Journal of Cell Science 108, 215-224 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

rab8 in retinal photoreceptors may participate in rhodopsin transport and in rod outer segment disk morphogenesis

Dusanka Deretic[1,*], Lukas A. Huber[2,†], Nancy Ransom[1], Michael Mancini[3], Kai Simons[2] and David S. Papermaster[1]

[1]Department of Pathology University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284-7750, USA
[2]Cell Biology Programme, EMBL, Heidelberg, Germany
[3]Institute of Biotechnology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284-7750, USA

*Author for correspondence
[†]Present address: Department of Biochemistry, University of Geneva, Geneva, Switzerland

SUMMARY

Small GTP-binding protein rab8 regulates transport from the TGN to the basolateral plasma membrane in epithelial cells and to the dendritic plasma membrane in cultured hippocampal neurons. In our approach to identify proteins involved in rhodopsin transport and sorting in retinal photoreceptors, we have found, using [^{32}P]GTP overlays of 2D gel blots, that six small GTP-binding proteins are tightly bound to the post-Golgi membranes immunoisolated with a mAb to the cytoplasmic domain of frog rhodopsin. We report here that one of these proteins is rab8. About 50% of photoreceptor rab8 is membrane associated and ~13% is tightly bound to the post-Golgi vesicles. By confocal microscopy, antibody to rab8 specifically labels calycal processes and the actin bundles of the photoreceptor inner segment that extend inward to the junctional complexes that comprise the outer limiting membrane. Anti-rab8 shows a striking periodicity of high density labeling at 1±0.12 μm intervals along the actin bundles. Rhodopsin-bearing post-Golgi membranes cluster around the base of the cilium where rab8 and actin are also co-localized, as revealed by confocal microscopy of retinal sections double labeled with anti-rab8 and phalloidin. Microfilaments have been implicated in rod outer segment (ROS) disk morphogenesis. Our data suggest that rab6, which we have previously localized to the post-Golgi compartment, and rab8 associate with the post-Golgi membranes sequentially at different stages of transport. rab8 may mediate later steps that involve interaction of transport membranes with actin filaments and may participate in microfilament-dependent ROS disk morphogenesis.

Key words: rab protein, small GTP-binding protein, rhodopsin, membrane transport and sorting

INTRODUCTION

To understand the molecular mechanisms that underline polarized sorting of newly synthesized rhodopsin to the rod outer segments (ROS) in retinal photoreceptors, we have isolated a population of low buoyant density vesicles that mediate its post-Golgi transport (Deretic and Papermaster, 1991). We found that these vesicles contain at least six membrane-bound small GTP-binding proteins that may be involved in regulating vesicular targeting. One of these proteins, rab6, is significantly enriched on the photoreceptor *trans*-Golgi network (TGN) and post-Golgi vesicle membranes (Deretic and Papermaster, 1993a). In the present study we have continued with the identification of the rab proteins associated with the post-Golgi membranes, since they, together with their accessory proteins, play a crucial role in the regulation of membrane traffic (reviewed by Simons and Zerial, 1993; Zerial and Stenmark, 1993; Ferro-Novick and Novick, 1993; Novick and Brennwald, 1993; Deretic and Papermaster, 1994).

rab proteins involved in endocytosis and exocytosis have been localized to the specific cellular compartments in several cell types where they control discrete steps of membrane traffic. However, it is becoming increasingly clear that regulation of membrane trafficking by rab proteins is more complex than initially thought. rab proteins and their associated proteins, by their combined interactions, appear to establish the specificity of vesicle-target recognition. Subcellular compartments, such as post-Golgi vesicles, may contain several rab proteins that cooperatively participate in different aspects of vesicle formation, targeting and fusion.

Post-translational modifications of rab proteins are crucial for their function. Isoprenylated rab proteins are escorted to the membrane compartment where they start their functional cycle by the subunit A of the rab geranylgeranyl transferase, now called REP1 (rab escort protein; Andres et al., 1993), which is homologous to the rab GDP dissociation inhibitor (GDI). REP1 is the protein that is mutated in human choroidoremia (Seabra et al., 1992, 1993). This mutation affects the choroidal vascularization of the retina and the retinal pigment epithelial cells in a fashion that ultimately leads to photoreceptor death

and blindness. A recently identified protein REP2, a REP1 homolog can substitute for REP1 in supporting geranylgeranylation of several rab proteins but its activity is much lower toward rab3A and rab3D (Cremers et al., 1994). This finding strongly suggests that REP2, or other REP proteins, substitute for REP1 in non-retinal cells of patients with choroidoremia, while in the retina this substitute is ineffective in preventing photoreceptor cell death and blindness. Therefore, lack of functional rab proteins may cause degeneration and death of the most sensitive cells, which suggests that rab proteins may play a critical role in the maintenance of functional retinal photoreceptors.

Small GTP-binding protein rab8 regulates post-Golgi transport to the basolateral plasma membrane in epithelial cells and to dendrites in cultured neurons (Huber et al., 1993a,b). Since photoreceptor cells are modified neurons and ROS, which contain mature rhodopsin, may represent modified dendrites, we wanted to determine if rab8 could possibly participate in the delivery of membrane proteins to the ROS. Indeed, we find that a significant fraction of membrane-bound rab8 in photoreceptors associates with the post-Golgi membranes that transport newly synthesized rhodopsin to the ROS. In addition, morphological and biochemical evidence indicates that rab8 is associated with actin filaments. This association suggests that rab8 may regulate some of the final steps in the post-Golgi transport of rhodopsin, which include microfilament-mediated rod outer segment disk 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) was from New England Nuclear (Boston, MA), Ampholines (pH 7-9, pH 5-7 and pH 3.5-9.5) from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ), urea (manufactured by BDH, Polle Dorset, UK) from Hoefer (San Francisco, CA), acrylamide from Accurate Chemical & Scientific Corp. (Westbury, NY), anti-actin antibody from Biomedical Technologies Inc. (Stoughton, MA), peroxidase-conjugated anti-rabbit and anti-mouse IgG from Kirkegaard and Perry (Gaithersburg, MD), the ECL Western Blotting Detection System and Hyperfilm ECL from Amersham Corporation (Arlington Heights, IL), 4% paraformaldehyde in 0.1 M phosphate buffer from Tousimis (Rockville, MD), Triton X-100 (for membrane research) from Boehringer Mannheim Diagnostics, Inc. (Houston, Tx), goat anti-rabbit IgG conjugated to Texas Red and fluorescein-phalloidin from Molecular Probes, Inc. (Eugene, OR).

Preparation of rabbit antiserum and affinity-purified antibody to the canine rab8 has been described previously (Huber et al., 1993a). Several antibodies were kindly provided for this study: polyclonal anti-ARF and monoclonal anti-ARF1 by Dr Richard Kahn (NIH, Bethesda, MD) and anti-Na,K-ATPase by Dr Robert Mercer (Washington University, St Louis, MO), respectively.

Retinal subcellular fractionation

Retinal subcellular fractionation was performed as described by Deretic and Papermaster (1991, 1993b). Briefly, frog retinas were isolated and ROS were separated and further purified on a step sucrose gradient. 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% to 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 was determined from a sample and the remainder was diluted with 10 mM Tris-acetate, pH 7.4, and centrifuged at 50,000 rpm for 40 minutes in a SW50.1 rotor. Pellets were resuspended in 10 mM Tris-acetate, pH 7.4, and sampled for analysis by SDS-PAGE or two-dimensional gel electrophoresis.

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 the method of 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-rab8 antiserum was diluted to 1:200, anti-actin antibody and anti-Na,K-ATPase antiserum were diluted 1:1000 in TTBS (TBS containing 0.05% Tween-20), incubated for 3 hours at 20°C, followed by peroxidase-conjugated anti-rabbit IgG. Bound antibodies were detected by the ECL Western Blotting Detection System (Amersham), according to the manufacturer's instructions. Hyperfilm ECL was scanned using the Image Analysis Program (Wayne Rasband, NIH).

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

Gels were blotted onto Immobilon-P membranes (Millipore) in a 10 mM CAPS, pH 11, 10% methanol buffer (Matsudaira, 1987). GTP-binding proteins were detected by [^{32}P]GTP overlays by the modified method of Lapetina and Reep (1987) as described (Deretic and Papermaster, 1993a). Blots were preincubated for 30 minutes with 1 μM ATP in binding buffer (50 mM phosphate buffer, pH 7.0, 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.

Immunofluorescence and confocal microscopy

Immunofluorescence and confocal microscopy were performed as described (Deretic and Papermaster, 1993a) by the method of Matsumoto and Hale (1993a). Frogs were dark-adapted and isolated retinas 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-rab8 antibody (dill 1:20) followed by goat anti-rabbit IgG conjugated to Texas Red. For double labeling, this was followed by fluorescein-phalloidin (dil. 1:20). All incubations were overnight at 4°C. Confocal optical sections at 0.25 μm intervals were obtained with a Zeiss Laser Scanning Confocal Microscope 310 (Carl Zeiss, Inc, White Plains, NY), using a 488 nm argon ion laser and 543 nm HeNe laser for fluorescein and Texas Red excitation, respectively. Digitized images of confocal optical sections 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) using Kodak Ektachrome 100 HC film.

High-resolution scanning electron microscopy (SEM)

Frog retinas were dehydrated and critical-point dried using a Bomar critical-point dryer with CO_2. Critical-point-dried tissue was sputter coated with gold-palladium and scanned in a JEOL 840A scanning electron microscope.

RESULTS

Several small GTP-binding proteins are associated with post-Golgi vesicles that transport newly synthesized rhodopsin. We have previously identified one of them as rab6 (Deretic and Papermaster, 1993a). Comparison of [^{32}P]GTP overlays of retinal subcellular fractions from sucrose density gradients demonstrates that the complexity of small GTP-binding protein content decreases as post-Golgi vesicles bud from the Golgi. Their GTP-binding protein content begins to resemble that of rod outer segment (ROS) membranes (Fig. 1). To determine if rab8 is also associated with rhodopsin-bearing vesicles, we immunoblotted the post-Golgi vesicle fraction from the sucrose density gradient with a polyclonal antibody raised against the C-terminal peptide of canine rab8 (described by Huber et al., 1993a). We find that anti-canine rab8 antibody also recognizes frog retinal rab8, one of the small GTP-binding proteins common to the post-Golgi vesicles and ROS membranes (Fig. 1D). Its binding is specifically inhibited by a peptide derived from the C-terminal, hypervariable domain of canine rab8 (data not shown). The estimated size of the frog retinal post-Golgi membrane-associated rab8 is ~23.5 kDa and its pI is ~5. The molecular mass is in good agreement with 23.7 kDa predicted from the nucleotide sequence of canine rab8 (Chavrier et al., 1990), but its apparent pI of ~5 is very different from the pI of 9.2 of the protein estimated from its sequence. However, canine rab8 also migrates identically to the frog protein in this 2-D gel system (Huber et al., 1993a, 1994).

rab8 is enriched on post-Golgi vesicles compared to the other subcellular fractions, since these membranes represent only a small fraction (0.03%) of the total retinal homogenate (Deretic and Papermaster, 1991). rab8 is tightly bound to rhodopsin-bearing vesicles, because membranes immunoisolated with a monoclonal antibody to the cytoplasmic domain of rhodopsin contain all of the small GTP-binding proteins present in the post-Golgi-enriched retinal subcellular fraction. Moreover, all of them partition into the detergent phase after Triton X-114 extraction and they cannot be removed by 4 M urea or high pH washes (Deretic and Papermaster, 1993a). Association of rab8 with rhodopsin-bearing vesicles resembles its tight binding to immunoisolated basolateral TGN-derived vesicles in MDCK cells (Huber et al., 1993a).

Confocal microscopy reveals co-localization of rab8 with actin bundles in photoreceptor cells

Confocal microscopy of retinal photoreceptors labeled with anti-rab8 antibody displays a pattern of labeling that suggests that some of the photoreceptor content of rab8 may be associated with cytoskeletal elements. A Z-series of confocal optical sections through the retinal photoreceptor cell layer at 0.25 μm

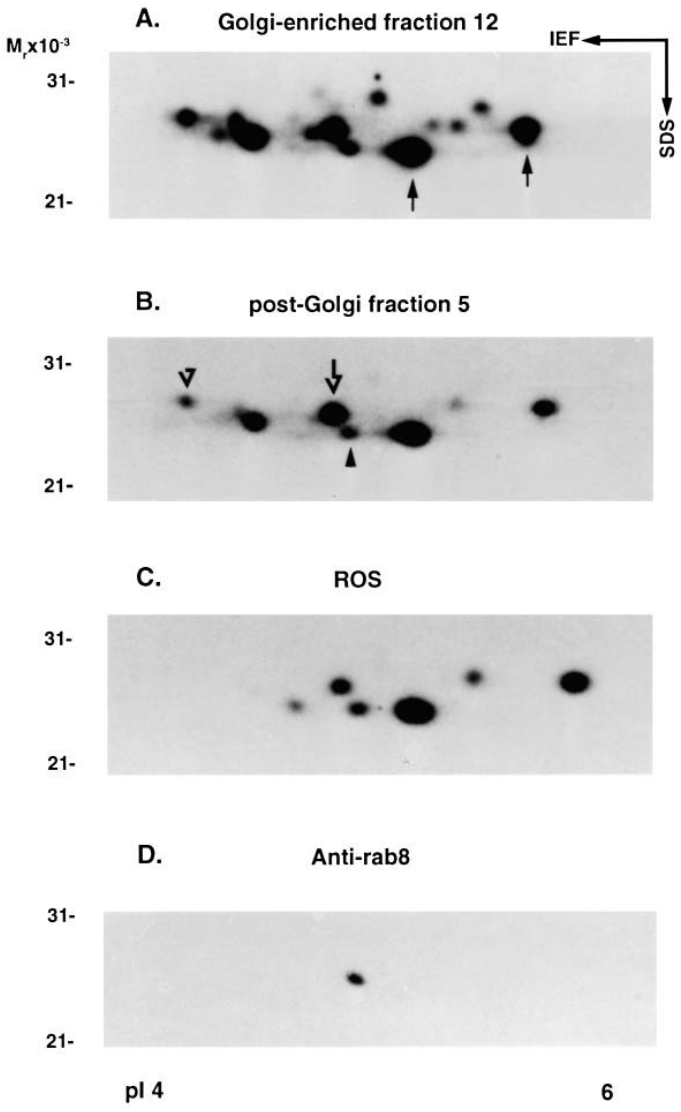
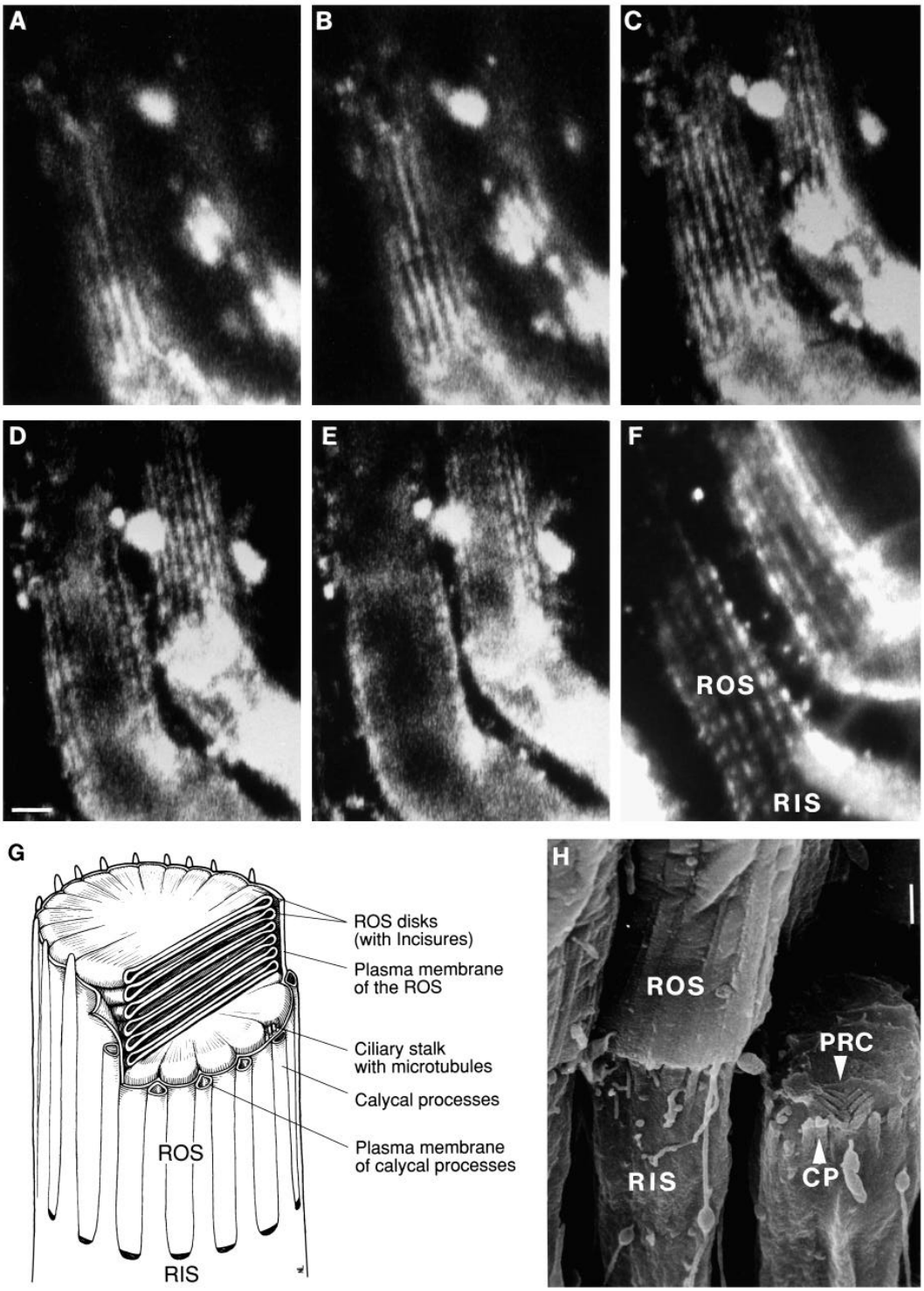


Fig. 1. GTP overlays of 2-D gels of membrane-associated small GTP-binding proteins from the Golgi-enriched fraction 12, post-Golgi vesicle fraction 5 and ROS. Antibody to rab8 recognizes one of the GTP-binding proteins common to the post-Golgi vesicles and ROS membranes. (A) The complex set of Golgi-associated small GTP-binding proteins (fr. 12) also includes GTP-binding proteins found on the post-Golgi membranes (fr. 5). Two of these proteins (arrows) are found in all cell types and most subcellular fractions studied (Huber et al., 1994). (B) The post-Golgi membranes (fr. 5) have a simpler GTP-binding protein composition. Many of the GTP-binding proteins have been identified by monospecific antibody binding. One of them is rab6 (open arrow) (Deretic and Papermaster, 1993a). The most acidic member of the set (open arrowhead) reacts with anti-rab3A (mAb 42.2; Matteoli et al., 1991). (C) The post-Golgi membranes share four GTP-binding proteins with the ROS membranes to which mature rhodopsin is delivered. Samples of retinal subcellular fractions isolated from two frog retinas were separated by 2-D gel electrophoresis and small GTP-binding proteins were detected by [^{32}P]GTP overlays. (D) Anti-canine rab8 antibody (described by Huber et al., 1993a) also recognizes frog retinal rab8, which migrates at ~23.5 kDa and an apparent pI ~5 (also indicated with an arrowhead in B).

intervals is shown in Fig. 2. Anti-rab8 intensely labels retinal photoreceptors and is probably detecting both soluble and membrane-bound forms, since the cytoplasmic pool of small GTP-binding proteins has not been extracted before fixation. Unlike rab6, whose distribution in retinal photoreceptors is ~70% membrane bound and 30% soluble, only slightly more than 50% of rab8 is membrane bound. This distribution is different from the distribution in MDCK cells where most of rab8 is membrane associated (Huber et al., 1993a). The significance of such a large soluble pool of rab8 in photoreceptors is not clear. In addition to the rab8 label clearly associated with the rod inner segments, it is also present in the villous structures called calycal processes that surround the base of the ROS (see panel G). A striking feature of anti-rab8 labeling is its periodicity along the cell axis. Intensity of label increases at 1±0.12 μm intervals, as shown in panel F, suggesting that rab8 may be associated with repetitive structures linked to the actin bundles. Panels G and H show a schematic diagram of



the region of photoreceptor cells where rab8 label is observed, and a SEM of the same region, to facilitate the understanding of the images generated by confocal microscopy of anti-rab8.

To test if the filamentous structures containing rab8 are indeed actin filaments we double-labeled retinal photoreceptors with anti-rab8 antibody detected with Texas Red, and phalloidin conjugated to fluorescein. Microfilament bundles, revealed by phalloidin binding, encircle photoreceptor inner segments and extend from the junctional complexes that form the outer limiting membrane outward to the calycal processes that evaginate from the inner segments and surround the ROS (Fig. 3). Partial colocalization of rab8 and photoreceptor microfilaments is particularly evident in the calycal processes (where the periodicity of label is observed), at the outer limiting membrane, and around the base of the photoreceptor cilium where rhodopsin-bearing vesicles cluster before their delivery to the outer segments. The Golgi complex does not appear to contain significant amounts of immunocytochemically detectable rab8. ROS disk membranes also appear to be unlabeled while rab8 is detected in the ciliary stalk, a cytoplasmic compartment of the ROS. Taken together these data indicate that rab8 may provide a mechanism for the attachment of rhodopsin-bearing vesicles to the microfilament bundles before their incorporation into ROS disk membranes.

The localization of rab8 in photoreceptors was also studied by EM immunocytochemistry. Labeling of LR gold-embedded glutaraldehyde-fixed retinas, previously used to localize rab6 in these cells (Deretic and Papermaster, 1993a), yielded inconclusive results due to the very low intensity of label. We interpret this result to be a consequence of antigen sensitivity to glutaraldehyde fixation. We next immunolabeled paraformaldehyde-fixed 100 μm thick sections by a pre-embedding protocol in the same manner as for confocal microscopy. After embedding them in LR gold and cutting thin sections we detected bound anti-rab8 antibody with a secondary antibody conjugated to 10 nm gold. This approach yielded specific labeling, which paralleled that obtained by confocal microscopy, although intensity of label was still very low (data not shown). This result is not surprising, since the intensity of rab8 label detected by immunoelectron microscopy of MDCK cells was also very low compared to confocal microscopy of the same cells using the same antibody (Huber et al., 1993a).

Fig. 2. By confocal immunolabeling, rab8 is localized to axially aligned structures that probably are actin bundles. Retinas from dark-adapted frogs were labeled with affinity-purified anti-rab8 antibody followed by second antibody conjugated to Texas Red. Z sections were taken every 0.25 μm parallel to the coverslip and they revealed that the structures containing rab8 surround the photoreceptor rod outer segments (ROS) (A-E). A merged image of a different series of optical sections is shown in F. It shows a striking periodicity of labeling at 1±0.12 μm along the cell axis, probably along the actin bundles. Actin was separately identified by phalloidin binding (see Fig. 3). RIS, rod inner segments. (G) A diagram that schematically represents the structural relations between the ROS and RIS (modified from Brown et al., 1963, with permission). Calycal processes evaginate from the RIS and surround the base of the ROS. They are labeled with anti-rab8 antibody (A-F) and contain actin filaments (see Fig. 3). The ciliary stalk lies along one side of the ROS. It is devoid of ROS disk membranes and includes ROS cytoplasm and ciliary microtubules. (H) An SEM of a frog retina. When the rod outer segments (ROS) are broken off at the connecting cilium (right cell), the structures involved in the fusion of rhodopsin-bearing post-Golgi vesicles are revealed. The rod inner segment (RIS) plasma membrane forms a characteristic structure, the periciliary ridge complex (PRC), which surrounds the connecting cilium and provides the docking site for rhodopsin-bearing vesicles (Papermaster et al., 1986; Peters et al., 1983). Calycal processes (CP), which are broken off in this cell, project from the inner segment and surround the base of the outer segment (left cell). Bars: (A-F) 2.5 μm; (H) 1 μm.

Rhodopsin-bearing post-Golgi vesicles are the principal sites of rab8 membrane association in photoreceptors

In order to determine the distribution of rab8 among density gradient fractions from retinal homogenates we have immunoblotted membrane proteins with an anti-rab8 antibody and quantitated the amount of antibody bound to the membranes from each fraction. Fractions 4-6 contain ~30% of the total membrane-associated rab8 (Fig. 4). The subcellular distribution of rab8 therefore coincides with a photoreceptor compartment that we have previously identified as rhodopsin-bearing post-Golgi membranes (Deretic and Papermaster, 1991). Anti-rab8 antibody binding to the post-Golgi membranes is completely inhibited by the C-terminal peptide of rab8 (data not shown).

To test if rab8 is also colocalized with membrane-bound microfilaments, we have immunoblotted the same fractions with the anti-actin antibody. Actin is concentrated in two major peaks: one in fractions 4-5 and the other, with ~50% of the total actin, in fractions 10-12. The less-dense peak coincides with the sedimentation of post-Golgi vesicles. The distribution of the more-dense actin peak completely parallels that of Na,K-ATPase, a membrane protein localized exclusively in the lateral plasma membrane of the photoreceptor inner segment (Schneider et al., 1991). This suggests that a portion of the microfilaments co-sedimenting to the high-density fractions may be associated with the lateral plasma membranes. These complexes, however, do not contain a significant portion of the total membrane-bound rab8.

Since multiple lines of evidence suggest that both rab6 and rab8 are present on the post-Golgi membranes, we wanted to compare their overall intracellular distribution and correlate it with the kinetics of transport of newly synthesized rhodopsin, anticipating that this may provide some insight into their mechanism of action. The distribution of rab6 predominantly coincides with the TGN marker sialyltransferase, but a considerable proportion is found in lighter fractions as well, indicating that rab6 is also bound to the post-Golgi rhodopsin-bearing membranes (Deretic and Papermaster, 1993a; and shown in Fig. 4). The distribution of ARF, a small GTP-binding protein involved in intra-Golgi transport (Stearns et al., 1990; Serafini et al., 1991) parallels that of the *trans*-Golgi marker galactosyltransferase in retinal photoreceptors (Fig. 4). Distribution of galactosyltransferase has been determined (Deretic and Papermaster, 1991). These data suggest that small GTP-binding proteins ARF, rab6 and rab8 associate predominantly with *trans*-Golgi, TGN and the post-Golgi membranes, respectively. Comparison of their distribution with that of radiolabeled rhodopsin at various times during the pulse-chase experiments (Deretic and Papermaster, 1991; and shown in

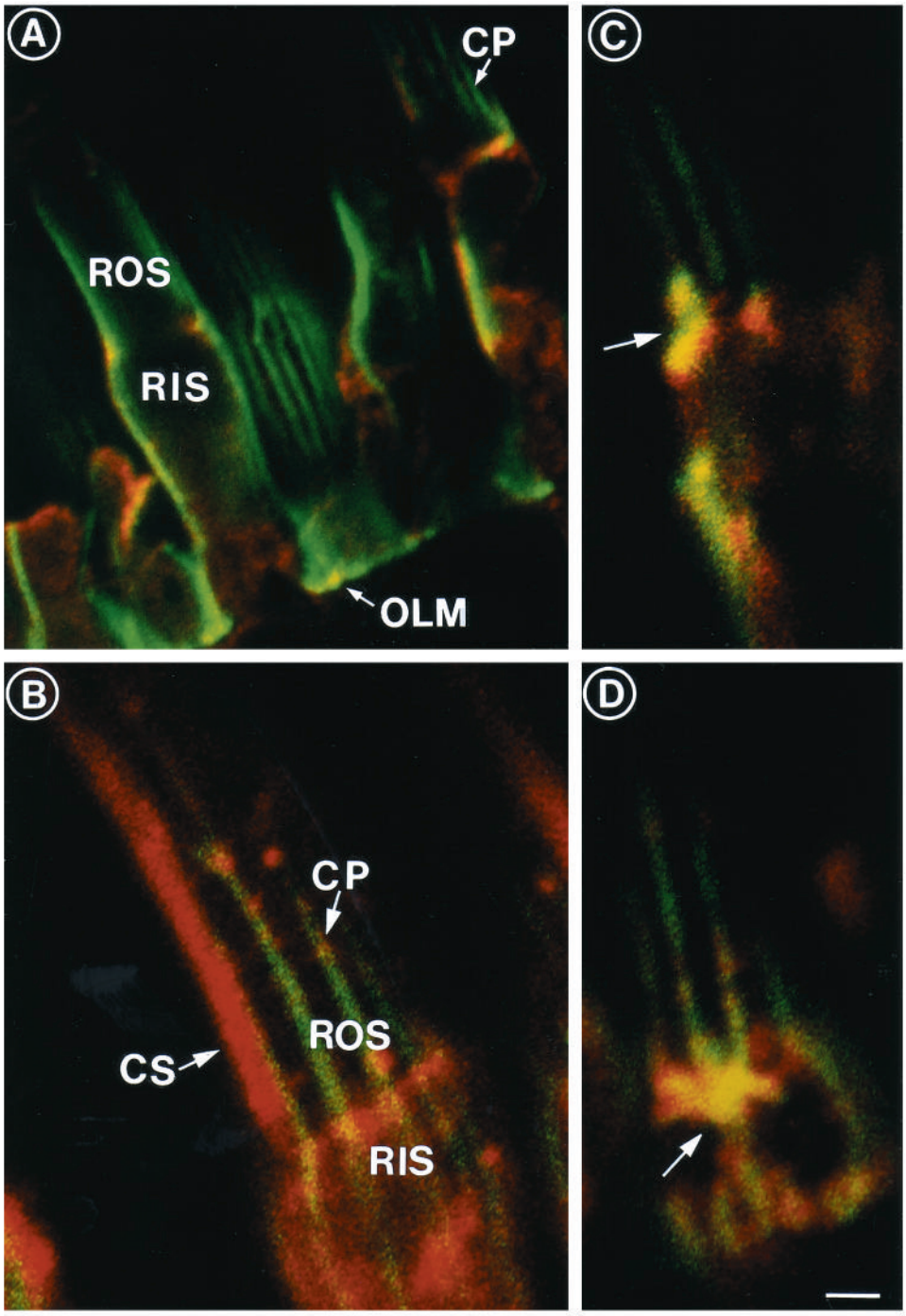


Fig. 3. Confocal microscopy of photoreceptor cells double-labeled with anti-rab8 antibody (red) and phalloidin (green) shows partial co-localization of rab8 with the actin bundles (yellow). Retinas from dark-adapted frogs were labeled with affinity-purified anti-rab8 antibody, followed by second antibody conjugated to Texas Red (red), followed by fluorescein-conjugated phalloidin (green). (A) Actin filaments in photoreceptor cells extend from the junctional complexes that comprise the outer limiting membrane (OLM) to the calycal processes (CP) that evaginate from the photoreceptor rod inner segments (RIS) and surround the rod outer segment (ROS). rab8 is partially colocalized with microfilaments underlining the inner segment plasma membrane. It is concentrated at the RIS/ROS junction, in the calycal processes (shown at higher magnification in B) and at the outer limiting membrane. Regions of overlapping distribution appear yellow. (B) rab8 is concentrated in the calycal processes (CP) and along the ciliary stalk (CS) in the ROS. Anti-rab8 labeling is not diffuse but it clusters at a periodicity of 1±0.12 μm along the actin bundles (see Fig. 2F). (C and D) Rhodopsin-bearing post-Golgi vesicles cluster around the base of the cilium (arrow) that connects the ROS to the RIS where rab8 and actin are also co-localized (yellow). Compare C and D with Fig. 2H, which shows a three-dimensional relationship between the connecting cilium, the PRC and the calycal processes with actin bundles viewed from a similar angle. Bars: (A) 5 μm; (B-D) 1 μm.

Fig. 4D) suggests that small GTP-binding proteins ARF, rab6 and rab8 may regulate successive and partially overlapping steps in rhodopsin transport. While after 90 minutes of radiolabeling rhodopsin co-distributes with ARF- and rab6-containing compartments, following an additional two hours of cold chase the distribution of radiolabeled rhodopsin exclusively parallels rab6- and rab8-enriched compartments. Therefore, the rab6-enriched compartment receives radiolabeled rhodopsin prior to its delivery to the rab8-enriched compartment, suggesting that rab6 and rab8 regulate discrete and successive steps in the post-Golgi transport of rhodopsin.

Taken together our data indicate that in photoreceptors rab8 associates with the post-Golgi vesicles at the late stages of transport and that a significant fraction of rab8 is associated with microfilament bundles. Since microfilaments have been implicated in ROS disk morphogenesis (Chaitin et al., 1984; Williams et al., 1988) our data therefore suggest that rab8 may mediate the attachment of newly synthesized membranes to the microfilaments that initiate new disk formation in the ROS.

DISCUSSION

The small GTP-binding protein rab8 regulates post-Golgi

transport in polarized epithelial cells and neurons (Huber et al., 1993a,b). It has high homology to yeast *Saccharomyces cerevisiae SEC4*, which, when mutated, blocks secretion and causes accumulation of the post-Golgi secretory vesicles (Salminen and Novick, 1987; Goud et al., 1988). Moreover, rab8 can complement the mutation in *ypt2*, a *SEC4* homolog in the fission yeast *Schizosaccharomyces pombe*, although it does not complement *SEC4* mutation (Craighead et al., 1993). This suggests a high degree of evolutionary conservation of the common machinery responsible for the late events in intracellular transport. In our studies of post-Golgi transport of newly synthesized rhodopsin and its polarized sorting to the photoreceptor ROS, we are interested in identifying proteins that may regulate late steps in rhodopsin transport. Here we show that the small GTP-binding protein rab8 specifically associates with rhodopsin-bearing post-Golgi membranes in retinal photoreceptors and is, therefore, one of the candidates for such a role in rhodopsin transport.

An interesting finding is that rab8-containing membranes also bind to microfilament bundles. This association with actin may provide a mechanism of attachment of rhodopsin-bearing membranes to actin filaments in a step that immediately precedes ROS disk membrane formation. ROS disk morphogenesis is dependent on intact microfilaments and their disruption by cytochalasin D causes disk membrane overgrowth (Williams et al., 1988). It has been suggested that microfilaments are necessary for initiation of new disks. Filamentous actin has been localized to the distal end of the connecting cilium at the sites of new disk initiation by ROS plasma membrane evagination (Chaitin et al., 1984; Vaughan and Fisher, 1987; Chaitin and Burnside, 1989). The delivery of newly synthesized rhodopsin to this site after fusion of the post-Golgi vesicles with the plasma membrane at the base of the connecting cilium is still poorly understood. Therefore, it is not clear at which point membranes carrying newly synthesized ROS proteins might first become associated with actin. One study of developing mouse photoreceptors indicates that rhodopsin and actin accumulate at the distal ciliary domains prior to the onset of ROS disc morphogenesis (Chaitin, 1992). This could provide a mechanism for the delivery and concentration of rhodopsin to the ciliary plasma membrane preceding the onset of disc synthesis. It is possible that the complex containing rab8, actin and other unidentified proteins, may define the site to which proteins destined for the ROS are recruited before their final localization.

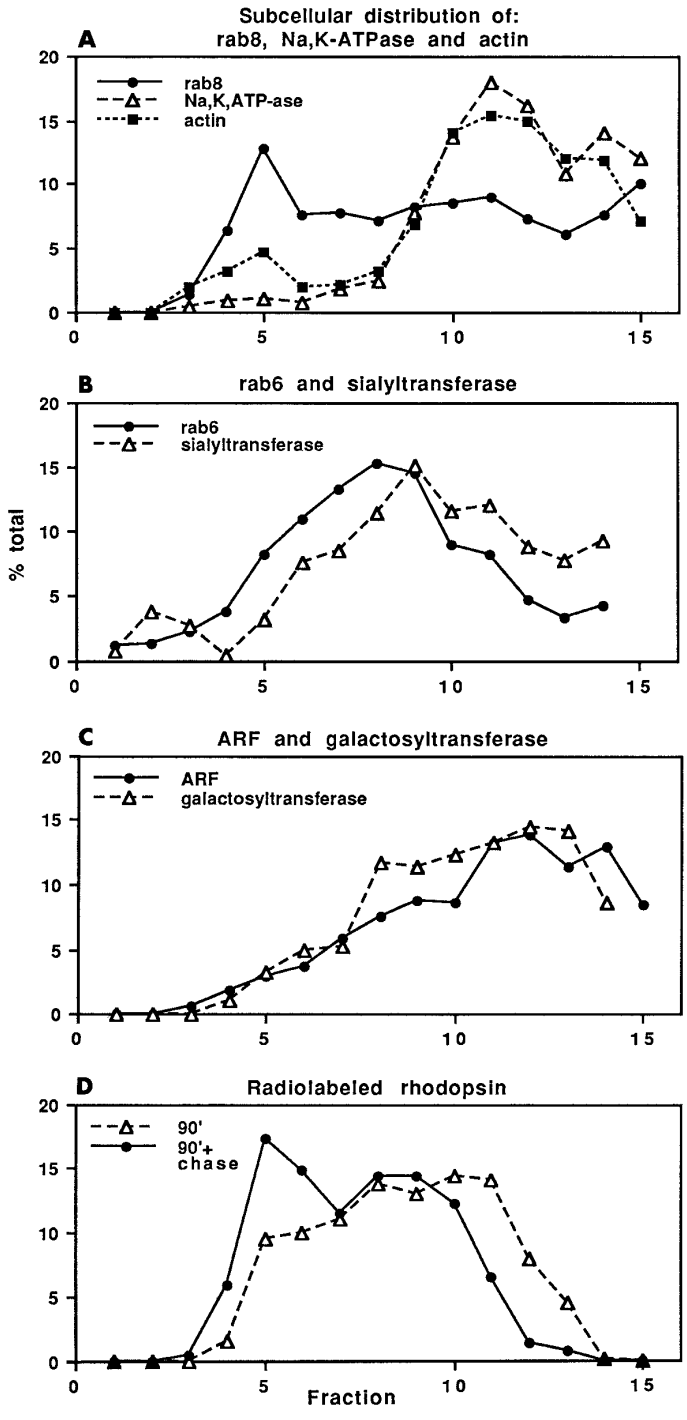


Fig. 4. About 13% of retinal membrane-bound rab8 is associated with the post-Golgi vesicles. The distribution of small GTP-binding proteins is compared with the fractionation of several other membrane-associated proteins separated by a linear sucrose gradient. Gradients were fractionated from the top. Membrane proteins were detected either by immunoblotting or by enzymatic activity. Bound antibodies were detected using the ECL System and quantitated by the Image Analysis Program. (A) A rab8-actin complex co-sediments with post-Golgi vesicles transporting newly synthesized rhodopsin (fraction 5). The more-dense peak of actin parallels the distribution of Na,K-ATPase, a marker for the lateral plasma membrane. (B) The distribution of rab6 and the TGN marker sialyltransferase coincides, but a significant portion of rab6 is also bound to the post-Golgi vesicles (modified from Deretic and Papermaster, 1993a). (C) ARF and galactosyltransferase co-sediment. ARF was detected by a polyclonal anti-ARF antiserum and a monoclonal anti-ARF1 antibody. Its distribution parallels that of the *trans*-Golgi marker galactosyltransferase (Deretic and Papermaster, 1991). (D) As newly synthesized radiolabeled rhodopsin moves through the photoreceptor cells, it shifts from the more-dense sucrose gradient fractions to the lighter fraction 5. After 90 minutes of isotope incorporation, it is predominantly distributed in two compartments: one containing galactosyltransferase and ARF, and the other containing sialyltransferase and rab6. After two hours of cold chase radiolabeled rhodopsin has left the ARF-galactosyltransferase compartment. In addition to the rab6-sialyltransferase compartment, the very light post-Golgi vesicle fraction (fr. 5) containing rab8 and rab6 becomes highly labeled. (This panel has been modified from Deretic and Papermaster, 1991, with permission).

Our finding that rab8 may mediate some of the initial steps in ROS disk morphogenesis extends the role of this small GTP-binding protein to yet another process that involves polarized delivery of newly synthesized membranes to their final cellular destination. It suggests that the specialized domain of retinal photoreceptors responsible for light capture is equivalent to the basolateral plasma membrane of epithelial cells and dendrites of neurons. A parallel between these two domains has previously been established by identification of common molecular mechanisms for protein sorting (Dotti and Simons, 1990; Simons et al., 1993). Although ROS may share a common sorting mechanism with dendrites of neurons, the issue becomes more complicated when ROS are compared with the basolateral membranes of epithelial cells. Photoreceptors clearly have at least three polarized plasma membrane domains: the ROS, the lateral plasma membrane and the synaptic terminal. Our finding that rab8 does not associate with the lateral plasma membrane containing Na^+,K^+-ATPase and the observation that it clearly associates with the basolateral plasma membrane of MDCK cells where this enzyme is also localized (Huber et al., 1993a) suggest that ROS and lateral plasma membrane in photoreceptors are two subdomains that are partially equivalent to the basolateral plasma membrane of epithelial cells, but are polarized even further.

The presence of rab8 in the repetitive structures along microfilament bundles, mostly in calycal processes, is not yet understood. These structures may represent signal transduction complexes that, in addition to rab8, contain other proteins and may regulate ROS disk assembly by an independent mechanism (see below). It remains to be determined if rab8 binds to photoreceptor microfilaments directly or as a part of a larger protein complex. Large protein complexes containing rab proteins have been found to associate with microfilaments (Kurzchalia et al., 1992). It is also possible that soluble rab8 uses actin filaments in the calycal processes for recycling back to the rod inner segment where it will bind post-Golgi vesicles and then participate in another round of vesicular transport. This binding is likely to be induced by the presence of a rab8-rabphilin-like molecule or nuceleotide exchange factor on the post-Golgi vesicles (Shirataki et al., 1991, 1993; Burstein and Macara,1992; Yamaguchi et al., 1993). This hypothesis can be tested when suitable reagents become available by using the procedures followed in those studies.

Our data support the notion that rab8 becomes involved in rhodopsin transport at a later stage than rab6. rab6 is probably recruited by its regulatory proteins at the TGN or *trans*-Golgi and may be a part of the sorting machinery that controls the post-Golgi vesicle budding. It stays bound to the post-Golgi membranes and may require interaction with additional regulatory complexes in order to dissociate from the membrane. We are currently investigating the localization of rab6 nucleotide exchange factor and establishing the role of rab6 in the budding of the rhodopsin-bearing post-Golgi vesicles. rab8 however, does not appear to associate with rhodopsin-bearing membranes prior to their budding from the TGN and is most likely recruited by the regulatory proteins sorted to the vesicles. Thus, the regulatory role of rab8 appears to be exerted at a later stage and it probably involves the initiation of ROS disk formation.

It is interesting that both rab6 and rab8 have been found in the ciliary stalk, a cytoplasmic compartment on one side of the ROS disks and plasma membrane. This suggests that both of these proteins dissociate from the membrane and presumably complete their regulatory function when newly synthesized rhodopsin is delivered to the ROS. Although rab6 associates with post-Golgi membranes earlier than rab8, it is possible that these two proteins together bring rhodopsin to its final destination. Interestingly, both rab6 and rab8 associate with the α granules in platelets, in the same sequential order, and they probably regulate thrombin-induced secretion (Karniguian et al., 1993). Taken together with our findings, these data suggest that a common pathway exists where both rab6 and rab8 may be necessary for post-Golgi vesicle transport and fusion with the plasma membrane.

Aside from the much earlier involvement of rab6 than rab8 in the post-Golgi transport of rhodopsin, another difference between the two is the absence of detectable rab6 in the highly repetitive microfilament-associated structures, although we have postulated that rab6 may also use microfilaments for the return to the rod inner segment (Deretic and Papermaster, 1993a). This suggests that the microfilament-associated structures containing rab8 may have functions other than mediating transport of soluble rabs. Since they are localized in the calycal processes that surround the ROS they may also guide the assembly of the ROS disks. It is interesting that the periodicity of these structures is the same as the light-dependent birefringence pattern observed by Kaplan (1981) and the periodicity of the 240 kDa ROS disk membrane protein recently reported by Matsumoto and Hale (1993b). Further studies should identify other components of these rab8-containing microfilament-associated structures in photoreceptors. An interesting feature of rab8 intracellular localization in epithelial cells is its non-uniform distribution along the basolateral plasma membrane, preferentially in the junctional complexes (Huber et al., 1993a). Junctional complexes may provide the docking site for the fusion of post-Golgi vesicles and rab8, together with rab13 (Zahraoui et al., 1994), may be a part of the machinery that regulates these processes. While we also find that rab8 colocalizes with actin filaments at the junctional complexes that comprise the photoreceptor outer limiting membrane, it is interesting to speculate that another equivalent domain has developed in photoreceptors by the sub-polarization of the basolateral membrane and includes the structures periodically associated with the microfilaments localized in calycal processes.

We have observed that rab proteins in photoreceptors have a very slow turnover. The amount of newly synthesized small GTP-binding proteins is too small to be detected either by incubation with radiolabeled amino acids, or by incorporation of [^{3}H]mevalonate as a precursor for rab protein isoprenylation. Although we were not able to confirm such a modification of frog retinal rab8, it has been recently shown that human rab8 is also geranylgeranylated (Joberty et al., 1993). Since rab8 terminates in a CAAX motif, this suggests that geranylgeranyl transferase I, or yet another transferase that recognizes the conformation of the substrate like geranylgeranyl transferase II, may be involved in the isoprenylation of rab8. From the standpoint of choroidoremia, a retinal degeneration caused by the mutation in the A subunit of geranylgeranyl transferase type II, e.g. REP1 (Seabra et al., 1992, 1993; Andres et al., 1993; Cremers et al., 1994), it would be important to identify this enzyme and its potential role in the preservation of pho-

toreceptor cells, which may give another dimension to the significance of rab8 for generation and maintenance of photoreceptor cell polarity.

We thank Belen Puleo-Scheppke and Claudia Velez for their skillful assistance, Dr Barbara Schneider for her helpful discussions and Drs Richard Kahn and Robert Mercer for their gifts of antibodies. This work was supported in part by NIH grant EY-6891.

REFERENCES

Andres, D. A., Seabra, M. C., Brown, M. S., Armstrong, S. A., Smeland, T. E. Cremers, F. P. M. and Goldstein, J. L. (1993). cDNA cloning of component A of rab geranylgeranyl transferase and demonstration of its role as rab escort protein. *Cell* **73**, 1091-1099.

Bravo, R. (1984). Two-dimensional gel electrophoresis: A guide for the beginner. In *Two-dimensional Gel Electrophoresis of Proteins* (ed. J. E. Celis and R. Bravo), pp. 3-36. Academic Press, Orlando, Florida.

Brown, P. K., Gibbons, I. R. and Wald, G. (1963). The visual cells and visual pigment of the mudpuppy*Necturus*. *J. Cell Biol.* **19**, 79-106.

Burstein, E. S. and Macara, I. G. (1992). Characterization of a guanine nucleotide-releasing factor and a GTPase-activating protein that are specific for the ras-related protein p25rab3A. *Proc. Nat. Acad. Sci. USA* **89**, 1154-1158.

Chaitin, M., Schneider, B., Hall, M. and Papermaster, D. S. (1984). Actin in the photoreceptor connecting cilium: Immunocytochemical localization to the site of outer segment disk formation. *J. Cell Biol.* **99**, 239-247.

Chaitin, M. H. and Burnside, B. (1989). Actin filament polarity at the site of rod outer segment disk morphogenesis. *Invest. Ophthalmol. Vis. Sci.* **30**, 2461-2469.

Chaitin, M. H. (1992). Double immunogold localization of opsin and actin in the cilium of developing mouse photoreceptors. *Exp. Eye Res.* **54**, 261-267.

Chavrier, P., Vingron, M., Sander, C., Simons, K. and Zerial, M. (1990). Molecular cloning of *YPT1/SEC4*-related cDNAs from an epithelial cell line. *Mol. Cell. Biol.* **10**, 6578-6585.

Craighead, M. W., Bowden, S., Watson, R. and Armstrong, J. (1993). Function of the ypt2 gene in the exocytic pathway of *Schizosaccharomyces pombe*. *Mol. Biol. Cell* **4**, 1069-1076.

Cremers, F. P. M., Armstrong, S. A., Seabra, M. C., Brown, M. S. and Goldstein, J. L. (1994). REP-2, a rab escort protein encoded by the choroideremia-like gene. *J. Biol. Chem.* **269**, 2111-2117.

Deretic, D. and Papermaster, D. S. (1991). Polarized sorting of rhodopsin on post-Golgi membranes in frog retinal photoreceptor cells. *J. Cell Biol.* **113**, 1281-1293.

Deretic, D. and Papermaster, D. S. (1993a). 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. *J. Cell Sci.* **106**, 803-813.

Deretic, D. and Papermaster, D. S. (1993b). Isolation of post-Golgi membranes transporting newly synthesized rhodopsin. In *Methods for the Study of Photoreceptor Cells. Methods in Neurosciences*, vol. 15 (ed. P. A. Hargrave), pp. 108-120. The Rockefeller University Press, New York, NY.

Deretic, D. and Papermaster, D. S. (1994). The role of small G-proteins in the transport of newly synthesized rhodopsin, In *Progress in Retinal and Eye Research* (ed. N. Osborne and G. Chader). Elsevier Science, Oxford, UK (in press).

Dotti, C. and Simons, K. (1990). Polarized sorting of viral glycoproteins to the axon and dendrites of hippocampal neurons in culture. *Cell* **62**, 63-72.

Ferro-Novick, S. and Novick, P. (1993). The role of GTP-binding proteins in transport along the exocytic pathway. *Annu. Rev. Cell Biol.* **9**, 575-599.

Goud, B., Salminen, A., Walworth N. C. and Novick P. J. (1988). A GTP-binding protein required for secretion rapidly associates with secretory vesicles and the plasma membrane in yeast. *Cell* **53**, 753-768.

Huber, L. A., Pimplikar, S., Parton, R. G., Virta, H., Zerial, M. and Simons, K. (1993a). Rab8, a small GTPase involved in vesicular traffic between the TGN and the basolateral plasma membrane. *J. Cell Biol.* **123**, 35-45.

Huber, L. A., de Hoop, M. J., Dupree, P., Zerial, M., Simons, K. and Dotti, C. (1993b). Protein transport to the dendritic plasma membrane of cultured neurons is regulated by rab8p. *J. Cell Biol.* **123**, 47-55

Huber, L. A., Ulrich, O., Takai, Y., Lütcke, A., Dupree, P., Olkkonen, V., Virta, H., de Hoop, M. J., Alexandrov, K., Peter, M., Zerial, M. and Simons, K. (1994). Mapping of ras-related GTP-binding proteins by GTP-overlay and 2D-gel analysis. *Proc. Nat. Acad. Sci. USA* (in press).

Joberty, G., Tavitian A. and Zahraoui A. (1993). Isoprenylation of rab proteins possessing a C-termina CaaX motif. *FEBS Lett.* **330**, 323-328.

Kaplan, M. W. (1981). Light cycle-dependent axial variations in frog rod outer segment structure. *Invest. Ophthalmol. Vis. Sci.* **21**, 395-402.

Karniguian, A., Zahraoui, A. and Tavitian, A. (1993). Identification of small GTP-binding rab proteins in human platelets: Thrombin induced phosphorylation of rab3B, rab6 and rab8 proteins. *Proc. Nat. Acad. Sci. USA* **90**, 7647-7651.

Kurzchalia, T. V., Gorvel J.-P., Dupree, P., Parton, R., Kellner, R., Houthaeve, T., Gruenberg, J. and Simons, K. (1992). Interaction of rab5 with cytosolic proteins. *J. Biol. Chem.* **267**, 18419-18423.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lapetina, E. G. and Reep, B. R. (1987). Specific binding of [α-^{32}P]GTP to cytosolic and membrane-bound proteins of human platelets correlates with the activation of phospholipase C. *Proc. Nat. Acad. Sci. USA* **84**, 2261-2265.

Matsudaira, P. (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262**, 10035-10038.

Matsumoto, B. and Hale, I. L. (1993a). Preparation of retinas for studying photoreceptors with the confocal microscope. In *Methods for the Study of Photoreceptor Cells. Methods in Neurosciences*, vol. 15 (ed. P. A. Hargrave), pp. 54-71. The Rockefeller University Press, New York, NY.

Matsumoto, B. and Hale, I. L. (1993b). Variations in the protein composition of the rod outer segment disc membranes. *Invest. Ophthalmol. Vis. Sci.* **34**, 1359.

Matteoli, M., Takei, K., Cameron, R., Hurlbut, P., Johnson, P. A., Südhof, T. C., Jahn, R. and De Camilli, P. (1991). Association of rab 3A with synaptic vesicles at late stages of the secretory pathway. *J. Cell Biol.* **115**, 625-633.

Novick, P. and Brennwald, P. (1993). Friends and family: the role of rab GTPases in vesicular traffic. *Cell* **75**, 597-601.

Papermaster, D. S., Schneider, B. G., Defoe, D. and Besharse, J. C. (1986). Biosynthesis and vectorial transport of opsin on vesicles in retinal rod photoreceptors. *J. Histochem. Cytochem.* **34**, 5-16.

Peters, K-R., Palade, G. E., Schneider, B. G. and Papermaster, D. S. (1983). Fine structure of a periciliary ridge complex of frog retinal rod cells revealed by ultrahigh resolution scanning electron microscopy. *J. Cell Biol.* **96**, 265-276.

Salminen, A. and Novick, P. J. (1987). A *ras*-like protein is required for a post-Golgi event in yeast secretion. *Cell* **49**, 527-538.

Schneider, B. G., Shyjan, A. W. and Levenson, R. (1991). Colocalization and polarized distribution of Na,K-ATPase α3 and β2 subunits in photoreceptor cells. *J. Histochem. Cytochem.* **39**, 507-517.

Seabra, M., Brown, M., Slaughter, C., Sudhof, T. and Goldstein, J. (1992). Purification of component A of Rab geranylgeranyl transferase: Possible identity with the choroideremia gene product. *Cell* **70**, 1049-1057.

Seabra, M., Brown, M. and Goldstein, J. (1993). Retinal degeneration in choroideremia: Deficiency of Rab geranylgeranyl transferase. *Science* **259**, 377-381.

Serafini, T., Orci, L., Amherdt, M., Brunner M., Kahn R. A. and Rothman J. E. (1991). ADP-ribosylation factor is a subunit of the coat of Golgi-derived COP-coated vesicles: a novel role for a GTP-binding protein. *Cell* **67**, 239-253.

Shirataki, H., Kaibuchi, K., Hiroyoshi, M., Isomura, M., Araki, S., Sasaki, T. and Takai, Y. (1991). Inhibition of the action of the stimulatory GDP/GTP exchange protein for smg p21 by the geranylgeranylated synthetic peptides designed from its C-terminal region. *J. Biol. Chem.* **266**, 20672-20677.

Shirataki, H., Kaibuchi, K., Sakoda, T., Kishida, S., Yamaguchi. T., Wada, K., Miyazaki, M. and Takai, Y. (1993). Rabphilin-3A, a putative target protein for smg p25A/rab3A p25 small GTP-binding protein related to synaptotagmin. *Mol. Cell Biol.* **13**, 2061-2068.

Simons, K., Dupree, P., Fiedler, K., Huber, L. A., Kobayashi, T., Kurzchalia, T., Olkkonen, V., Pimplikar, S., Parton, R. G. and Dotti, C. (1993). Biogenesis of cell-surface polarity in epithelial cells and neurons. *Cold Spring Harbor Symp. Quant. Biol.* **57**, 611-619.

Simons, K. and Zerial, M. (1993). Rab proteins and the road maps for intracellular transport. *Neuron* **11**, 789-799.

Stearns, T., Willingham, M. C., Botstein, D. and Kahn, R. A. (1990). ADP-

ribosylation factor is functionally and physically associated with the Golgi complex. *Proc. Nat. Acad. Sci. USA* **87**, 1238-1242.

Vaughan, D. K. and Fisher S. K. (1987). The distribution of F-actin in cells isolated from vertebrate retinas. *Exp. Eye Res.* **44**, 393-406.

Williams, D. S., Linberg, K. A., Vaughan, D. K., Fariss, R. N. and Fisher., S. K. (1988). Disruption of microfilament organization and deregulation of disk membrane morphogenesis by cytochalasin D in rod and cone photoreceptors. *J. Comp. Neurol.* **272**, 161-176.

Yamaguchi, T. Shirataki, H., Kishida, S., Miyazaki, M., Nishikawa, J., Wada, K., Numata, S., Kaibuchi, K. and Takai, Y. (1993). Two functionally different domains of rabphilin-3A, rab3A p25/smg p25A-binding and phospholipid- and Ca^{2+}-binding domains. *J. Biol. Chem.* **268**, 27164-27170.

Zahraoui, A., Joberty, G., Arpin, M., Fontaine, J. J., Hellio, R., Tavitian, A. and Louvard, D. (1994). A small rab GTPase is distributed in cytoplasmic vesicles in non-polarized cells but colocalizes with tight junction marker ZO-1 in polarized epithelial cells. *J. Cell Biol.* **124**, 101-115.

Zerial, M. and Stenmark, H. (1993). Rab GTPases in vesicular transport. *Curr. Opin. Cell Biol.* **5**, 613-620.

(Received 30 May 1994 - Accepted 30 August 1994)