Transport between cis and medial Golgi Cisternae Requires the Function of the Ras-related Protein Rab6*

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The small GTP-binding protein Rab6, a member of the Ras superfamily, is localized on the membranes of the Golgi apparatus and the trans Golgi network. Recent studies revealed that the Rab6 protein might be involved in the transit of proteins through the Golgi complex. In this report we demonstrate the essential function of the Rab6 protein in a direct step of reconstituted Golgi transport. Polyclonal antibodies and Fab fragments directed against the C-terminal part of the Rab6 protein inhibit transport between the cis and the medial Golgi cisternae. Inhibition also occurred when a trans-dominant mutant form of the Rab6 protein (N126I) was added to the reconstituted transport. Furthermore, Rab6 antibodies inhibit uncoupled fusion of Golgi membranes. From these data we conclude that Rab6 is involved in a process related to membrane fusion at the cisternal membranes of the Golgi apparatus and therefore is needed for the consumption of Golgi-derived vesicles by their target membranes.

The transit between the different endocytic and exocytic membrane compartments in the eucaryotic cell is mediated by carrier vesicles that bud from a donor compartment and fuse with the proximal acceptor membrane. Transport is regulated by an array of distinct proteins, which are conserved from man to yeast (for review see Ref. 1).

Functions of GTP-binding proteins in different intracellular transport processes have been demonstrated by using the non-hydrolyzable GTP analogue GTPγS in different in vitro systems (2, 3). Genetic and biochemical analyses have led to the discovery of two Ras-like GTP-binding proteins, SEC4 and YPT1, which are necessary for protein export in the yeast Saccharomyces cerevisiae (4–6). In mammalian cells an increasing number of SEC4/YPT1 related proteins have been described (7). These so-called Rab proteins form an independent subfamily of the Ras-like GTPases, which can replace the corresponding YPT protein function in yeast (8–10). Localization studies on different Rab proteins have shown that Rab1 is present on the ER (11), Rab2 on the intermediate compartment (12), Rab4 and Rab5 on early endosomes and the plasma membrane (12, 13), Rab6 on the medial and trans cisternae of the Golgi apparatus and the membranes of the trans Golgi network (14), and Rab7 and Rab9 on late endosomes (12, 15). Rab3 is restricted to synaptic vesicle membranes of neuronal and chromaffin cells (16). Rab15 is also restricted to brain-specific tissue, and Rab17 is found only in polarized epithelial cells (17, 18). It was postulated that Rab proteins have important functions in targeting or fusion of transport vesicles (19). Studies using different in vitro transport assays have shown the important role of Rab1b in ER to Golgi and cis to medial Golgi transport (11), of Rab5 in the fusion of early endosomes (20), and of Rab9 in the transport from late endosomes to the trans Golgi network (15). The Rab6 protein is expressed ubiquitously in mammalian cells and is found also in plants and yeast (14, 21). In a previous study we have used the guanine nucleotide dissociation inhibitor to prove that cis to medial Golgi transport needs the function of Rab proteins (22).

Gound and co-workers proposed that the transit of proteins through the Golgi depends on Rab6 protein (23). To study the function of Rab6, we have generated specific antibodies against the hypervariable region of Rab6 protein. These antibodies were used to study the function of Rab6 in the well established cis to medial in vitro Golgi transport system. We report here that Rab6 is: 1) an essential factor for the vesicular transport between two Golgi cisternae and 2) involved in a step related to membrane fusion. The data presented here define for the first time a Rab6-dependent step on a molecular level.

EXPERIMENTAL PROCEDURES

Materials—Golgi donor-acceptor membranes and CHO cytosol were prepared as described previously (24). The affinity-purified ARF1 antibodies were provided by Dr. Bernd Helms. Chemicals and DNA modifying enzymes were from Boehringer Mannheim and Sigma. Protein G-agarose was from Pharmacia Biotech Inc. Western blotting was performed using the ECL System from Amersham. Supercarrer was from Pierce, and Amicon microconcentrator was from Millipore.

Expression of Recombinant Rab6 Proteins—Point mutations were introduced into the Rab6 cDNA inserted in the plasmid pT79 (Bio-Rad) using oligonucleotide-based site-directed mutagenesis. The individual mutations are summarized in Table I. An oligonucleotide coding for the amino acids NH2-Met-Glu-Gln-Leu-Ile-Ser-Glu-Glu-Asp-Arg using polymerase chain reaction, a BamHI site was added to the 3′ and 5′ ends of the cDNA. The modified cDNA was inserted into the expression plasmid pQE9, which provides a stretch of 6 histidines in front of the Rab6 cDNA (Qiagen, Düesseldorf, Germany). The structure of the resulting Rab6 cDNA and the Rab6 mutants are depicted in Table I. After transfection of the resulting plasmid into Escherichia coli XL1 Blue (Stratagene), Rab6 expression was induced with isopropyl-1-thio-β-D-galactopyranoside (1 mM) for 3 h at 37 °C. The mutant protein Rab6 N126I, which accumulates in inclusion bodies, was induced for 1 h at 37 °C or for 3 h at 30 °C. After harvesting, the E. coli cells were lysed by two passages through a French press cell. Rab6 protein was isolated from the cell lysate by binding to a nickel-agarose column. Protein was eluted with 250 mM imidazole, pH 7.0. For the mutant protein Rab6...
Western blots were used to establish the specificity of the antibodies. In total CHO or bovine brain cytosol and in Golgi membrane preparations, a single band of 26 kDa comigrating with the native Rab6 band was detected. No cross-reaction with other bands in the 20–30 kDa range could be detected. Using bacterially expressed Rab6 protein, the detection limit was 1–2 ng with an antibody concentration of 4 μg/ml. In ELISA neither of the two affinity-purified antibodies showed any cross-reactivity with Rab1b, the second member of the Rab family found in the Golgi apparatus.

Rab6 Antibodies Inhibit Transport Between cis and medial Cisternae—Affinity-purified peptide- or protein-specific antibodies were added to in vitro Golgi transport reactions, and the effects of the two different antibodies were studied. Both types of antibodies affected intra-Golgi transport. Comparison of different antibody preparations revealed that the preparations with the highest inhibitory potency could be generated by affinity-purifying antibodies raised against total Rab6 recombinant protein with the synthetic Rab6 peptide. In Fig. 1A the effect of these C-terminal specific antibodies on an in vitro transport assay is shown. Increasing amounts of antibody inhibited the assay. Maximum inhibition was achieved with 10 μg of antibody in a 50-μl assay. The half-maximal inhibition was observed at antibody concentrations of 40 μg/ml of IgG/mL. Precubation of the transport reaction for 1 h on ice before shifting the reaction to 37 °C enhanced the inhibitory effect of the antibodies. To exclude interference of the anti-Rab6 antibodies with the antibodies used for the immunoprecipitation that quantifies the in vitro transport, anti-Rab6 antibodies were added at different points in time after the start of the transport reaction. Transport inhibition by the anti-Rab6 antibodies decreased when they were added after starting the transport reaction. Inhibition was negligible when the anti-Rab6 antibodies were added 40 min after starting the transport reaction (data not shown).

Addition of rabbit IgG (Sigma) to the transport reaction also did not result in an inhibition of transport, thus excluding inhibition by IgG molecules. Further control experiments were performed with affinity-purified antibodies against ARF1. ARF1 is a related Golgi-associated small GTP-binding protein that is found in much higher concentrations in the cytosol than Rab6. As can be seen in Fig. 1A, no inhibition of the transport assay occurs, proving that antibodies to Golgi localized antigens have no inhibitory effect per se.

After blocking of the Rab6 inhibitory antibodies by preincubation with the Rab6 peptide, an increase of transport was observed. A total recovery of transport activity by preincubation with the Rab6 peptide could not be achieved. This can be explained by two facts. 1) The amount of peptide that could be added to the transport reaction was limited, since the peptide itself showed an inhibitory effect on the transport. This inhibition may be due to an interference of the peptide with the interaction of Rab6 and Rab6 accessory factors. 2) The Rab6 antibodies were prepared from antisera raised against Rab6 protein and subsequently purified using the Rab6 peptide as an affinity matrix. It is a known fact that antibodies have a much greater affinity for affinity matrices than for free peptides. This enhanced affinity may have resulted in a faster preincubation of the Rab6 antibodies with the Rab6 peptide.
higher avidity to the corresponding native protein than to an artificial peptide. In the assay the peptide prebound to the antibody can so be exchanged against the endogenous Rab6 protein, resulting in a new inhibition of transport activity. The specificity of the peptide block is shown by the fact that preincubation of the Rab6 antibodies with a C-terminal Rab1 peptide did not result in a rescue of transport (Fig. 1B).

To exclude cross-linking of the Golgi membranes by bivalent IgGs as the underlying event of the anti-Rab6 transport inhibition, monovalent Fab fragments were prepared from Rab6-specific sera. In Fig. 1C the inhibitory effect of Rab6-specific Fab fragments was demonstrated. The Fab fragments are not as potent as the intact IgGs, which could be due to partial denaturation of the molecules during the papain treatment. Control reactions in the presence of Fab fragments prepared from unspecific rabbit IgG (Sigma) showed no effect on the transport activity.
TABLE I

| Mutation       | Localization | Phenotype          |
|----------------|--------------|--------------------|
| T27N (Thr 27 – Asn) | Loop I       | GDP $\rightleftharpoons$ GTP binding |
| Q72R (Gln 72 – Arg) | Loop IV      | no GTPase activity |
| N126I (Asn 126 – Ile) | Loop VIII    | no GTP/GDP binding |

Rab6 in Golgi Transport

A Transdominant Mutant of the Rab6 Protein Interferes with Interacisternal Golgi Transport—To gain further proof of the essential function of the Rab6 gene product in intra-Golgi transport, interferant mutations were generated. Previous mutational analyses of Ras and Ras-related proteins have shown that certain mutations in the GTP-binding domains of small GTP-binding proteins result in a so-called trans dominant effect. Trans dominant mutations neutralize the function of the wild type gene product in trans (28).

Based on the knowledge of the structure-function relationship in the Ras superfamily, three different point mutations were introduced into the GTP-binding sites of the Rab6 protein. Table I summarizes the constructed mutations in the Rab6 gene. The hydroxy amino acid Thr-27 was changed to Asn. The corresponding mutation in the Ras protein results in a mutant that prefers to bind GDP instead of GTP (29). The replacement of Gln-72 by Arg in the Rab6 protein leads to a mutant that shows a drastically reduced GTPase activity (28). The change of Asn-126 to Ile results in an inability to bind any nucleotide in the Ras protein and in the closely related YPT1 and Rab gene products (20, 28).

The mutations were introduced into the Rab6 cDNA, and the mutant proteins were expressed in E. coli using the pQE9 vector system, which provides a His tag to facilitate purification by nickel-agarose chromatography. The structure of the Rab6 expression vector is shown schematically in Table I.

Fig. 2A shows the effect of Rab6 wt, Rab6 Q72R, and Rab6 T27N proteins on cis and medial Golgi transport. No significant effect could be obtained; neither the wild type nor the T27N or Q72R mutation showed any interference with the transport reaction in concentrations up to 3 μg/50-μl assay. In contrast, the N126I mutation inhibited transport by more than 60% with almost maximal concentration of about 60 ng in a 50-μl transport reaction (Fig. 2B). An inhibitory effect resulting from E. coli proteins copurifying with the Rab6 N126I could be excluded, since neither the Rab6 wt nor the T27N or the Q72R proteins, purified with the same protocol, showed an inhibitory effect. Wild type and mutant proteins were isolated from E. coli, so they lacked the C-terminal geranylgeranyl modification. This lipid modification is necessary for membrane binding of Ras proteins (20, 30, 31). Membrane binding studies using isolated rabbit liver Golgi membranes showed that Rab6 protein purified from overexpressing E. coli cells binds only very poorly (data not shown). It can therefore be concluded that the Rab6 N126I mutation inhibits transport as a soluble agent. Whether the T27N and Q72R mutants have no effect on transport at all or may have an effect in a membrane-bound form awaits further investigations.

Rab6 Is Involved in a Process Close to Membrane Fusion—The experiments shown above clearly illustrate the essential function of Rab6 in Golgi transport. Vesicle formation on Golgi membranes requires ARF and the soluble coatamer complex (32, 33). Membrane binding studies using inhibitory Rab6 antibodies showed that the Rab6 antibodies had at most a slight effect on the binding of the coatamer complex to the membranes, as judged by the amount of p120 bound to the membranes in the presence of GTP-γ-S. These data make Rab6 participation in the formation of coated vesicles highly unlikely. The fact that we could not detect the Rab6 protein on Golgi-derived coated vesicles with Western blot analysis (data not shown) is an additional hint that Rab6 is functional in vesicle consumption rather than formation. Since Rab6 seems to be absent from coated vesicle membranes, it is unlikely that Rab6 functions as a factor that facilitates targeting or docking of Golgi-derived vesicles to their acceptor compartments.

It has been postulated previously that Rab proteins mediate the fusion of vesicles with intracellular membranes. To gain further proof of this hypothesis, we used brefeldin A-treated or salt-washed transport assays. These assays are permutations of the in vitro Golgi transport assays (27, 34). Addition of the fungal metabolite brefeldin A to cultured mammalian cells results in fusion of Golgi membranes with membranes of the rough endoplasmic reticulum. Similar effects are observed on isolated membranes only (34). Movement and glycosylation of the VSV-G protein in a brefeldin A-treated cis to medial Golgi transport assay are mediated by uncoupled fusion between the Golgi cisternae rather than by vesicle formation and vesicle fusion (35). Thus, brefeldin A uncouples fusion from vesicle formation by removing the coat from the Golgi membranes. Brefeldin A itself has little effect on the glycosylation of the VSV G marker protein. This can be explained by the fact that in the standard in vitro transport assay most of the G protein is transported and glycosylated by vesicular transport. The same is true for the brefeldin A-treated assay, but now glycosylation is achieved by colocalization of the G protein and the glycosyltransferase through uncoupled fusion induced by the cellular fusion machinery. Since under both conditions most of the G protein is glycosylated, only a minor difference in the total amount of glycosylation could be observed. Pretreatment of standard transport reactions with 200 μM brefeldin A made the transport resistant to GTP-γ-S. This shows that brefeldin A-treated transport assays measure membrane fusion rather than vesicular transport, since GTP-γ-S blocks uncoating of transport vesicles by arresting ARF on the coated vesicle membrane (34, 35). In Fig. 3A the effect of anti-Rab6 antibodies on brefeldin A transport assays is shown. Pretreatment of transport assays with both 10 μM of anti-Rab6 antibodies and brefeldin A resulted in an inhibition of glycosylation similar to that observed in transport assays pretreated with anti-Rab6 antibodies only (Fig. 1).

An additional proof for a function of Rab6 in fusion came...
from the use of salt-washed assays. Treatment of donor and acceptor membranes with 1 M KCl results in the loss of peripheral membrane proteins, which makes the membranes competent for fusion with each other (27). Fusion in brefeldin A and salt-treated transport depends on the cytosolic fusion proteins NSF and SNAPs (27, 34). Fig. 3 shows the effect of anti-Rab6 antibodies on the standard and salt-washed fusion assays. 10 μg of Rab6-specific antibodies inhibit the transport assay by more than 50%. These data are similar to those obtained from the standard transport assay (Fig. 1A). Thus, there is strong evidence for a role of Rab6 in fusion of vesicle membranes with their acceptor compartments.

**DISCUSSION**

At present, three different subclasses of GTP-binding proteins involved in intra-Golgi transport have been characterized. In addition to the ARF proteins (36) and heterotrimeric G-proteins (37), Rab1b (11) and Rab6 (23), two members of the Ras-related Rab protein family, are involved in membrane trafficking through the Golgi complex.

Recently Martinez et al. (23) were able to demonstrate that overexpression of Rab6 or the Rab6 Q72L mutant reduced the transport of different proteins through the Golgi apparatus.

In the investigations presented here, we used a cell-free system and pursued two different approaches to analyze possible functions of Rab6 in more detail.

1) Inhibitory antibodies specific for the hypervariable region of Rab6 were found to block transport between cis and medial cisternae of the Golgi apparatus. Inhibitory antibodies have already been used as a tool to study Rab function in various cell-free systems (11, 20, 38). The data presented in Fig. 1 show the anti-Rab6 antibody effect to be specific for Rab6 inhibition.

2) The effects of different mutations in the GTP binding regions of Rab6 on cis to medial Golgi transport were analyzed (Table I). One mutant, Rab6 N126I, showed a transdominant interferant effect on intra-Golgi transport (Fig. 2B), thus providing further evidence for the essential function of Rab6 in intra-Golgi transport. The analogous mutations in SEC4 (30) and Rab5 (20), with a substitution of Ile for Asn in the conserved NKXD motif, also resulted in a loss of GTP binding activity and a neutralization of the corresponding wild type protein.

Since the Rab6 N126I mutant was purified from E. coli, the resulting protein lacked the C-terminal geranylgeranyl modification. Under the conditions used in our assay system, an efficient modification of the Rab6 proteins seems unlikely, since low molecular weight components were removed from the cytosol by gel filtration. The corresponding Rab1 N124I mutant had a similar effect on vesicle transport. Pind et al. showed that an non-isoprenylated form of this mutant inhibits the fusion of ER-derived vesicles with Golgi membranes (38). While for Rab1 and Rab6, isoprenylation of the N126I mutant seems optional, Stahl and co-workers demonstrated that for an analogous mutant protein of Rab5, Rab5 N133I, this lipid modification is needed for the trans dominant inhibitory effect (39). It was reported that the Rab6 N126I mutant did not affect exocytosis after expression in HeLa cells (40). This can be explained by the fact that the resulting protein product might be unstable after transfection into HeLa cells.

Recombinant Rab6 wt, Rab6 T27N, and Rab6 Q72R had no effect in our assay system. However, overexpression of Rab6 wt and Rab6 T27N in mammalian cells resulted in a slowdown of protein transfer in the Golgi complex (23). A possible explanation for this lack of inhibition might be that the mutants can only show an effect after modification and subsequently binding to the cisternal membranes. Recently Nuoffer et al. reported that a mutant of Rab1 with the same amino acid exchange as in Rab6 T27N inhibits protein export from the endoplasmic reticulum and cis to medial Golgi transport (41). In contrast to Rab1 N124I and Rab6 N126I, the inhibitory activity of this mutant depends on the C-terminal isoprenylation.

To study the function of Rab6, we used an established cell-free system, which has been used in the past to get fundamental insights into the molecular level of vesicular traffic between two consecutive Golgi apparatus compartments. Therefore, we cannot exclude the possibility that Rab6 may be involved in
additional cellular transport events.

To demonstrate that Rab6 is specifically involved in a step close to fusion in intracisternal transport, we used salt-washed membranes or the fungal drug brefeldin A. Treatment of highly purified Golgi preparations with high salt removes peripheral proteins from the membranes. The contents of previously distinct Golgi stacks are thus mixed by uncoupled fusion rather than by coat-controlled formation and fusion of transport vesicles (34, 35). Treatment with brefeldin A results in the formation of an extensive tubular network from the Golgi cisternae, and thereby proteins are transported. Under both conditions, salt-washed membranes or brefeldin A treatment, the VSV G protein becomes colocalized with the UDP-GlcNAc glycosyltransferase I by a vesicle-independent fusion step. The fusion mechanism is the same as in vesicular transport, since it needs ATP and depends on the same fusion proteins, namely NSF and SNAPs. The same is true for the results from the salt-washed assays. While our data clearly suggest that Rab6 is an important factor in the fusion event, there is no proof whether Rab6 is required at only one step or at different steps in intracisternal transport. Beside the fact that cisternal transport between the cis and the medial Golgi compartments needs Rab6 function, a better understanding of the Golgi apparatus is crucial to decide whether anterograde, retrograde, or bidirectional transport events require the intact Rab6 protein.

Another question that remains unanswered as yet is whether the targeting, the docking, or the fusion step of vesicle consumption is regulated by Rab6.

Further investigations are necessary to identify the proteins that directly interact with Rab6 and to delineate the role of Rab6 in interaction with those proteins already known to mediate membrane fusion, such as NSF, the SNAPs, and the SNAP receptors (42–44). At present, it is not clear whether the three known Golgi-localized Rab proteins, Rab1a/1b, Rab6, and Rab12, have different functions or just regulate the same molecular reaction at different stages of the vectorial passage through the Golgi complex.

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