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Rab6 is associated with a compartment that transports rhodopsin from the *trans*-Golgi to the site of rod outer segment disk formation in frog retinal photoreceptors

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SUMMARY

The biogenesis of light sensitive membranes in retinal rod photoreceptors involves polarized sorting and targeting of newly synthesized rhodopsin to a specialized domain, the rod outer segment (ROS). We have isolated and characterized the population of post-Golgi membranes that mediate intracellular transport of rhodopsin. In the present study we have examined the association of small (20-25 kDa) GTP-binding (G) proteins with these membranes. We found that one of the small G proteins, rab6, behaves like an integral membrane protein of the post-Golgi vesicles, although ~30% of rab6 is soluble. The distribution of the membrane-associated and the soluble forms is highly polarized. By confocal and EM immunocytochemistry it can be seen that most of rab6 is associated with the photoreceptor *trans*-Golgi cisternae, *trans*-Golgi network (TGN) and post-Golgi vesicles. The photoreceptor axon and synaptic terminal are unlabeled, but dendrites of deeper retinal layers are labeled. The distribution of rab6 across sucrose density gradient fractions parallels the distribution of sialyltransferase (a TGN marker) activity. About 9% of membrane-bound rab6 is associated, however, with the rhodopsin-bearing sialyltransferase-free post-Golgi vesicles, which represent a very small fraction (<1%) of the total retinal membranes. Rab6 is absent from the mature ROS disk membranes but it is present at the sites of new ROS disk formation and in the ROS cytoplasm. This suggests that rab6 becomes soluble upon disk membrane formation. Therefore, rab6 may function not only as a component of the sorting machinery of photoreceptors that delivers rhodopsin to its appropriate subcellular domain but may also participate in some aspects of ROS disk morphogenesis.

Key words: rab proteins, small G proteins, rhodopsin, membrane transport and sorting, TGN

INTRODUCTION

In frog rod photoreceptors newly synthesized rhodopsin is sorted from the Golgi to the rod outer segment (ROS) by a population of very low buoyant density (1.09 g/ml) vesicles whose protein content is relatively simple (Deretic and Papermaster, 1991). Since delivery of these membranes only to the ROS is accomplished with high fidelity, we have begun to dissect vesicle components to identify candidates that confer the information for the vectorial transport of rhodopsin. Among these is a set of low molecular mass GTP-binding proteins that associate with the photoreceptor post-Golgi membranes.

Small (20-25 kDa) GTP-binding (G) proteins belong to a growing family that appears to play a crucial role in providing signals for membrane protein sorting among subcellular compartments (for reviews see Hall, 1990; Balch, 1990; Goud and McCaffrey, 1991; Valencia et al., 1991; Takai et al., 1992; Goud, 1992; Pfeffer, 1992). Substantial evidence for the role of small G proteins has been obtained from the studies of yeast mutants. The *SEC4* gene product participates in post-Golgi transport (Salminen and Novick, 1987; Goud et al., 1988; Walworth et al., 1989) while proteins YPT1 and Sar1 regulate ER-to-Golgi traffic (Segev et al., 1988; Bacon et al., 1989; Segev, 1991; Nakano and Muramatsu, 1989; Rexach and Schekman, 1991).

Rab proteins are mammalian counterparts of the *SEC4/YPT1* family (Zahraoui et al., 1989). As in yeast, these proteins have been localized to specific compartments of mammalian cells (Kim et al., 1989; Chavrier et al., 1990a; Darchen et al., 1990; Fischer von Mollard et al., 1990; van der Sluijs et al., 1991). The C-terminal domain of rab proteins appears to contain signals for targeting to specific membranes (Chavrier et al., 1991). Compartmentalization enables rab proteins to regulate specific steps of membrane traffic (Gorvel et al., 1991; Plutner et al., 1991; Fischer von Mollard et al., 1991; Bucci et al., 1992; van der Sluijs et al., 1992; Lombardi et al., 1993). In particular, rab6 has been localized to the cytoplasmic surface of medial and *trans*-Golgi cisternae in NRK cells (Goud et al.,

1990) and, recently, to the TGN in NIH/3T3 and BHK cells (Antony et al., 1992). In *Torpedo marmorata* electrocytes a major fraction of the cell's complement of rab6 is associated with post-Golgi vesicles (Jasmin et al., 1992). This suggests that rab6 may be involved in the regulation of several steps in the post-Golgi membrane traffic.

Members of the *SEC4/YPT1/rab* subfamily contain a consensus sequence at the C-terminal domain (Chavrier et al., 1990b), which is a signal for post-translational modifications that increase the protein's hydrophobicity and allow it to bind directly to membranes. Rab proteins are geranylgeranylated at their C-terminal cysteines (Khosravi-Far et al., 1991; Kinsella and Maltese, 1992) by rab geranylgeranyl transferase (Seabra et al., 1992a). The enzyme subunit responsible for substrate recognition is homologous to the human choroideremia gene (Seabra et al., 1992b, 1993). The mutation of this gene causes blindness by an obscure effect on the choroidal vascularization of the retina and the retinal pigment epithelial cells that eventually leads to photoreceptor death. This disease indicates that a lack of functional rab proteins may cause degeneration and death of the most sensitive cells. Isoprenylation of rab proteins is also essential for their interactions with regulatory proteins: GDP dissociation inhibitor (GDI) (Araki et al., 1990; Regazzi et al., 1992) and the stimulatory GDP/GTP exchange protein (GDS) (Shirataki et al., 1991).

Interaction of rab proteins with appropriate effectors on the target membrane may direct polarized sorting of membrane proteins from donor to acceptor compartments. Membrane compartments involved in a number of vesicle-mediated trafficking pathways, such as the *trans*-Golgi network (TGN), probably contain numerous rab proteins that direct budding from, or fusion with their membranes. We have focused on the post-Golgi compartment of retinal photoreceptors since the sorting of proteins and their vectorial transport occurs in this compartment. Rhodopsin-bearing post-Golgi membranes contain a subset of rab proteins also present in retinal Golgi membranes. Rab6 is one of the proteins enriched on the post-Golgi membranes and on the TGN. In addition, we have found that rab6 is localized to the sites of ROS disk morphogenesis. This suggests that rab6 is a part of the sorting machinery that ensures polarized sorting and delivery of newly synthesized rhodopsin from the TGN to the site of new ROS disk membrane formation.

MATERIALS AND METHODS

Southern leopard frogs, *Rana berlandieri,* (100-250 g) purchased from Rana Co. (Brownsville, Tx), were maintained in a 12 hour light/dark cycle and fed live crickets. [^{32}P]GTP (3000 Ci/mmol) and [^{3}H]-CMP-*N*-acetyl-neuraminic acid (5-35Ci/mmol) were from New England Nuclear (Boston, MA), asialofetuin, *N*-acetyl-neuraminic acid and rabbit anti-goat IgG conjugated to 10 nm gold from Sigma Chemical Co. (St. Louis, MO), Ampholines pH 7-9, pH 5-7 and pH 3.5-9.5 from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ), urea (manufactured by BDH, Poole, Dorset, UK) from Hoefer (San Francisco, CA), Eupergit-C1Z beads (manufactured by Rohm Pharma, Weitezstadt, Germany) and acrylamide from Accurate Chemical & Scientific Corp. (Westbury, NY), peroxidase conjugated anti-rabbit IgG from Kirkegaard and Perry (Gaithersburg, MD), goat anti-rabbit IgG from Jackson Immuno Research Laboratories, Inc. (Avondale, PA), goat anti-rabbit IgG conjugated to Texas Red from Molecular Probes, Inc. (Eugene, OR), Triton X-100 (for membrane research) from Boehringer Mannheim Diagnostics, Inc. (Houston, TX), 4% paraformaldehyde in 0.1 M phosphate buffer from Tousimis (Rockville, MD), and the ECL Western Blotting Detection System and Hyperfilm ECL from Amersham Corporation (Arlington Heights, IL).

Rabbit antiserum and affinity-purified antibody to the human rab6 expressed in *E. coli* were kindly provided for this study by Dr Bruno Goud (Institut Pasteur, Paris).

Retinal subcellular fractionation

Retinal subcellular fractionation was performed as described in Deretic and Papermaster (1991, 1993). Briefly, frog retinas were isolated and ROS were separated and further purified on a step sucrose gradient as described by Papermaster and Dreyer (1974). Retinal pellets were re-homogenized in 0.25 M sucrose and spun at 4,000 rpm (1,250 $\boldsymbol{g}_{av}$) (JA20 rotor, Beckman Instruments, Inc. Palo Alto, CA) for 4 minutes. Supernatants (3 ml) were overlaid on 10 ml linear 20-39% (w/w) sucrose gradients containing protease inhibitors in 10 mM Tris-acetate, pH 7.4, and 1 mM $MgCl_2$, above a 0.5 ml cushion of 49% (w/w) sucrose in the same buffer. After centrifugation at 28,000 rpm (100,000 $\boldsymbol{g}_{av}$) in an SW40 rotor (Beckman) for 13 hours at 4°C, 0.9 ml fractions were reproducibly collected from the top of the gradient. For each fraction the refractive index and sialyltransferase activity were determined from a sample and the remainder was diluted with 10 mM Tris-acetate, pH 7.4, and centrifuged at 50,000 rpm (240,000 $\boldsymbol{g}_{av}$) for 40 minutes in a SW50.1 rotor. Pellets were resuspended in 10 mM Tris-acetate, pH 7.4, and aliquoted for analysis by SDS-PAGE or two-dimensional gel electrophoresis.

Sialyltransferase assay

Sialyltransferase was assayed by a modification of the method described by Brandli et al. (1988). The reaction mixtures contained the following in a total volume of 100 μl: 50 mM Na-cacodylate, pH 6.6; 10 mM $MnCl_2$; 54 mM NaCl; 0.5% w/v Triton X-100; 0.25 mg of asialofetuin, 0.2 μCi of [^{3}H]-CMP-*N*-acetyl-neuraminic acid (25 μM final) and 50 μl of sucrose gradient fraction. After 1 hour at room temperature (RT), the reaction was terminated with 1 ml of ice cold 5% (w/v) phosphotungstic acid in 2 M HCl. The precipitates were collected onto Whatman GF/A glass fiber filters with a Millipore apparatus (Millipore Corp., Bedford, MA). Filters were washed once with 5% (w/v) phosphotungstic acid in 2 M HCl, twice with 1% (v/v) ethanol and dried. Then 10 ml of Optifluor was added and radioactivity was determined in a Beckman LS7000 scintillation counter.

SDS-polyacrylamide gel electrophoresis, two-dimensional gel electrophoresis and immunoblotting

Membranes were pelleted from sucrose gradient subcellular fractions as described above, solubilized and separated by 12% SDS-PAGE according to Laemmli (1970). For two-dimensional gel electrophoresis the samples were solubilized in 9.8 M urea, 4% (w/v) NP-40, 2% (v/v) Ampholines, pH 7-9, (Pharmacia) and 100 mM DTT (lysis buffer). A combination of isoelectric focusing (IEF) and SDS-PAGE was performed as described by Bravo (1984).

Gels were blotted onto Immobilon-P membranes (Millipore) according to Matsudaira, (1987). Blots were blocked in 5% nonfat dry milk, 1% BSA and 0.1% Tween-20 in TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) for 1 hour. Anti-rab6 antiserum was diluted to 1:400 in TTBS (TBS containing 0.05% Tween-20), and incubated for 2 hours at 20°C, followed by peroxidase-conjugated anti-rabbit IgG for 1 hour. Bound antibodies were detected using

diaminobenzidine as a substrate or by the ECL Western Blotting Detection System (Amersham), according to the manufacturer's instructions. Hyperfilm ECL was scanned using the Image processing and analysis program (Wayne Rasband, NIH).

Immunoisolation of the rhodopsin-bearing post-Golgi vesicles

Immunoisolation using mAb 11D5 (anti-rhodopsin C-terminal domain) and murine IgG1 was performed by the method of Burger et al. (1989) as modified by Deretic and Papermaster (1991). Antibody-coated beads (2.5 mg; ~7 μg antibody/mg of beads) were blocked for 1 hour with 5% nonfat dry milk and 1% BSA in PBS to reduce nonspecific binding and incubated for 2 hours at 4°C with 20 μg of fraction 5 (equivalent to membranes isolated from 4 retinas). After several washes with 0.25 M sucrose containing protease inhibitors, immunoisolated membranes were solubilized in lysis buffer and analyzed by two-dimensional gel electrophoresis as described above.

Triton X-114 extraction and phase separation

Triton X-114 extraction and phase separation were performed as described by Wandinger-Ness et al. (1990). Post-Golgi membranes isolated from 4 frog retinas were resuspended in 50 μl of 1% Triton X-114 (w/v) in PBS. The mixture was incubated for 15 minutes on ice and 5 minutes at 37°C to promote phase separation. After 1 minute of centrifugation, the detergent phase and the proteins precipitated from the aqueous phase were solubilized in SDS-PAGE sample buffer. Aliquots equal to 2 retinas were separated by SDS-PAGE and blotted onto Immobilon-P membranes as described above.

Detection of the GTP-binding proteins on the Immobilon-P blots

GTP-binding proteins were detected as described by Lapetina and Reep (1987). Blots were preincubated for 30 minutes with 1 μM ATP in binding buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, and 0.3% Tween-20), [α-^{32}P]GTP, 50 μCi/blot (1 μCi/ml), was added and blots were incubated for 2 hours, washed with binding buffer for 2 hours, dried and autoradiographed at −70°C using Kodak X-Omat film with intensifying screens. In some experiments, to further reduce background, 50 mM Tris-HCl in the binding buffer was substituted for 50 mM phosphate, pH 7.0. This resulted in markedly increased affinity (~5 fold) of rab6 for [α-^{32}P]GTP.

Immunofluorescence and confocal microscopy

Immunofluorescence and confocal microscopy were performed as described by Matsumoto and Hale (1993). Frogs were dark-adapted and eyecups were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, overnight and embedded in 5% agarose. Agarose blocks were sectioned at 100 μm thickness on a Vibratome (Technical Products International, Kansas City, MO). Sections were treated with 1% Triton X-100 and labeled with affinity-purified anti-rab6 antibody followed by goat anti-rabbit IgG conjugated to Texas Red. Cells were visualized in a Zeiss Laser Scanning Confocal Microscope 310 (Carl Zeiss, Inc, White Plains, NY). Digitized images of confocal optical sections (0.25 μm) were merged using VoxelView software (Vital Images, Inc. Des Moines, IA) on an SGI Indigo workstation running Elan Graphics (Silicon Graphics, Inc., Sunnyvale, CA). Optical sections and merged images were photographed directly with an ImageCorder (Focus Graphics, Inc., Foster City, CA).

Electron microscopy

Retinas were embedded in LR gold and labeled with anti-rab6 according to the procedure of Berryman and Rodewald (1990), except that post-fixation with OsO_4 was omitted. Bound affinity-purified anti-rab6 antibody was detected by goat anti-rabbit IgG and rabbit anti-goat IgG conjugated to 10 nm gold. Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips 301 electron microscope.

RESULTS

A set of small G proteins is associated with the *trans*-Golgi and post-Golgi membranes transporting newly synthesized rhodopsin in photoreceptor cells.

Several small G proteins are detected when frog retinal membrane proteins are separated by two-dimensional gel electrophoresis, blotted onto Immobilon membranes and blots are overlaid with [^{32}P]GTP (Fig. 1). A set of small G proteins that co-isolated with the Golgi-enriched membrane fraction, which was previously identified by its galactosyl-

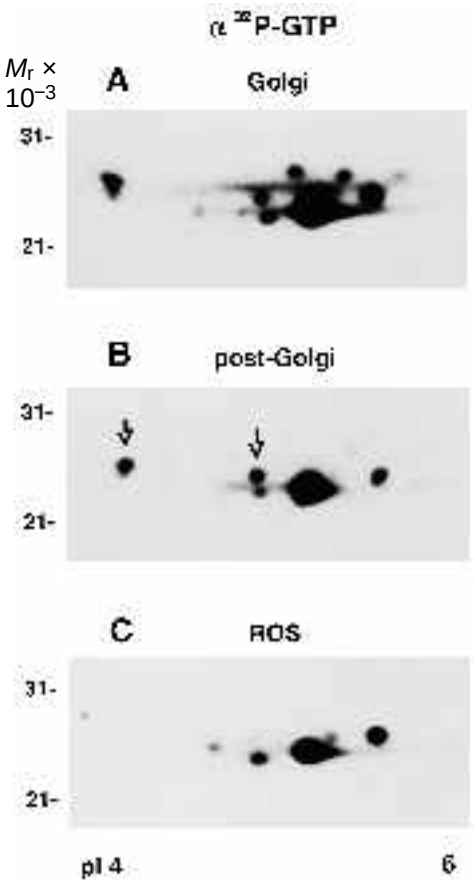

Fig. 1. Post-Golgi membranes in photoreceptor cells contain a set of small G proteins revealed by [^{32}P]GTP binding. A Golgi-enriched membrane fraction (A), post-Golgi membranes (B), and isolated ROS membranes (C) from two frog retinas were separated by two-dimensional gel electrophoresis, transferred to Immobilon membranes and incubated in the presence of [^{32}P]GTP. Bound GTP was detected by autoradiography. Arrows indicate two proteins that are absent from the mature ROS membranes and present on the post-Golgi membranes. These two G proteins are therefore candidates for specific participation in rhodopsin post-Golgi transport.

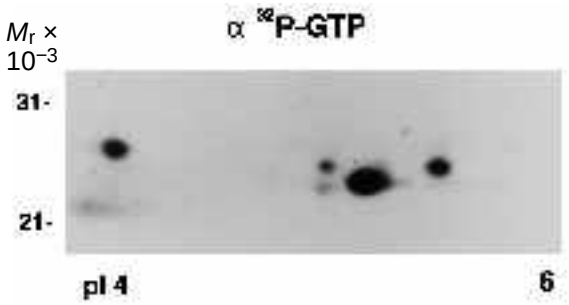

Fig. 2. G proteins are present in the post-Golgi membrane fraction, which has been immunoisolated with anti-rhodopsin mAb 11D5. Immunoisolated membranes were treated as described in the legend for Fig. 1. Two G proteins that may participate in rhodopsin transport (Fig. 1B, arrows) are also present on the immunoisolated membranes.

transferse activity (Deretic and Papermaster, 1991), is shown in Fig. 1A. The Golgi-associated set of small G proteins also includes G proteins found on the post-Golgi membranes, which have a simpler composition (Fig. 1B). The post-Golgi membranes share only three G proteins with the ROS membranes to which mature rhodopsin is delivered (Fig. 1C). This suggests that proteins absent from the mature ROS disk membranes and present on the Golgi and post-Golgi membranes may be involved in sorting and transport of newly synthesized rhodopsin. Comparison of the autoradiograms points to two candidate GTP-binding proteins indicated by the arrows in Fig. 1B. Although they are also present on the Golgi membranes, they are not delivered to the ROS membranes and therefore might regulate post-Golgi membrane traffic in retinal photoreceptors.

To determine if these small G proteins are associated with the membranes that contain rhodopsin or with a population of membranes co-sedimenting in the same retinal subcellular fraction, we have immunoisolated rhodopsin-bearing post-Golgi membranes by using immunobeads coated with mAb 11D5, which binds to the cytoplasmic domain of rhodopsin. Using [^{32}P]GTP overlays (Fig. 2) we find that the content of small G proteins of immunoisolated membranes is indistinguishable from the starting retinal subcellular fraction (as shown in Fig. 1B). Thus, at least two low molecular mass GTP-binding proteins may specifically regulate post-Golgi rhodopsin transport and others may participate in a less specific manner.

Rab6 is present on the *trans*-Golgi and post-Golgi membranes in photoreceptor cells

Rab6 is a G protein that has been shown, by immunocytochemistry, to be present on the medial and *trans*-Golgi membranes of NRK cells (Goud et al., 1990) and, in addition, on the post-Golgi vesicles in *Torpedo marmorata* electrocytes (Jasmin et al., 1992). In order to determine if rab6 is one of the small G proteins associated with the photoreceptor post-Golgi membranes, we have immunoblotted retinal subcellular fractions with an anti-rab6 antibody (Goud et al., 1990). Fig. 3 shows that rab6 is detectable on immunoblots of the retinal *trans*-Golgi and post-Golgi membranes and is undetectable on the ROS membranes. Comparison with the autoradiograms of the GTP overlays

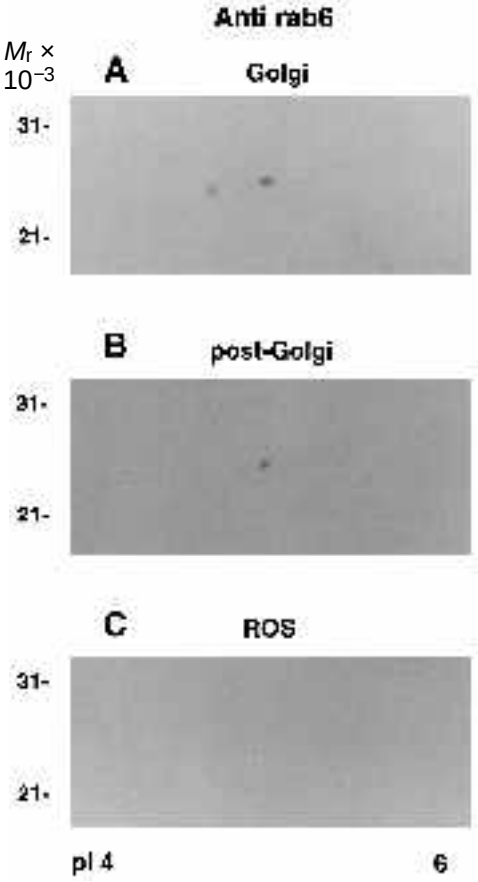

Fig. 3. Antibody to rab6 recognizes one of the GTP-binding proteins on the post-Golgi membranes (B) and two GTP-binding proteins in the Golgi-enriched fraction of the frog retinas (A). Rab6 is undetectable on ROS membranes (C). Membranes isolated from two frog retinas were separated by two-dimensional gel electrophoresis, transferred to the Immobilon membranes and immunoblotted with anti-rab6 antiserum (Goud et al., 1990). Bound antibody was detected by peroxidase-conjugated anti-rabbit IgG and diaminobenzidine as a substrate.

indicates that rab6 is one of the two proteins, with a more basic pI, previously indicated above by the arrows in Fig. 1B. The estimated size of the post-Golgi membrane-associated-rab6 is ~24 kDa and its pI is ~5. This is in good agreement with the molecular mass of 23.5 kDa and pI of 5.03 predicted from the nucleotide sequence of human rab6 (Zahraoui et al., 1989)

Interestingly, antibody to rab6 binds to two different GTP-binding proteins on the Golgi membranes, only one of which is present on the post-Golgi membranes. This may be a consequence of post-translational modification, since no cross-reactivity with other rab proteins has been reported for the anti-rab6 antiserum (Goud et al., 1990). Cross-reaction with another member of the rab family associated with the frog retinal Golgi membranes cannot be excluded, however, since the antibody was generated to a human antigen. Rab6 is geranylgeranylated at its C-terminal cysteines (Kinsella and Maltese, 1992). Two different membrane-bound forms of rab6 were identified when it was expressed in baculovirus/insect cell systems due to different isoprenylation

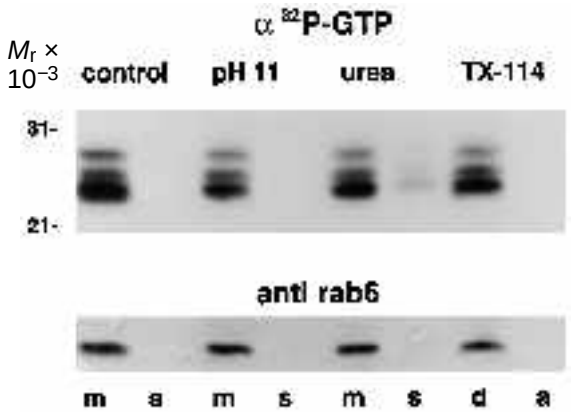

Fig. 4. Rab6 is tightly bound to the rhodopsin-bearing post-Golgi membranes. Aliquots of the post-Golgi membranes isolated from two retinas were incubated with buffer alone (control), with 0.1 M Na_2CO_3, pH 11, 4 M urea, or 1% Triton X-114. Membranes were sedimented (m) and eluted supernatant proteins were TCA precipitated (s). After Triton X-114 partitioning, the detergent phase (d) was separated from the aqueous phase (a). Proteins were separated by SDS-PAGE and blotted onto Immobilon membranes. Blots were first incubated with [^{32}P]GTP (top panel) followed by an anti-rab6 antiserum (bottom panel). Bound anti-rab6 antibody was detected using the ECL Detection System. Rab6 remains membrane-bound under each condition tested.

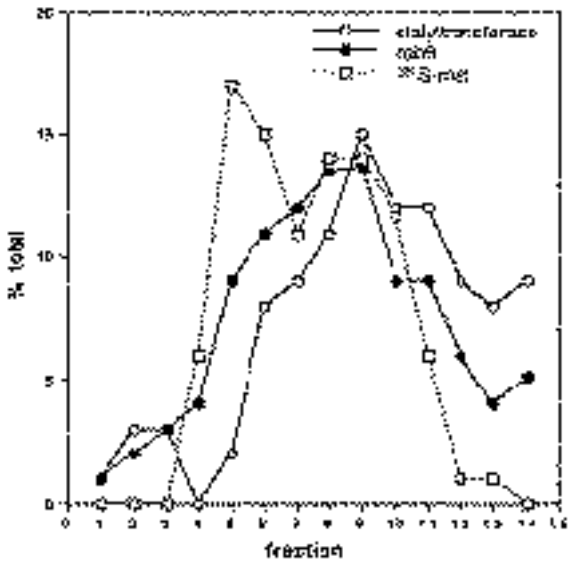

Fig. 5. Distribution of rab6 among the retinal subcellular fractions separated on the linear sucrose density gradients coincides with the distribution of sialyltransferase activity, a marker for TGN. Aliquots of retinal subcellular fractions were assayed for sialyltransferase activity (open circles). Membranes were then sedimented, proteins were separated by SDS-PAGE, immunoblotted with anti-rab6 antiserum and the amount of bound antibody revealed by the ECL Detection System was quantfied by the Image analysis program (solid circles). Membranes of fractions 8 and 9 contain the major portion of cellular rab6. In a pulse-chase experiment under conditions when the majority of newly synthesized rhodopsin leaves the Golgi and accumulates in the post-Golgi membrane fraction 5, an additional peak of radiolabeled rhodopsin also includes fractions 8 and 9 (open squares) suggesting that these fractions also contain post-Golgi membranes, probably arising from sialyltransferase-enriched TGN membranes. Data from the pulse-chase experiment are modified from Deretic and Papermaster, 1991, with permission.

states (Yang et al., 1992). However, it is not known if these modifications alter the pI of the protein or if another post-translational modification generates a more acidic, *trans*-Golgi-associated form of rab6.

Rab6 is tightly bound to post-Golgi membranes

To test the nature of the association of rab6 with the post-Golgi membranes, they were treated with 4 M urea and high pH washes. Both treatments remove extrinsic membrane proteins such as α-crystallin (D. Deretic, R. H. Aebersold, D. S. Papermaster and H. D. Morrison, unpublished) but fail to strip rab6 from the membrane (Fig. 4). Rab6 behaves like an integral membrane protein on the post-Golgi membranes and partitions into the detergent phase after Triton X-114 extraction (Fig. 4). These data suggest that the form of rab6 that is associated with the post-Golgi membranes is isoprenylated. We have attempted to determine if rab6 can be isoprenylated in isolated retinas in vitro, using [^{3}H]mevalonate as a substrate, but have been unable to demonstrate this modification due to the very low level of mevalonate incorporation into retinal proteins. After treatment of the Golgi-enriched membrane fraction with 4 M urea and high pH washes, the more acidic form of rab6 also remains membrane bound, suggesting that both forms present on these membranes are isoprenylated (data not shown).

Although membrane-bound rab6 behaves like an integral membrane protein, a significant fraction (~ 30%) of the total cellular pool of rab6 is found in the soluble form. The fraction of soluble rab6 has been estimated by comparison of the amount of the antibody bound to the membranes from all subcellular fractions combined to that bound to the soluble antigen by densitometry of immunoblots (data not shown).

The bulk of rab6 in photoreceptor cells is associated with the TGN

To determine rab6 distribution within photoreceptors, we have immunoblotted membrane proteins from the retinal subcellular sucrose density gradient fractions with an antibody to rab6 and quantfied the amount of antibody bound to the membranes from each fraction. Fig. 5 shows that fractions 8 and 9 contain ~30% of the total rab6 present in the cell. The subcellular distribution of rab6 does not coincide with any specific photoreceptor membrane compartment that we have identified so far (Deretic and Papermaster, 1991). The vesicles that transport proteins to the cell surface in other cells bud from the TGN (Griffiths and Simons, 1986). To determine if rab6 is localized in this compartment we have compared its distribution to that of sialyltransferase activity, a membrane protein of the TGN (Roth et al., 1985). The distribution of the enzyme's activity across the sucrose gradient coincides with the peak of rab6 immunoreactivity (Fig. 5). This suggests that the bulk of rab6 in photoreceptors is associated with the TGN. Post-Golgi membranes (fraction 5) contain ~9% of the cellular pool of rab6, yet these membranes represent only 0.03% of the total protein from the retinal homogenate (excluding

ROS). These membranes are therefore significantly enriched in rab6 compared to the Golgi complex.

We have also compared rab6 distribution to that of newly synthesized rhodopsin after 90 minutes of [^{35}S]methionine incorporation followed by 2 hours of cold chase (Deretic and Papermaster, 1991, and shown in Fig. 5). At this time point the Golgi is relatively depleted of radiolabeled rhodopsin, which is, by then, transported to the post-Golgi membrane fraction 5. Along with its accumulation in fraction 5, newly synthesized rhodopsin appears in fractions 8 and 9, which kinetically defines these membranes as part of the post-Golgi compartment. As shown in Fig. 5, fractions 8 and 9 contain TGN, as determined by their sialyltransferase activity, and a large portion of the total cellular pool of rab6. This suggests that rab6 may be involved in the regulation of the formation of the post-Golgi vesicles carrying newly synthesized rhodopsin from the photoreceptor TGN.

Confocal immunofluorescence microscopy reveals the polarized distribution of rab6 in retinal photoreceptors

Given the biochemical evidence for rab6 association with the post-Golgi membranes, we have used confocal microscopy to determine the distribution of rab6. Fig. 6(A-I) shows a series of confocal optical sections through the photoreceptor cells at 0.25 μm intervals and Fig. 6J shows their composite image. Areas shown span from the photoreceptor outer segments (at the top) to the nuclei at the base of the inner segments (at the bottom). Rab6 immunoreactivity is associated with (i) the Golgi-complex (Fig. 6C-E, open arrows), (ii) the network of post-Golgi membranes, which traverses through the photoreceptor's ellipsoid region that is tightly packed with mitochondria (Fig. 6F, arrow) and (iii) the post-Golgi periciliary vesicles, which accumulate near the cilium that connects inner segments with the ROS (Fig. 6G-I, arrowheads). The ciliary stalk, a cytoplasmic domain of the ROS, is also intensely labeled by anti-rab6 antibody (Fig. 6G and H, open arrowheads). The appearance of rab6 in this domain and the low intensity of labeling throughout the ROS, in the absence of detectable membrane-bound rab6 in ROS by immunoblotting, suggest that, upon delivery of rhodopsin, rab6 dissociates from the rhodopsin-bearing vesicular membranes and becomes soluble. Fig. 6K shows strongly polarized distribution of rab6 immunoreactivity in retinal cells where the Golgi (open arrows) and post-Golgi structures (arrowheads) contain the bulk of the total cellular pool, while the outer nuclear layer with axons and rod synaptic terminals (large bracket) is apparently devoid of rab6. This region is shown at the higher magnification in Fig. 6L. It appears that the polarization of rab6 in photoreceptors is a feature of other retinal neurons as well, since rab6 is found in the dendritic region of bipolar and horizontal cells that form synapses with photoreceptors in the outer plexiform layer (Fig. 6K and L, below the photoreceptor synaptic terminals).

Volume rendering of confocal optical sections has enabled us to quantify the amount of anti-rab6 antibody bound to the photoreceptor membranes. Images obtained by this analysis have indicated that rab6, although detectable in the Golgi complex, is concentrated at two sites: on one side of the Golgi complex in structures revealed by punctate label, which may represent the TGN, and on the periciliary post-Golgi vesicles. This indicates that these two sites are the major domains of rab6 accumulation in retinal photoreceptors.

Fig. 6. Confocal immunocytochemistry reveals that the distribution of rab6 in retinal photoreceptors is highly polarized: while Golgi and post-Golgi membranes involved in the delivery of membrane proteins to the ROS are heavily labeled, axons and synaptic terminals are devoid of detectable rab6. Retinas from dark-adapted frogs were labeled with anti-rab6 antibody followed by a second antibody conjugated to Texas Red. Z sections were taken every 0.25 μm, parallel to the coverslip. Individual optical sections are shown in (A-I) and the merged image in (J). (K) An overall view of the retina indicating that the distribution of rab6 immunoreactivity is clearly polarized in the photoreceptors. (L) The anti-rab6 label is absent from the region extending from the outer nuclear layer to the photoreceptor axons and synaptic terminals (indicated by a large bracket, and the bracket in (K)). The anti-rab6 label appears again in the dendritic processes of the outer plexiform layer (OPL) (below the photoreceptor cell layer). Open arrows indicate the Golgi complex, solid arrows indicate post-Golgi membranes intercalated between mitochondria, solid arrowheads indicate post-Golgi vesicles clustered near the cilium and open arrowheads indicate the ciliary stalk, a cytoplasmic domain of the base of the ROS. Bars: (A-J and L), 5 μm; (K), 20 μm.

EM immunocytochemistry shows abundant rab6 label on the post-Golgi membranes and indicates that rab6 is also present at the sites of new ROS disk formation

By EM immunocytochemistry rab6 is localized on *trans*-Golgi membranes, vesicles that may be a part of a tubular network (the TGN), inter-mitochondrial vesicles (Fig. 7A and C), post-Golgi vesicles and sites of new ROS disk formation (Fig. 7B). Immunoreactivity with rab6 antibody is detected along the pathway of the post-Golgi transport membranes we have previously shown to be involved in the polarized sorting of rhodopsin to the photoreceptor outer segment (Papermaster et al., 1986; Deretic and Papermaster, 1991). Post-Golgi vesicles carrying newly synthesized rhodopsin cluster between the mitochondria and near the cilium that connects the rod outer segment to the inner segment. These vesicles bind several anti-rhodopsin antibodies (Papermaster et al., 1986), including mAb11D5, an anti-rhodopsin C-terminus-specific antibody obtained after immunization with the isolated post-Golgi membranes (Deretic and Papermaster, 1991). Labeling of the inter-mitochondrial and periciliary vesicles with anti-rab6 antibody, as shown in Fig. 7, strongly suggests that rab6 associates with the rhodopsin-bearing membranes as they exit from the Golgi complex, where the sorting occurs. Rab6 remains bound to these membranes until they reach their final destination within the inner segment, where they fuse with the plasma membrane at the base of the connecting cilium before delivery of rhodopsin to the outer segment.

Labeling of the distal connecting cilium (Fig. 7B), which has been implicated in ROS disk morphogenesis (Steinberg et al., 1980; Peters et al., 1983), and labeling of the ciliary stalk (which is clearly seen by confocal microscopy) suggest that after rhodopsin-bearing membranes are incor-

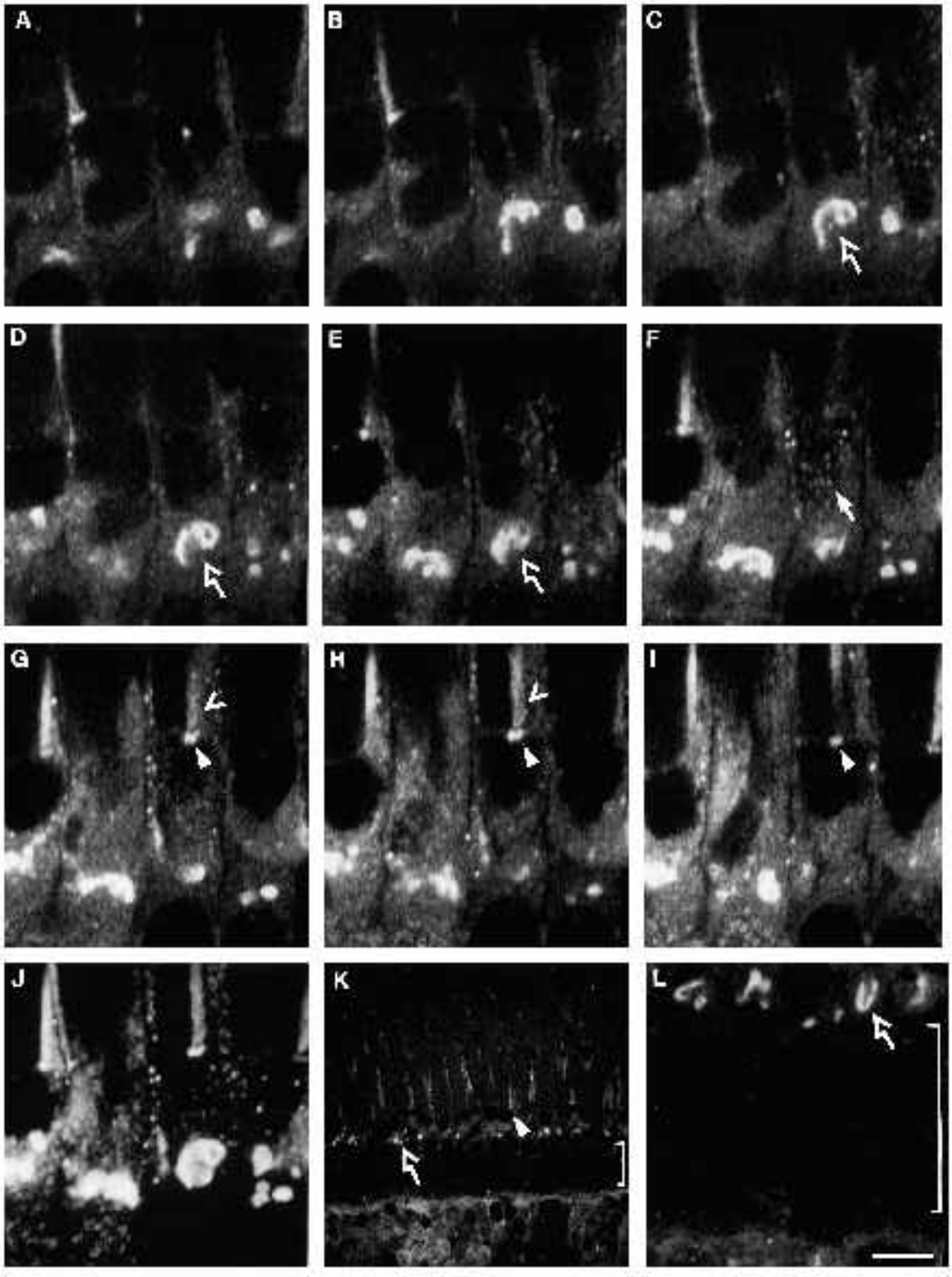

Fig. 6

porated into newly formed ROS disks, rab6 dissociates to a soluble form. It probably recycles back to the inner segment since its turnover is very slow (D. Deretic and D. S. Papermaster, unpublished observation). Patchy label along the inner segment plasma membrane, particularly in the calycal processes that evaginate from the frog rod inner segments and surround outer segments (Fig. 7B), suggests that these structures may be involved in recycling. These regions

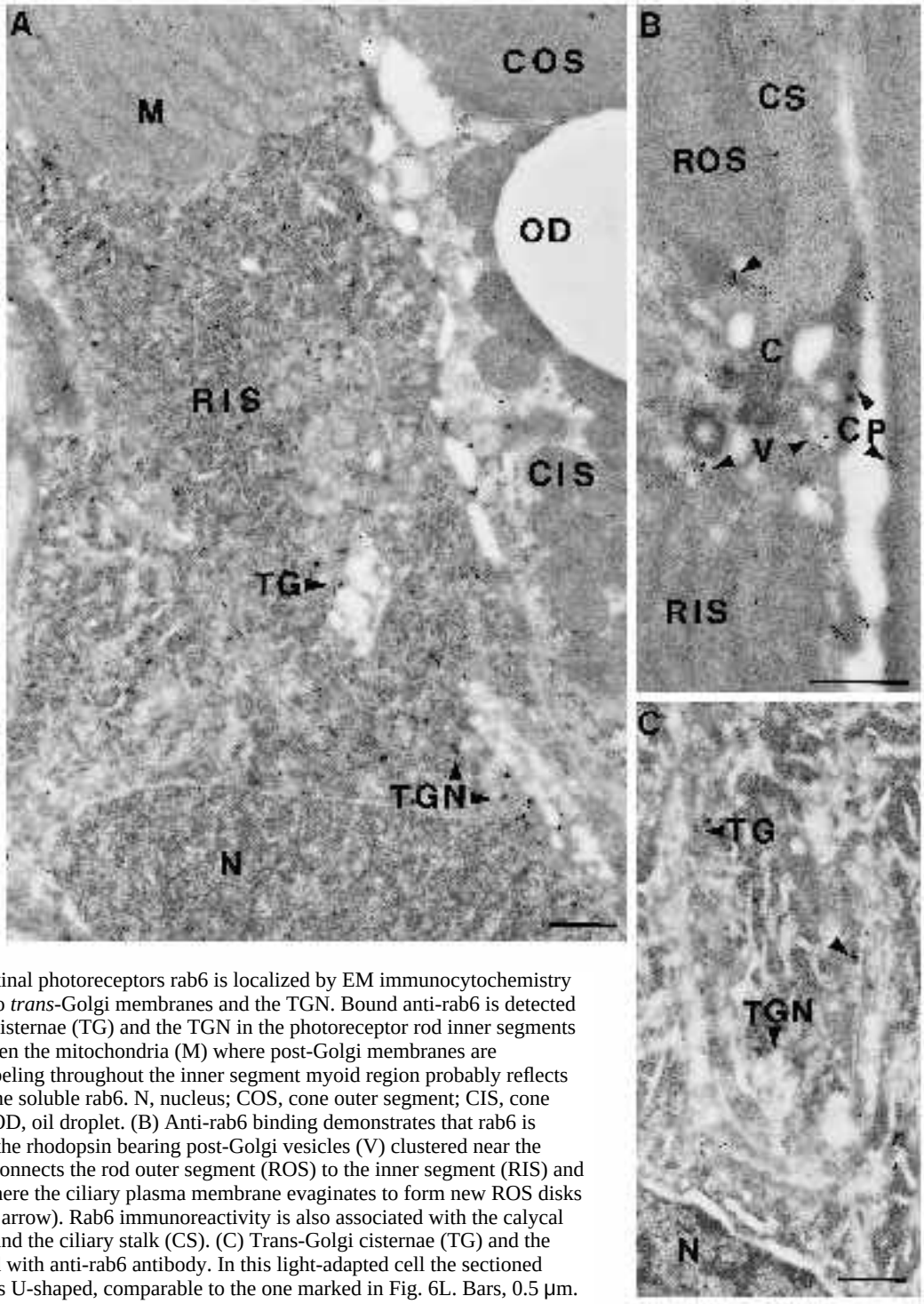

Fig. 7. (A) In retinal photoreceptors rab6 is localized by EM immunocytochemistry predominantly to *trans*-Golgi membranes and the TGN. Bound anti-rab6 is detected on *trans*-Golgi cisternae (TG) and the TGN in the photoreceptor rod inner segments (RIS) and between the mitochondria (M) where post-Golgi membranes are intercalated. Labeling throughout the inner segment myoid region probably reflects localization of the soluble rab6. N, nucleus; COS, cone outer segment; CIS, cone inner segment; OD, oil droplet. (B) Anti-rab6 binding demonstrates that rab6 is associated with the rhodopsin bearing post-Golgi vesicles (V) clustered near the cilium (C) that connects the rod outer segment (ROS) to the inner segment (RIS) and with the sites where the ciliary plasma membrane evaginates to form new ROS disks (indicated by an arrow). Rab6 immunoreactivity is also associated with the calycal processes (CP) and the ciliary stalk (CS). (C) Trans-Golgi cisternae (TG) and the TGN are labeled with anti-rab6 antibody. In this light-adapted cell the sectioned Golgi complex is U-shaped, comparable to the one marked in Fig. 6L. Bars, 0.5 μm.

do not contain rhodopsin but are rich in actin (Chaitin et al., 1984), which may facilitate retrograde transport of rab6.

Curiously, anti-rab6 antibody also prominently labels outer segments of green rods, a minor rod cell subpopulation in frogs that accounts for ~5% of total rods (data not shown). It is not clear if this labeling is due to the cross-reactivity with an unrelated antigen, or if the mechanism of rhodopsin transport in these cells differs from the pathway observed in the major population of red rods. Labeling of the green rod inner segments resembles that of the red rods. Therefore, it is likely that the green rod outer segment labeling is a cross-reaction with one of its major proteins. The nature of this protein is not revealed by immunoblots of the ROS, since green ROS are shorter and therefore contribute only ~2% of the protein in an ROS preparation.

Our biochemical and morphological data therefore suggest that protein rab6 in retinal photoreceptor cells participates in the complex regulation that directs post-Golgi

transport and polarized delivery of newly synthesized rhodopsin to the rod outer segments.

DISCUSSION

We have analyzed small G proteins that are tightly associated with the post-Golgi membranes of retinal photoreceptors as part of a search for molecules that may regulate their intracellular traffic. Subcellular distribution of two of these proteins suggests that they may participate in the post-Golgi transport and sorting of newly synthesized rhodopsin.

One of these proteins is rab6, which was previously localized to the medial and *trans*-Golgi in NRK cells (Goud et al., 1990). By combined biochemical and immunocytochemical analysis we have localized this protein to the *trans*-Golgi cisternae, TGN, post-Golgi membranes carrying newly synthesized rhodopsin and newly formed ROS disks at the base of the outer segment. The differences in subcellular localization of rab6 in the photoreceptor, compared to NRK cells, suggest that these two cells may utilize rab6 for slightly varied, but, nevertheless, late stages of the exocytic membrane biosynthetic pathway. Our interpretation is further supported by the recent study of Jasmin et al. (1992), which determined, by immunocytochemical analysis, that in electrocytes of *Torpedo marmorata*, rab6 has a polarized distribution and associates mostly with the post-Golgi membranes delivering proteins, particularly acetylcholine receptor (AChR), to the post-synaptic membranes, a domain homologous to the dendritic surface of neurons. While this manuscript was in preparation, Antony et al. (1992) also localized rab6 by confocal microscopy to the TGN of various cells. Rab6 in photoreceptors appears to be specifically associated with rhodopsin-bearing membranes since rhodopsin-laden vesicles, prior to their fusion with the inner segment plasma membrane near the cilium, as well as the sites of new ROS disk formation, contain rab6 while immunoreactivity with this antibody is absent from the synaptic terminal at the opposite end of the cell. The presence of rab6 on immunoisolated rhodopsin-bearing vesicles further strengthens this assignment.

The observed differences in rab6 distribution among different cell types in various species may result from the engagement of cells like photoreceptors and electrocytes in the predominant synthesis and transport of a single protein: rhodopsin is the major protein synthesized by the photoreceptors and AChR is a major protein delivered to the post-synaptic membranes by the electrocytes. This probably results in the relative hypertrophy of the post-Golgi compartments involved in the sorting of these proteins, which makes localization of rab6 to the post-Golgi membranes possible in these two cell types.

Association of rab6 with rhodopsin-bearing membranes and its subcellular localization fulfill the criteria for a protein that contains, or contributes to, the sorting signal for rhodopsin transport. The highly polarized photoreceptor cell sorts rhodopsin and synaptophysin, proteins destined for the opposite ends of the cell, after they exit the *trans*-Golgi cisternae (Schmied and Holtzman, 1989). We have now shown that rab6 is one of the first proteins to join rhodopsin-bearing membranes in the *trans*-Golgi cisternae. Localization of rab6 in the photoreceptor TGN strongly suggests that the function of this protein is to participate in directing the sorting of the membranes destined for delivery to the ROS.

How does rab6 find its way back after it has delivered its cargo protein? The mechanism for recycling rab6 back to the TGN is not clear at present. The post-Golgi compartment extends throughout a significant portion of the elongated photoreceptor cell. We have observed significant anti-rab6 labeling of the calycal processes and distal ends of the connecting cilium stalk at the base of the ROS, greater than that of the reminder of the ROS. These sites were previously identified as actin-rich domains (Chaitin et al., 1984). In addition, the distal connecting cilium is involved in the ROS disk morphogenesis (Steinberg et al., 1980; Peters et al., 1983; Chaitin and Burnside, 1989). It is possible that rab6 dissociates from the post-Golgi membranes at the site of disk formation and recycles back to the TGN via a microfilament-dependent mechanism.

Recently, Nilsson et al. (1993) have shown that different cisternae in the Golgi complex of HeLa cells are defined not by unique sets of glycosyltransferases but by different mixtures of these enzymes. Our current and previous results (Deretic and Papermaster, 1991) suggest that the post-Golgi compartment in photoreceptor cells can be further subfractionated into several different fractions defined by the mixtures of glycosyltransferases and/or rab6 association. Partial overlap of sialyltransferase and galactosyltransferase (which peaks in fractions 11-13; Deretic and Papermaster, 1991) and partial overlap of rab6 and sialyltransferase distribution (which peaks in fractions 8-9) suggest that newly synthesized rhodopsin travels as follows: from the compartment that is very rich in galactosyltransferase, also contains sialyltransferase and has a small fraction of rab6 (*trans*-Golgi cisternae), to the compartment that is very rich in sialyltransferase and rab6 (TGN), to the compartment that is still rich in rab6 but has lower sialyltransferase activity (fractions 7 and 8, distal TGN ?), and finally to the post-Golgi vesicles (fraction 5) that contain rab6 but very little sialyltransferase. Distribution of these compartments on the sucrose gradient suggests that maturation of post-Golgi vesicles in photoreceptors also involves an increase in the lipid/protein ratio that results in the lower buoyant densities of the successive compartments. Distribution of anti-rab6 label by EM and confocal immunocytochemistry suggests that the photoreceptor TGN is in close proximity to the Golgi complex, while the intermitochondrial post-Golgi membranes may be a part of the 'distal' TGN. Identification of additional components of the sorting machinery and their subcellular localization should further define the post-Golgi compartment.

Since rab6 appears to be a part of a sorting machinery that ensures proper delivery of rhodopsin, it probably acts in concert with its regulatory proteins, which may also involve other small or heterotrimeric G proteins, some of which may be other rab proteins. We have shown that post-Golgi membranes contain several other G proteins that may be candidates for these interactions. We have recently identified one of these proteins as rab8 (D. Deretic, L. A. Huber, K. Simons and D. S. Papermaster, unpublished). With this in mind, it is important that our post-Golgi membrane preparations do not contain endosome-specific proteins

rab4, rab5 and rab7 (L. Huber, personal communication). This is remarkable since endosomes in hepatocytes have the same sucrose gradient sedimentation profile as the photoreceptor post-Golgi membranes (Dunn and Hubbard, 1984).

Although rab6 is absent from the synaptic terminal, its involvement in the initial steps of sorting of the synaptic proteins is not ruled out. If the sorting mechanism for synaptophysin in frog retinal photoreceptors resembles that of PC 12 cells (Regnier-Vigouroux et al., 1991), separation of rhodopsin from synaptophysin may occur at the stage even later than the TGN. We have found small amounts of synaptophysin in the post-Golgi fraction. Moreover, another small G protein, a member of the rab3 family, is associated with the post-Golgi membranes that had been immunoisolated with antibody to rhodopsin. The more acidic of the two proteins indicated in Fig. 1B reacts with anti-rab3A mAb 42.2 (Matteoli et al., 1991) (D. Deretic and D. S. Papermaster, unpublished observations). Since most of rab3 is also on the synaptic vesicles (Kim et al., 1989; Fischer von Mollard et al., 1990, 1991), we believe that, in photoreceptors, proteins destined for the axonal domain begin their post-Golgi transport on the same membranes as rhodopsin. If this is true, rab6 may direct membrane traffic to the ROS and another rab protein may act at the later stage for the separation of these two pathways and direct delivery of proteins to the axon and the synapse.

It remains to be determined if sorting of the post-Golgi membranes in retinal photoreceptors involves a distinct post-translational modification of rab6 and whether this presents a signal, or is a consequence of a separate sorting signal for the proper delivery of rhodopsin. It is clear from our study that rab6 is present on several succesive compartments involved in the delivery of newly synthesized rhodopsin to the ROS in retinal photoreceptors. In addition to participating in post-Golgi sorting events, rab6 is localized to a site where it may contribute to ROS disk morphogenesis. This suggests that rab6, like rab1b (Plutner et al., 1991) may function at several transport steps and may regulate both formation and docking of the rhodopsin-bearing post-Golgi membranes.

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