

Rab9 functions in transport between late endosomes and the trans Golgi network

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Rab proteins represent a large family of ras-like GTPases that regulate distinct vesicular transport events at the level of membrane targeting and/or fusion. We report here the primary sequence, subcellular localization and functional activity of a new member of the rab protein family, rab9. The majority of rab9 appears to be located on the surface of late endosomes. Rab9, purified from *Escherichia coli* strains expressing this protein, could be prenylated *in vitro* in the presence of cytosolic proteins and geranylgeranyl diphosphate. *In vitro*-prenylated rab9 protein, but not C-terminally truncated rab9, stimulated the transport of mannose 6-phosphate receptors from late endosomes to the trans Golgi network in a cell-free system that reconstitutes this transport step. Rab7, a related rab protein that is also localized to late endosomes, was inactive in the *in vitro* transport assay, despite its efficient prenylation and capacity to bind and hydrolyze GTP. These results strongly suggest that rab9 functions in the transport of mannose 6-phosphate receptors between late endosomes and the trans Golgi network. Moreover, our results confirm the observation that a given organelle may bear multiple rab proteins with different biological functions.

Key words: late endosomes/mannose 6-phosphate receptor/rab proteins/ras-like GTPases/trans Golgi network

Introduction

Rab proteins are ras-like GTP binding proteins that serve as key regulators of vesicular transport (Balch, 1990; Bourne *et al.*, 1991; Goud and McCaffrey, 1991; Pfeffer, 1992). Members of this family include the *SEC4* and *YPT1* gene products, which regulate yeast secretory vesicle fusion and endoplasmic reticulum (ER)-to-Golgi transport, respectively (Gallwitz *et al.*, 1983; Salminen and Novick, 1987; Schmitt *et al.*, 1988; Segev and Botstein, 1988). Rab proteins are thought to function in vesicle targeting and/or fusion events, because *SEC4* mutant yeast strains accumulate secretory vesicles (Novick *et al.*, 1980), anti-*YPT1* antibodies inhibit ER-derived transport vesicle fusion (Plutner *et al.*, 1991; Rexach and Schekman, 1991; Segev, 1991), and anti-rab5 antibodies block early endosome fusion (Gorvel *et al.*,

1991). Moreover, the discovery that most organelles of the endocytic and secretory pathways bear distinct rab proteins on their surfaces implies that rab proteins may function to ensure the accuracy of vesicle targeting events.

We study the transport of mannose 6-phosphate receptors (MPRs) between late endosomes and the trans Golgi network (TGN; see Kornfeld and Mellman, 1989 for review). MPRs carry newly synthesized lysosomal enzymes from the TGN to late endosomes, where the luminal low pH leads to the release of receptor-bound hydrolases. MPRs then return to the TGN to complete this cycle of protein delivery. We have established a cell-free system that reconstitutes the transport of MPRs from late endosomes to the TGN (Goda and Pfeffer, 1988). Transport requires ATP, cytosolic factors, and appears not to involve clathrin-coated vesicles (Draper *et al.*, 1990; Goda and Pfeffer, 1991).

Given the importance of rab proteins in intracellular transport events, we initiated a search for a specific rab protein that functions in endosome-to-TGN transport. We show here that rab9, a recently identified rab protein (Chavrier *et al.*, 1990a), appears to reside primarily on the surface of late endosomes. In addition, rab9 stimulates the recycling of MPRs from late endosomes to the TGN *in vitro*. Since protein sorting to lysosomes and recycling of regulated secretory vesicle membrane proteins are likely to involve transport between late endosomes and the TGN, rab9 may participate in both of these physiologically important events.

Results

Rab9 was first identified in a screen for *YPT1* and *SEC4* protein-related cDNA clones (Chavrier *et al.*, 1990a). The partial available sequence was found to be 54% identical to that of rab7, a constituent of late endosomes (Chavrier *et al.*, 1990b). We rescreened the initial cDNA library to obtain clones encompassing the missing N-terminal portion (Figure 1). The sequence of the full-length protein is 57% identical to rab7 (Chavrier *et al.*, 1990a) and 39% to *YPT1* protein (Gallwitz *et al.*, 1983).

Specific anti-rab9 antibodies were generated using a fusion protein comprised of the bulk of rab9 sequences linked to the MS2 polymerase fragment (Chavrier *et al.*, 1990b). The specificity of the antibodies was confirmed by immunoblot analysis (not shown) and by indirect immunofluorescence (Figure 2). Anti-rab9 antibody immunofluorescence revealed a striking perinuclear staining (Figure 2A, B and D), which matched the distribution of MPRs in these cells as determined by confocal immunofluorescence microscopy (compare Figure 2F and G). The distribution of rab9 in cells overexpressing the protein, after vaccinia virus infection and plasmid transfection (Figure 2B), matched that seen in control, non-transfected cells (Figure 2A). Thus, as has been observed for other rab proteins, rab9 appears to be accurately targeted, even when overexpressed ~50-fold. Specificity of the antibody was further confirmed by the lack of antibody

| | |
|--|-----|
| ATGGCAGAAAACTTCACTTTTAAAGTAATTCTCCTGGAGATGGTGGAGTGGGAAG | 60 |
| MetAlaGlyLysSerSerLeuPheLysValIleLeuLeuGlyAspGlyGlyValGlyLys | 20 |
| AGCTCTCTAATGAACAGATATGTGACCAATAAGTTTGATACCCAGCTCTCCATACAATA | 120 |
| SerSerLeuMetAsnArgTyrValThrAsnLysPheAspThrGlnLeuPheHisThrIle | 40 |
| GGGTAGAAATTTTAAATAAAGATTTGGAGGTGGATGGACATTTTGTACCATGCAGATT | 180 |
| GlyValGluPheLeuAsnLysAspLeuGluValAspGlyHisPheValThrMetGlnIle | 60 |
| TGGGACACAGCCGGTCAAGAGCGATTGAGAAGCCTGAGGACGCCGTTTACAGAGGTCT | 240 |
| TrpAspThrAlaGlyGlnGluArgPheArgSerLeuArgThrProPheTyrArgGlySer | 80 |
| GACTGTTGCCTGCTCACTTTTAGTGTGATGATCTCAGAGCTTCCAGAACTTGAGTAAC | 300 |
| AspCysCysLeuLeuThrPheSerValAspAspSerGlnSerPheGlnAsnLeuSerAsn | 100 |
| TGGAAGAAAGAAATTCATATATTATGCAGATGTGAAAGAGCCCGAAAGCTTTCTTTTGTG | 360 |
| TrpLysLysGluPheIleTyrTyrAlaAspValLysGluProGluSerPheProPheVal | 120 |
| ATTTTGGGCAACAAGATCGACATAAGTGAACGACAAGTGTCTACAGAAGAAGCCCAAGCT | 420 |
| IleLeuGlyAsnLysIleAspIleSerGluArgGlnValSerThrGluGluAlaGlnAla | 140 |
| TGGTGCAGGGACAACGGCGACTATCCTTACTTTGAAACAAGTGCAAAAGATGCCACAAAT | 480 |
| TrpCysArgAspAsnGlyAspTyrProTyrPheGluThrSerAlaLysAspAlaThrAsn | 160 |
| GTGCAGCAGCCTTTGAGGAAGCTGTTTGAAGAGTGCTTACTGACTGAGGATAGGTGAGAT | 540 |
| ValAlaAlaAlaPheGluGluAlaValArgArgValLeuAlaThrGluAspArgSerAsp | 180 |
| CACCTGATTCAGACAGACAGTCAGCCTGCACCGAAAGCCCAAGCCTAGCTCATCTTGC | 600 |
| HisLeuIleGlnThrAspThrValSerLeuHisArgLysProLysProSerSerSerCys | 200 |
| TGTTGA | 606 |
| Cys * | 201 |

Fig. 1. Nucleotide and predicted amino acid sequences of rab9 protein.

cross-reactivity with rab2, rab5 or rab7 in BHK cells overexpressing these proteins (data not shown).

The perinuclear distribution of rab9 was consistent with its presence in late endosomes and the Golgi complex. Failure of the rab9 protein to redistribute to the ER upon addition of brefeldin-A (Figure 2D) confirmed that the stained structures were at or distal to the TGN (Chege and Pfeffer, 1990). Considering the fact that the vast majority of MPRs reside in late endosomes (Griffiths *et al.*, 1988), rab9 colocalization with MPRs strongly suggests that most rab9 protein is also present on the surface of that organelle.

Rab9 stimulates endosome-to-TGN transport *in vitro*

The localization of rab9 to late endosomes suggested that this protein may play a role in membrane traffic between late endosomes and the TGN. To test this possibility, we used a cell-free system that reconstitutes the transport of the 300 kDa MPR from late endosomes to the TGN (Goda and Pfeffer, 1988). Briefly, the assay measures the sialylation of radiolabeled MPRs that occurs after they are transported from late endosomes to an exogenously added, Golgi-enriched fraction. As shown in Figure 3, anti-rab9 antibodies inhibited endosome-to-TGN transport ~50%, in a concentration-dependent manner, under conditions in which anti-rab7 antibodies had no effect. The inability of anti-rab7 antibodies to block transport suggests that the anti-rab9 antibody inhibition was specific, because both rab7 and rab9 are present on the surface of late endosomes. Although anti-rab9 antibodies inhibited the overall reaction by ~50%, cytosol-stimulated transport was almost completely inhibited under these conditions.

To analyze further the possible role of rab9 in endosome-to-TGN transport, we expressed the protein in BHK cells using the vaccinia virus/T7 RNA polymerase expression system (Fuerst *et al.*, 1986). As shown in Figure 4, cytosols containing overexpressed rab9 protein stimulated the overall transport reaction ~2.5-fold. In contrast, no stimulation was observed with cytosols containing overexpressed rab7 or

rab4b, a highly homologous relative of rab4 (Chavrier *et al.*, 1990a) that is localized to early endosomes (D.Lombardi and M.Zerial, unpublished). Similarly, cytosols from mock-transfected cells had control levels of activity. Thus, overexpression of rab9 in BHK cells increased the specific activity of the cytosol fraction.

To confirm that rab9 protein itself was responsible for the increase in cytosol activity, we expressed rab7 and rab9 in *Escherichia coli* and purified the proteins to >95% homogeneity (Figure 5A). We then tested the ability of the purified proteins to stimulate the transport of MPRs. As shown in Figure 5B, addition of ≤ 3 ng of purified rab9 protein was sufficient to yield the level of stimulation observed with BHK cytosol containing overexpressed rab9. Maximal stimulation was observed in reactions that were supplemented with geranylgeranyl diphosphate; addition of geranylgeranyl diphosphate alone was without effect.

We utilized a C-terminally truncated rab9 protein (rab9 Δ C), which lacks ~30 amino acid residues at its C-terminus, to demonstrate that the ability of rab9 to stimulate endosome-to-TGN transport requires an intact C-terminus. [³H]GTP binding assays (Northup *et al.*, 1982) confirmed that rab9 Δ C, purified from *E. coli* lysates (Figure 5A), bound GTP as well as control rab7 or rab9 proteins (data not shown). Despite their normal GTP binding capacities (~0.9 mole GTP bound per mole protein), neither purified rab9 Δ C nor rab7 proteins stimulated endosome-to-TGN transport *in vitro* (Figure 5B and C). Rab5, which functions in early endosome fusion, was also without activity (data not shown).

The data presented in Figure 5 suggested that rab9 was prenylated during the endosome-to-TGN transport reaction. To test this directly, rab9 protein was incubated with [³H]geranylgeranyl diphosphate in the presence of cytosol and ATP under transport assay conditions. As shown in Figure 6B, under these conditions, the *E. coli*-produced rab9 protein incorporated the ³H-labeled prenyl group. Rab7 was also prenylated *in vitro* under the same reaction conditions (data not shown). In contrast, no incorporation was observed

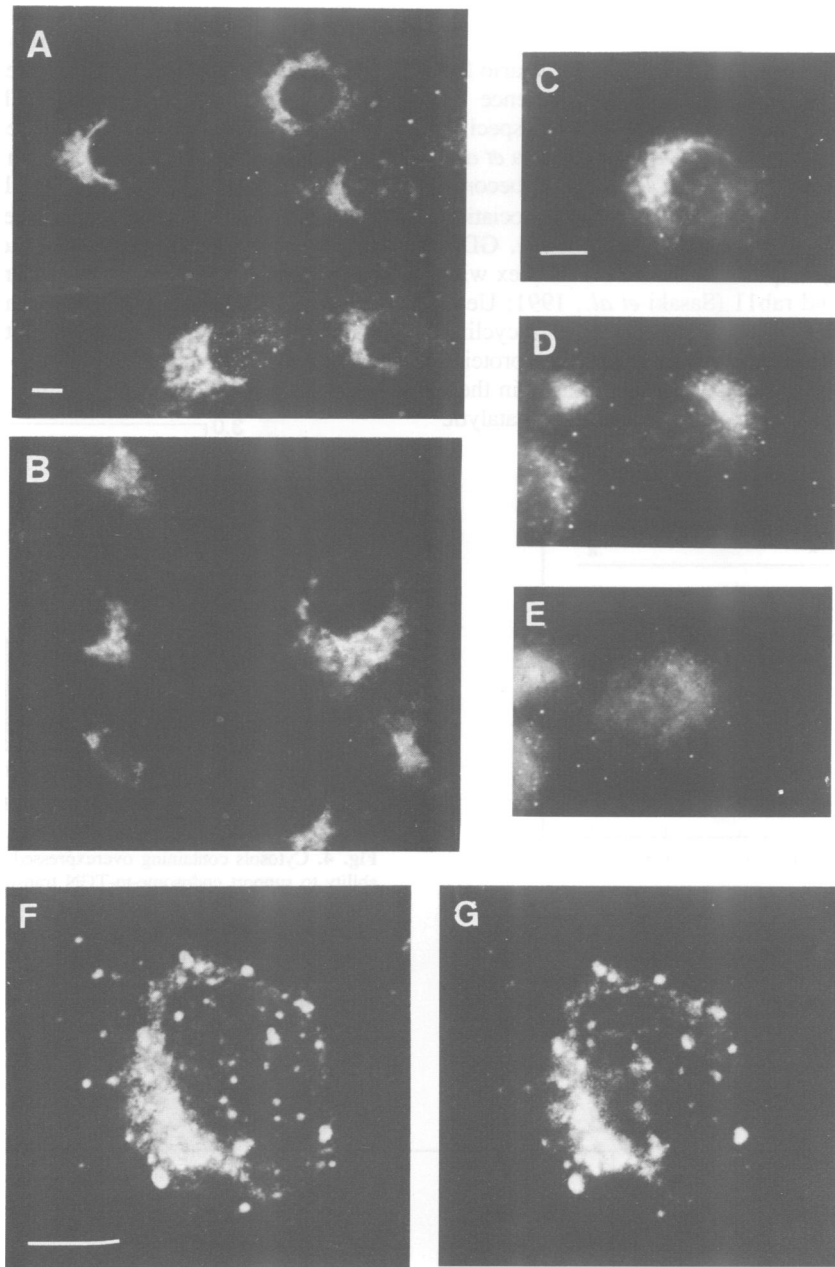


Fig. 2. Localization of rab9 protein by immunofluorescence microscopy. (A and B) Anti-rab9 antibody staining of untransfected BHK cells (A) or BHK cells infected with a T7 RNA polymerase-recombinant vaccinia virus and transfected with a rab9-encoding construct (B). Antibodies were diluted either 1:5 (A) or 1:20 (B). (C–E) Mannosidase II, but not rab9, returns to the ER in brefeldin-A: (C) anti-mannosidase II antibody staining of untreated BHK cells; (D) anti-rab9 antibody staining after brefeldin-A; (E) anti-mannosidase II antibody staining in the same cell as in (D) after brefeldin A. (F and G) Confocal microscopic localization of rab9 (F) or the 300 kDa mannose 6-phosphate receptor (G) in a HeLa cell infected with the T7 RNA polymerase-recombinant vaccinia virus and transfected with a rab9-encoding plasmid. Bars represent 10 μm .

for rab9 Δ C (Figure 6A). These results strongly suggest that rab9 prenylation is essential for its *in vitro* activity, consistent with previous genetic and biochemical findings (Molenaar *et al.*, 1988; Walworth *et al.*, 1989; Chavrier *et al.*, 1991; Farnsworth *et al.*, 1991; Gorvel *et al.*, 1991; Khosravi-Far *et al.*, 1991).

Discussion

We have shown here that the bulk of rab9 is localized to the surface of late endosomes. Preliminary electron microscopic analysis supports this conclusion, and also

detects a small amount of rab9 at the TGN. This localization is consistent with our observation that rab9 can facilitate vesicular transport from late endosomes to the TGN.

Rab9 protein, in cytosol from BHK cells overexpressing this protein, or in purified form, halved the requirement for cytosolic proteins in the endosome-to-TGN transport assay. The ability of rab9 protein to stimulate transport depended upon the presence of an intact C-terminus, and more than likely its prenylation, since maximal stimulation was observed in reactions supplemented with geranylgeranyl diphosphate. Indeed, reactions containing [^3H]geranylgeranyl diphosphate yielded [^3H]rab9, which demonstrates

that rab prenylation occurred after its addition to *in vitro* reaction mixtures.

Our results are consistent with the following scenario for rab action. *Escherichia coli* rab9, in the presence of geranylgeranyl diphosphate, is prenylated by a rab-specific prenyl transferase that is present in the cytosol (Seabra et al., 1992). After prenylation, the protein is likely to become complexed with a protein termed 'GDI' or GDP dissociation inhibitor (Matsui et al., 1990; Sasaki et al., 1990). GDIs represent a small group of proteins shown to complex with rab3a, SEC4 protein and rab11 (Sasaki et al., 1991; Ueda et al., 1991). Such proteins can be thought of as 'recycling factors' that help to solubilize doubly prenylated rab proteins and also, to retrieve rab proteins from membranes in their GDP-bound forms, after they have completed their catalytic cycles (Araki et al., 1990; Sasaki et al., 1990).

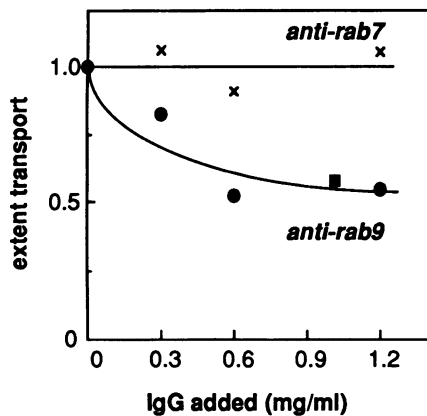


Fig. 3. Effect of anti-rab antibodies on endosome-to-TGN transport. ×, polyclonal anti-rab7 IgG; •, ■, two different polyclonal anti-rab9 IgGs. Standard reactions contained ~1.5 mg/ml cytosol proteins. An extent of transport of 1.0 represents the acquisition of sialic acid by 15% of CHO cell MPRs in 2 h.

Fractionation experiments indicated that the exogenously added rab protein becomes membrane associated after addition to *in vitro* transport reactions (T.Soldati and S.R.Pfeffer, in preparation). The proposed cytosolic rab9-GDI complex is probably delivered to the surface of late endosomes by interaction with proteins on the surface of that organelle. Organelle localization might be accomplished by a stoichiometric receptor, or instead might utilize a protein that stimulates the exchange of GTP for rab9-bound GDP. Nucleotide exchange would trigger release of the GDI, exposing the two prenyl groups, which might then spontaneously partition into the late endosome membrane (Pfeffer, 1992).

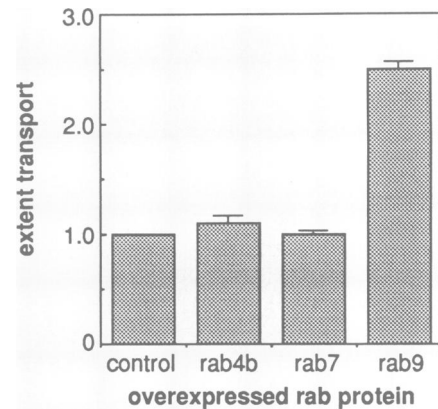


Fig. 4. Cytosols containing overexpressed rab proteins differ in their ability to support endosome-to-TGN transport. Vaccinia virus-infected BHK cell cytosol was employed at 1.0 mg/ml in reactions supplemented with 0.25 mg/ml CHO cytosol; immunoblot analyses showed that the infected cell cytosols contained ~100 ng of the respective rab proteins. The values represent the average (and standard deviation) of duplicate measurements; an extent of transport of 1.0 represents the acquisition of sialic acid by 5.4% of CHO cell MPRs in 2 h.

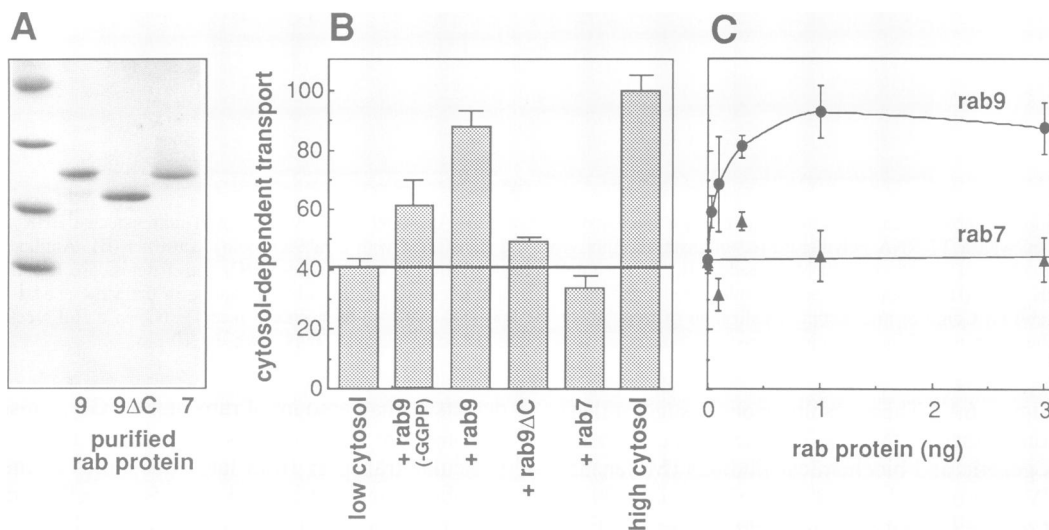


Fig. 5. Purified rab9 protein stimulates endosome-to-TGN transport. (A) SDS-PAGE of purified rab proteins. Lane 1, mol. wt markers: ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). Three μg of the indicated rab proteins were analyzed on a 15% gel, stained with Coomassie blue. (B) Purified rab9, but not rab7 or rab9ΔC, stimulates endosome-to-TGN transport. Reactions contained either low cytosol (0.5 mg/ml), high cytosol (1.0 mg/ml) or low cytosol plus 3 ng of the respective proteins and 1 μg/ml geranylgeranyl diphosphate (unless specified otherwise). (C) The extent of transport stimulation by rab9 is proportional to the amount of purified protein added. Reactions contained 0.54 mg/ml cytosol and the indicated amounts of rab proteins. The values presented in (B) and (C) represent the average (and standard deviation) of duplicate measurements. One hundred percent transport represents the acquisition of sialic acid by 16.9% (B) or 15.4% (C) of CHO cell MPRs in 2 h.

Immunoblot analyses indicated that *in vitro* reactions contain on the order of $\sim 1-2$ ng endogenous rab9 protein, $\sim 90\%$ of which is membrane-associated (not shown). The ability of an additional nanogram of exogenous rab9 to stimulate transport demonstrates that it is a limiting constituent in transport reactions, and as such, may function as a key regulator of vesicular transport. Other components become limiting for transport in reactions containing more than 1 ng exogenous rab9 protein (Figure 5C). This latter conclusion is supported by the observation that exogenous rab9 cannot stimulate transport above the level seen when high levels of cytosol are employed.

The finding that a small increase in the level of rab9 protein yielded a large increase in transport implies that some of the endogenous rab9 protein may be present in an inactive form. This inactive pool could represent membrane-bound rab9 that is for some reason sequestered at the target membrane and not available for another round of transport. Nevertheless, physiological levels of purified rab9 are sufficient to stimulate transport.

Of the two rab proteins now localized to late endosomes (rab7 and rab9), only rab9 stimulates endosome-to-TGN transport. Although the physiological function of rab7 is not yet known, this finding supports the notion that multiple rab proteins on the surface of a given organelle can have different physiological roles. This is analogous with results obtained by Bucci *et al.* (1992) and van der Sluijs *et al.* (1992) who have shown that rab4 and rab5, two early endosomal rab proteins, have completely different functions.

In analogy with current models for other rab proteins, rab9 may be incorporated into nascent transport vesicles, and function in vesicle targeting and/or fusion. After these vesicles deliver their cargo to the TGN, rab9 must be recycled to the membrane from which it originated. The ability of rab9 to stimulate transport in an *in vitro* assay will permit us to investigate the mechanism by which this protein acts to facilitate the recycling of membrane proteins from late endosomes to the TGN of eukaryotic cells.

Materials and methods

Cloning of full-length rab9

Clones encompassing the missing N-terminal portion of rab9 were obtained by further screening of the oriented λ MDCK cDNA library cloned in UNI-

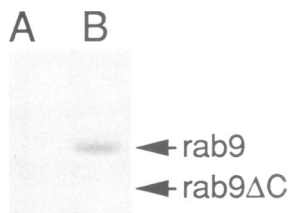


Fig. 6. Rab9 but not rab9 Δ C is prenylated *in vitro*. Shown is an autoradiogram of a 12.5% SDS-polyacrylamide gel. Rab9 protein (1 μ g) was added to cytosol (5.6 mg/ml) under the conditions of *in vitro* transport except that the incubation was supplemented with 8 μ M geranylgeranyl diphosphate and 0.1 μ M (1 μ Ci) [3 H]geranylgeranyl diphosphate (American Radiolabeled Company, St Louis, MO) and lacked semi-intact cells and rat liver Golgi. It is important to note that these reactions contained 1000 times the amount of endogenous rab9 protein normally present in cytosol, and it is not yet clear whether this high level of cytosol is required to detect prenylation. The cytosolic products were resolved by gel filtration, and the chromatographic profile of rab9 determined by immunoblotting. (A) a fraction enriched in rab9 Δ C; (B) a fraction enriched in rab9.

ZAP XR (Stratagene). The library was screened with the *Eco*RI fragment corresponding to the 5' end of the available rab9-encoding cDNA, cloned in the polylinker of the Bluescript vector restricted with *Eco*RI. Duplicate filters were prehybridized for 2 h at 65°C in 6 \times SSC, 5 \times Denhardt's, 1% SDS; hybridization was performed for 12 h at 65°C in the same solution, but containing the cDNA fragment labeled by random priming (1 \times 10⁶ c.p.m./ml). Filters were then washed for 3 h in 0.1 \times SSC, 0.5% SDS. *In vivo* excision of the cDNA inserts from the UNI-ZAP vector was performed according to the manufacturer. Phagemid DNAs were prepared and used directly for double-stranded DNA sequencing with the T7 sequencing kit (Pharmacia).

Antibody production and immunofluorescence

Rabbit anti-rab9 antiserum was raised against a bacterially expressed rab9-MS2 polymerase fusion protein. Antibody affinity purification was performed using rab9 fusion protein immobilized on nitrocellulose filters. Anti-rab IgGs were purified by protein A-Sepharose (Pharmacia) as described by the manufacturer. Monoclonal antibody 53FC3 was used to label mannosidase II in the Golgi complex (23); a monoclonal anti-bovine 300 kDa MPR antibody (2G11) was used to label late endosomes. Cells were fixed with 3% formaldehyde for 15 min. Vaccinia virus infection, transfection and other procedures for immunofluorescence were as described (Chavrier *et al.*, 1990b). Brefeldin-A was employed at 10 μ g/ml for 60 min.

Purification of rab proteins

Rab proteins were expressed in *E. coli* BL21(DE3) using the pET8c expression system (Studier and Moffatt, 1986). Expression was induced with IPTG for 3 h at 37°C; cells were then harvested in lysis buffer (64 mM Tris-Cl, pH 8.0, 8 mM MgCl₂, 2 mM EDTA, 0.5 mM DTT, 10 μ M GDP, 10 mM benzamidine, 1 mM PMSF, 2 mM *o*-phenanthroline, 1 mM NaN₃), containing 0.8 μ g/ml egg white trypsin inhibitor and 1 \times protease inhibitors (Goda and Pfeffer, 1988) and processed as described (Tucker *et al.*, 1986). Rab proteins were purified by Q-Sepharose ion exchange and Sephacryl S-100 gel filtration chromatography; purified proteins were stored in lysis buffer containing 100 mM NaCl at -80°C. Rab9 Δ C was generated by an endogenous protease when *E. coli* extracts were processed in the absence of egg white trypsin inhibitor; its identity was confirmed by direct N-terminal sequencing which yielded the sequences: $_2$ HN-Ala-Gly-Lys-Ser-Ser for both authentic rab9 and the C-terminally truncated product. From the mass of rab9 Δ C, it appears to lack ~ 30 C-terminal amino acid residues, perhaps due to the presence of two basic amino acids at that location in the rab9 sequence.

In vitro determination of endosome-to-trans Golgi network transport

The assay was carried out precisely as described by Goda and Pfeffer (1988). Briefly, CHO clone 1021 (CHO¹⁰²¹) cells were labeled with [35 S]methionine and cysteine and chased for at least 4 h to permit export of newly synthesized man6P receptors to points beyond the medial Golgi. Labeled cells are then gently broken, and aliquots of the semi-intact cell extract are mixed with 'wild type' rat liver Golgi complexes, a crude cytosol fraction, ATP and an ATP regenerating system. Mixtures are incubated at 37°C, after which man6P receptors are isolated by affinity chromatography using pentamannosyl-6-phosphate Sepharose. If CHO¹⁰²¹ cell man6P receptors are transported to the wild type trans Golgi or TGN, radiolabeled receptors acquire sialic acid, which can be detected by chromatography of the isolated receptors on a column of the sialic acid-specific, *Limax flavus* lectin. Transported receptors bind to such columns and elute in the presence of excess sialic acid; non-transported proteins bearing galactose-terminating oligosaccharides do not bind, and are collected in the flow-through fractions. Samples were then analyzed by either direct scintillation counting or SDS-PAGE. Reactions (100 μ l) were supplemented with geranylgeranyl diphosphate as indicated in the figure legends. The extent of transport was determined by Phosphorimager quantitation (Molecular Dynamics) of autoradiograms of sialic acid-containing, [35 S]methionine-labeled, 300 kDa MPRs. The values presented were corrected for the efficiency of slug lectin binding as described (65% efficiency; Goda and Pfeffer, 1988).

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