and Clapham, 1988; Casey and Gilman, 1988; Boyer et al., 1989; Lotersztajn et al., 1992; reviewed in Brown and Birnbaumer, 1990) one interpretation of our results is that inhibition of ER export is a consequence of trapping of free G_{α} subunits. Alternatively, recent evidence suggests that $\beta\gamma$ subunits alone can serve as direct modulators of downstream effector function (Federman et al., 1992; Tang and Gilman, 1992). There are now recognized to be at least two to three isoforms of β subunits and four isoforms of γ subunits (Simon et al., 1990). If these isoforms have distinct affinities for different G_{α} subunits as suggested recently (Kleuss et al., 1992), then the actual concentration of the relevant $\beta\gamma$ subunits in the mixed brain pool necessary to inhibit transport will undoubtedly be considerably lower.

It remains to be established which G_{α} subunit regulates VSV-G protein export from the ER. To begin to address this issue, we examined the effects of PtX and CtX on ER to Golgi transport. Pertussis toxin catalyzes the ADP-ribosylation of the carboxyl terminus of a restricted class of G_{α} subunits. Ribosylation blocks receptor interaction and GDP to GTP exchange, preventing G protein activation (Ross, 1989; Simon et al., 1991; Bommakanti et al., 1992). It will also antagonize the effects of mastoparan on $G_{\alpha i}$ (Mousli et al., 1990; Weingarten et al., 1990). Pretreatment of cells with PtX neither inhibited nor stimulated the transport of VSV-G protein through the Golgi complex in vivo or in vitro, nor antagonized the inhibition of transport in vitro by mastoparan based on our ability to detect both intermediate and mature (complex) oligosaccharide containing forms of VSV-G. The lack of effect of PtX on VSV-G protein transport contrasts with a recent report which suggests that PtX both stimulates the flow of glycosaminoglycans through the secretory pathway in LLC-PK epithelial cells and blocks the inhibitory effects of overexpression of $G_{\alpha 2}$ on glycosaminoglycan transport (Stow et al., 1991). In addition, association of ARF and β -COP with Golgi membranes is sensitive to PtX (Ktistakis et al., 1992). While our results suggest that a PtX-(CtX) insensitive G protein might be involved in regulation of transport between the ER and the Golgi, we cannot exclude the possibility that ADP-ribosylation of the key G protein(s) may be either incomplete, or that ADP-ribosylation per se may not generate a strong trans-dominant phenotype. Given the recent recognition of the diversity of PtX and CtX sensitive and insensitive G proteins involved in signal transduction (reviewed in Ross, 1989; Simon et al., 1990), more direct approaches will be necessary to identify the presumptive G protein(s) involved.

Multiple GTP-binding Protein Function in Vesicular Trafficking between the ER and the Golgi: The Concept of Gated Flow

It is now evident that multiple GTP-binding proteins are re-

quired for transit of protein between the ER and the *cis*-Golgi compartment. The combined results using reagents in this and previous studies (Beckers and Balch, 1989; Plutner et al., 1990, 1991, 1992; Balch et al., 1992) suggest that a heterotrimeric G protein(s), ARF and rab1 are likely to be involved in vesicle formation during export from the ER. While a number of reagents including AIF₍₃₋₅₎, mastoparan, and $\beta\gamma$ subunits strongly hint at an important role for a G protein(s) at an early step (Fig. 15), direct proof of G-protein involvement remains to be established. One possibility is that these reagents have a global effect on ER function, rapidly rendering it incompetent for general export through a G protein coupled event. The mechanism(s) basic to this phenomenon would have an important impact on our understanding of the ER as secretory organelle.

The early requirement for ARF and its essential role in the recruitment of β -COP to Golgi membranes (Donaldson et al., 1992) suggests that coat assembly is an initial event in export from the ER affected by these reagents. On the other hand rab1, while acquired early, may function at a late step in targeting or fusion (Fig. 15), temporally equivalent to the function of NSF and Ca²⁺ in targeting or fusion with pre-Golgi intermediates and the *cis*-Golgi complex (Plutner et al., 1992) (Fig. 15). Although our experimental strategy was largely based on following the transport of tsO45 VSV-G protein (taking advantage of its thermosensitive phenotype to precisely control its distribution before export from the ER), we have reached identical conclusions by following the transport of either wild-type VSV-G protein or an endogenous lysosomal membrane glycoprotein, lgp58 (Balch, W. E., R. Schwaninger, and S. L. Schmid, unpublished results).

The possible regulation of vesicle formation and exit from the ER by a heterotrimeric G protein(s) provides a novel and intriguing twist to the principles governing function of the secretory pathway. In classical signal transduction, G proteins link a diverse range of external (extracellular) agonists (A) which bind specific transmembrane receptors (R) to a more restricted range of intracellular effectors (E) (A-R→G→E) (reviewed in Bourne et al., 1990; Bourne et al., 1991; Ross, 1989; Simon et al., 1991). Abstracting this signal transduction model to vesicular transport, one potential implication is that a G protein regulated by a transmembrane receptor (R) may be critical for defining the site of vesicle formation from the ER (R→G→E). In so doing this R→G complex would serve to "gate" export by controlling coat assembly (Fig. 15). The effectors (E) in this case would include components of the transport machinery involved in vesicle formation (i.e., ARF, β -COP, and rab proteins), although the ubiquitous role of G proteins in regulating ion channels (Brown and Birnbaumer, 1990) and second messengers (Ross, 1989) may also play an unsuspected role in ER export function. Recently, $\beta\gamma$ subunits have been demonstrated to prevent the GTP γ S stimulated reassociation of β -COP to Golgi membranes (Donaldson et al., 1991) through an AIF₍₃₋₅₎ sensitive, ARF-dependent mechanism (Donaldson et al., 1992). Although these results do not address the biological role of the putative G protein(s), ARF or β -COP in vesicular transport per se, they certainly lend support to the interpretation that a G protein(s) regulates coat assembly as predicted by our studies with export of VSV-G protein from the ER. Although mastoparan and $\beta\gamma$ subunits have opposite effects on the activation state of G

proteins, they do have the common property of disrupting the coupling between a G protein and its cognate transmembrane receptor. If uncoupling prevents coat assembly, this would provide a plausible explanation for the identical effects of mastoparan and $\beta\gamma$ subunits on ER export.

Given the signal transduction model, can transported proteins per se also serve as the agonists ultimately triggering G protein directed coat assembly? Our studies were based on analysis of the transport of tsO45 VSV-G, a transmembrane protein thought to be a prototypical member of a large class of molecules which move to the cell surface via a default or "bulk flow" pathway (Pfeffer and Rothman, 1987). At the restrictive temperature, tsO45 VSV-G is unable to initiate export from the ER due to its inability to achieve the proper oligomeric structure (Doms et al., 1987; de Silva et al., 1990). If transport is gated, then incubation of cells at the permissive temperature, conditions which lead to homooligomerization of VSV-G to form the mature trimer (Doms et al., 1987; de Silva et al., 1990) may generate an essential signal to initiate export through a G-coupled event. A prediction of this hypothesis is that mutants should be detected which fold normally, but fail to be exported from the ER. This is indeed the case for VSV-G. Mutations in the cytoplasmic tail, while having no effect on oligomerization, prevent export from the ER (Doms et al., 1989). Whether VSV-G can serve as a true agonist (by activating a G protein-coupled receptor) or function directly as a novel "activated" receptor remains to be determined. In a more general sense, gated flow could represent an additional basis for the "quality control" processes which insure that only mature, oligomerized proteins are exported from the ER (reviewed in Klausner, 1989; Hurtly and Helenius, 1989; Gething and Sambrook, 1992).

Given the complexities associated with organelle structure and function in the endocytic and exocytic pathways, the potential for signal transducing G proteins to couple a diverse collection of ligands to be exported from the ER to a common biochemical machinery involving multiple GTP-binding proteins make them well suited for the task of regulating intracellular vesicular trafficking.

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