A Novel Rab9 Effector Required for Endosome-to-TGN Transport

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Abstract. Rab9 GTPase is required for the transport of mannose 6-phosphate receptors from endosomes to the trans-Golgi network in living cells, and in an in vitro system that reconstitutes this process. We have used the yeast two-hybrid system to identify proteins that interact preferentially with the active form of Rab9. We report here the discovery of a 40-kD protein (p40) that binds Rab9–GTP with roughly fourfold preference to Rab9–GDP. p40 does not interact with Rab7 or K-Ras; it also fails to bind Rab9 when it is bound to GDI. The protein is found in cytosol, yet a significant fraction (~30%) is associated with cellular membranes. Upon sucrose density gradient flotation, membrane-associated p40 cofractionates with endosomes containing mannose 6-phosphate receptors and the Rab9 GTPase. p40 is a very potent transport factor in that the pure, recombinant protein can stimulate, significantly, an in vitro transport assay that measures transport of mannose 6-phosphate receptors from endosomes to the trans-Golgi network. The functional importance of p40 is confirmed by the finding that anti-p40 antibodies inhibit in vitro transport. Finally, p40 shows synergy with Rab9 in terms of its ability to stimulate mannose 6-phosphate receptor transport. These data are consistent with a model in which p40 and Rab9 act together to drive the process of transport vesicle docking.
conformation and can stimulate the transport of mannosese 6-phosphate receptors (MPRs) from endosomes to the TGN in vitro.

Materials and Methods

Rab9 cDNA clones (Shapiro et al., 1993; Riederer et al., 1994), anti-Rab9 antibodies (Shapiro et al., 1993), monoclonal anti-mannose 6-phosphate receptor antibodies (Lombardi et al., 1993), prenyl Rab9, bovine brain GDI, and Rab9/GDI complexes were as described (Dirac-Svejstrup et al., 1994; Soldati et al., 1994). Anti-p40 antisera was prepared in rabbits using recombinant, His-tagged p40. Endosome-to-TGN transport assays were carried out by a modification (Itin, C., C. Rancaño, Y. Nakajima, and S.R. Pfeffer, manuscript submitted for publication) of the standard procedure (Godde and Pfeffer, 1988) using K562 cytosol. p40 and Rab9/GDI were diluted in cytosol before addition to the assay. Cytosol-dependent transport was determined by subtracting the cpm obtained in the absence of cytosol (~200 cpm). Transport in the presence of 1 mg/ml cytosol was defined as 100%. Rab9 IgG was purified using protein A Sepharose (Sigma Chemical Co., St. Louis, MO). Protein assays were carried out using reagent (Bio Rad; Hercules, CA) and bovine serum albumin as standard.

Yeast Two Hybrid Screen

Yeast strain Y190 (MATα gal4 gal80 his3 trp1-901 ade2-11 ura3-52 leu2-3,112 ß GAL→lacZ, LYS2::GAL→HIS3) was transformed with the pASI-CYH-Rab9acc plasmid (Schiestl and Giest, 1989) and colonies selected by growth on plates lacking tryptophan. Rab9 fusion protein expression was verified by immunoblotting. These cells were then transformed with library DNA (human mature B cell cDNA in pACT) and plated on synthetic medium lacking tryptophan, leucine, and histidine and containing 25 mM 3′-aminotriazole (Sigma Chemical Co.). Colonies were picked after 5 d at 30°C and β-galactosidase activity was detected by colony filter assay (Vojtek et al., 1993) or quantitative liquid culture assay (Rose and Botstein, 1983). Positive clones (His+, LacZ+) were forced to lose the library plasmid by growth on YPAD containing 10 µg/ml cyclohexamide and were mated to yeast strain Y187 (MATα gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,112 GAL→lacZ) expressing pASI-CYH-Rab9S21N or -Rab7acc and tested for β-galactosidase activity. Several clones displayed Rab9-GTP-dependent β-galactosidase activity. Library plasmids were recovered in Escherichia coli XL1-blue cells and purified on a column (Qiagen, Chatsworth, CA) for subsequent sequencing.

Cloning and Expression

A human Jurkat lymphoma cDNA library (1010 plaque-forming units) in λZAPII (Stratagene, La Jolla, CA) was screened with a 5′-labeled probe made by random-primer labeling (Boehringer Mannheim, Indianapolis, IN) of a 405 bp BglII–PvuII fragment representing the most 5′ region of the two hybrid clone. Plasmid DNA was recovered by in vivo excision of the pBluecript plasmid from the pAZAPII vector in E. coli XL1-blue cells. Cells (Qiagen, Chatsworth, CA) were forced to lose the library plasmid by growth on YPAD containing 10 µg/ml cyclohexamide and were mated to yeast strain Y187 (MATα gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,112 GAL→lacZ) expressing pASI-CYH-Rab9S21Nacc or -Rab7acc and tested for β-galactosidase activity. Several clones displayed Rab9-GTP-dependent β-galactosidase activity. Library plasmids were recovered in Escherichia coli XL1-blue cells and purified on a column (Qiagen, Chatsworth, CA) for subsequent sequencing.

In Vitro Binding Assay

Reactions were in 20 mM Hepes, pH 7.4, 50 mM NaCl, 20 mM imidazole, 5 mM MgCl2, 100 µg/ml BSA. Each prenylated GT-Pase (1 µg = 571 nM in complex with BSA or GDI) was incubated with 1 µg (357 nm) recombinant p40 and 100 µM GDP or GTP at 37°C for 1.5 h. p40-bound GT-Pase was recovered by copurification on NTA-agarose (Qiagen; 20 µl of a 50% slurry) and elution in 20 µl, 100 mM EDTA; eluted amounts were quantitated by immunoblot analysis. GT-Pase standards (1–40 ng) were analyzed in parallel; the amount of GT-Pase bound to the resin in the absence of p40 (≤2 ng) was subtracted.

Abbreviation used in this paper: MPR, mannosese 6-phosphate receptor.

GTPase Assays

Rab9 (50 nM) GTPase activity was measured as described (Shapiro et al., 1993); reactions were analyzed by thin layer chromatography.

Sucrose Gradient Floation

K562 cell postnuclear supernatant (PNS) was fractionated by sucrose gradient flotation according to Balch et al. (1984). The PNS (6 ml) in 1.4 M sucrose was overlaid with 3 ml, 1.2 M sucrose and 3 ml, 0.8 M sucrose in an SW41 tube. Gradients were centrifuged for 3 h at 36,000 rpm. Fractions (0.5 ml) were collected from the top. Marker protein distributions were determined by immunoblot after trichloroacetic acid precipitation of 20 µl samples and 12% SDS-PAGE separation.

p40-depleted Cytosol

IgG from preimmune or anti-p40 serum (0.25 ml) was precipitated with 50% ammonium sulfate and pelleted at 95,000 rpm for 10 min in a centrifuge (TLA100; Beckman Instr., Fullerton, CA). Pellets were dissolved in 1 ml K562 cytosol (5 mg/ml) and incubated 5 h at 4°C; protein-A Sepharose (0.4 ml) was then added for 30 min at 4°C. The slurry was poured into a column, and the flow through was collected as “depleted cytosol.”

Results

We used the yeast two hybrid system (Fields and Song, 1989) to identify proteins that interact with Rab9 in its active, GTP-bound form. A GAL4 DNA-binding domain hybrid was constructed using wild-type Rab9 lacking the two COOH-terminal cysteine residues (Rab9acc) to avoid interference due to protein prenylation. To enrich for proteins that interacted specifically with active Rab9-GTP, we discarded clones that interacted with a mutant of Rab9 (Rab9S21Nacc) that binds GDP with >50-fold preference to GTP (Riederer et al., 1994) or a related Rab family member (Rab7acc). Two hybrid screening of 1.4 × 106 GAL4 activation domain hybrid transformants led to the identification of clone 361, which interacted preferentially with Rab9acc but not Rab9S21Nacc or Rab7acc in a quantitative, β-galactosidase liquid culture assay (Fig. 1). Clone 361 showed at least fourfold higher β-galactosidase activity with Rab9acc than with Rab7acc (Fig. 1), even though these proteins are 54% identical (Chavrier et al., 1990).

A Jurkat cDNA library was screened to obtain a full length 361 cDNA. Of 6 independent clones identified, clone 361.6 extended the 5′ region of the two hybrid clone 361 by 589 bp (Fig. 2) and contained a potential ATG start codon (Kozak, 1987). The 5′ region of this clone was virtually identical to a human EST (these sequence data are available from GenBank/EMBL/DDBT under accession number N36641). Furthermore, two in-frame stop codons

Figure 1. Discovery of a yeast two hybrid cDNA clone encoding a peptide that preferentially binds Rab9–GTP, β-galactosidase activity of yeast strains co-expressing the clone 361-GAL4 activation domain hybrid and GAL4 DNA binding domain hybrids of either Rab9 (black), Rab9S21N (white), or Rab7 (gray).
were found upstream of the potential initiator codon (Fig. 2, underlined). Thus, clone 361.6 represents a full length cDNA.

Clone 361 encodes a hydrophilic protein of 372 amino acids with a predicted molecular weight of 40,566. We have termed this protein p40. p40 shares a 50 amino acid stretch (44% identity) with the *Saccharomyces pombe* protein, Ral2p (these sequence data are available from GenBank/EMBL/DDBJ index accession number M30827). Ral2p shows genetic interaction with *S. pombe* RAS1 and is thought to be involved in the activation of Ras1p (Fukui et al., 1989); thus, p40 contains a domain in common with another small GTPase activator.

The p40 sequence is comprised almost entirely of six internally repeated sequences of ~50 amino acids in length (Fig. 3A). These represent so-called kelch repeats, which were first detected in the *Drosophila* kelch protein (Xue and Cooley, 1993) and are found in a wide variety of proteins of completely unrelated function (Bork and Doolittle, 1994). Kelch repeats are predicted to form four-stranded, anti-parallel β sheets that assemble into propeller-like barrel structures. The repeat is characterized by a pair of glycine residues at positions 15 and 16, immediately preceded by two hydrophobic amino acids, a tyrosine, and a fourth hydrophobic residue (Bork and Doolittle, 1994). In p40, phenylalanine is found at position minus one relative to the glycine pair in four of six of the repeats, and valine or isoleucine is always present at position minus two. However, only two of the p40 kelch repeats contain the upstream tyrosine residue. A tryptophan residue (found in other kelch motif-containing proteins) and a proline (unique in p40) are found in all of the repeats at position 120 and 130, respectively, downstream of the glycine pair. Due to the kelch repeats, the entire p40 structure can be predicted as drawn in Fig. 3B.

In this representation, the sequences homologous to *S. pombe* Ral2p are shown in bold; they fit readily into a connected pair of β strands that may comprise a portion of the Rab9-interacting region. Circular dichroism analysis of p40 failed to reveal the presence of any predominant α helices, consistent with the structure shown (data not shown). In kelch proteins of known tertiary structure, enzyme active sites are created by the loops located at the top of the barrel structure (Bork and Doolittle, 1994). Thus, p40 is predicted to fold into a compact β barrel that may interact with Rab9 via the β sheet connecting loops.

**Purified p40 Binds Rab9–GTP**

P40 was expressed in *E. coli* as an NH₂-terminally His-tagged protein and purified to homogeneity (Fig. 4A, lane 1). Antibodies raised against the recombinant protein rec-
recognized a 44-kD band on immunoblots of human cell extracts (Fig. 4A, lanes 2–4). Fractionation of HeLa cells revealed that p40 is predominantly cytosolic, but a significant fraction (~30%) was found associated with membranes (Fig. 4A, lanes 3 and 4).

Fig. 4B shows that a portion of p40 cofractionates with Rab9 and mannose 6-phosphate receptors upon sucrose gradient flotation of K562 cell postnuclear supernatant. The top of the gradient is at the left.

The majority of the protein was found in the lower half of the density gradient (fractions 11–19).

To test whether the full length p40 protein interacted directly with Rab9, the proteins were mixed in the presence of GTP or GDP, and after a period of incubation, p40 and bound proteins were collected via p40's histidine tag using Ni-agarose; bound proteins were then identified by immunoblot. As shown in Fig. 5, purified, recombinant p40 bound Rab9–GTP in preference to Rab9–GDP. No Rab9 was detected bound to the resin if p40 was omitted from the reactions (Fig. 5A).

Rab9 in complex with GDI bound much less p40 than Rab9–GDP (Fig. 5B, white bars). Since GDI retains Rab proteins in their GDP-bound conformations, this result confirms the lack of interaction of p40 with Rab9–GDP; GDI could equally well mask a p40 binding site. Consistent with the original two hybrid screen results, p40 bound very little Rab7 (Fig. 5B, light gray bars); it also failed to bind K-Ras (Fig. 5B, dark gray bars). These experiments demonstrate that the Rab9-interaction domain detected by the two-hybrid screen is competent for Rab9–GTP association, when present within the structure of the full length p40 protein. Moreover, the nucleotide-state prefer-
ence of the interaction was also recapitulated with the full length protein.

Binding of p40 did not influence the intrinsic nucleotide exchange rate of Rab9 (Fig. 6 A). In contrast, at micromolar concentrations, p40 inhibited the intrinsic rate of GTP hydrolysis by Rab9 (Fig. 6 B). While the physiological significance of this observation is not known, this result provides independent confirmation of a direct interaction between p40 and Rab9. Indeed, GTPase inhibition may not reflect the true role of p40, since Rab9 has a very low intrinsic rate of GTP hydrolysis (Shapiro et al., 1993). Together, these data demonstrate that p40 interacts directly with Rab9–GTP.

**p40 Is a Potent Transport Factor**

To explore the function of p40, we tested its influence on an in vitro transport assay that reconstitutes MPR transport from endosomes to the TGN (Goda and Pfeffer, 1988). Transport requires Rab9 (Lombardi et al., 1993; Dirac-Svejstrup et al., 1994), NSF, and α SNAP (Itin, C., C. Racaño, Y. Nakajima, and S.R. Pfeffer, manuscript submitted for publication) and shows all of the biochemical characteristics unique to this transport process (Goda and Pfeffer, 1988).

Our most satisfying result was obtained when we tested the activity of purified, recombinant p40. As shown in Fig. 7 A, p40 greatly stimulated the overall extent of endosome-to-TGN transport. Under conditions of limiting cytosol to provide other essential transport factors, nanomolar concentrations of p40 enhanced transport to 150% of the level seen with saturating concentrations of crude cytosolic proteins (Fig. 7 A). Thus, p40 represents a novel and potent transport factor that stimulates MPR transport.

If p40 functions by virtue of direct interaction with Rab9,
the two proteins might be expected to act synergistically in transport. In reactions containing limiting cytosol, low concentrations of either p40 or Rab9 (added as an active complex with GDI) alone showed very little stimulation. However, addition of both of these components stimulated transport to maximum levels (Fig. 7 B). These data strongly suggest that p40 functions in concert with Rab9 to facilitate MPR transport.

The experiments described above showed that p40 can stimulate MPR transport from endosomes to the TGN. Evidence that the molecule normally functions in this process came from antibody inhibition experiments. Anti-p40 IgG inhibited transport by almost 50% compared with preimmune IgG (Fig. 8). Furthermore, the inhibition was neutralized by addition of recombinant p40 (Fig. 8). These data demonstrate that p40 is normally required for efficient transport. The fact that only partial inhibition was observed may reflect the fact that our antibodies were raised against a human antigen while the assay utilizes CHO cell components.

Membrane Association Accompanies p40 Function

A number of independent lines of evidence support the conclusion that membrane-associated p40 represents the active form of the protein. As discussed earlier, ~30% of p40 is present on membranes. In addition, p40 acts in a synergistic fashion with presumably membrane-associated Rab9-GTP. Moreover, recombinant p40 can also bind directly to endosome-enriched membranes (E. Díaz, unpublished observation). Is this the active form of the protein? While anti-p40 IgG inhibited the overall transport reaction (Fig. 8), cytosol lacking p40 as a consequence of immunodepletion was still fully active in supporting in vitro transport (Fig. 9), consistent with this possibility. Moreover, preincubation of cytosol alone with anti-p40 IgG did not inhibit transport in reactions containing untreated membranes (not shown). Together, these data imply that anti-p40 IgG blocks transport by inactivating an active, membrane-associated p40 pool. At minimum, these data show that membrane-associated p40 is sufficient for MPR transport.

How, then, does soluble, recombinant p40 stimulate transport? It is important to note that p40 stimulated transport under conditions of limiting cytosolic proteins. We have shown elsewhere that α SNAP is among the most limiting cytosolic factors under these conditions (Itin C., C. Racaño, Y. Nakajima, and S.R. Pfeffer, manuscript submitted for publication). Presumably, by driving more p40 onto membranes, the reaction can bypass limitations of other factors.

Further experiments will be needed to identify directly the proteins to which p40 binds on membranes.

Discussion

We have reported here the discovery of a novel transport factor, p40, that acts together with the active Rab9 GTPase to drive MPR trafficking from endosomes to the TGN. p40 is present in cytosol and on membranes, a feature common to a variety of important transport factors including NSF (Block et al., 1988), α SNAP (Clary and Rothman, 1990), p115 (Waters et al., 1992), and Rabaptin 5 (Stenmark et al., 1995). An important characteristic of p40 is its specific interaction with the active conformation of the Rab9 GTPase. Since Rabs likely function in the process of vesicle docking, we infer that p40 also functions at this stage of vesicular transport.

Figure 7. (A) Anti-p40 antibodies inhibit MPR transport. Immune or preimmune IgG (75 μg/ml) was added to MPR transport reactions containing 1 mg/ml cytosol and where indicated, p40 (100 ng/ml). Error bars represent standard error of the mean (n = 2). Control reactions yielded 724 cpm.

Figure 8. Anti-p40 antibodies inhibit MPR transport. Immune or preimmune IgG (75 μg/ml) was added to MPR transport reactions containing 1 mg/ml cytosol and where indicated, p40 (100 ng/ml). Error bars represent standard error of the mean (n = 2). Control reactions yielded 724 cpm.

Figure 9. Depletion of cytosolic p40 does not inhibit MPR transport. (A) Anti-p40 immunoblot analysis of cytosols (100 μg each) preadsorbed with either preimmune IgG (control) or anti-p40 immune IgG (depleted). (B) Transport activity of the cytosols shown in A. Cytosols were assayed at 0.4 mg/ml; 100% transport of control cytosol represents 556 cpm. Values shown represent the average of duplicate determinations (standard error ± 5%) in a representative experiment.

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significant, local concentration effects on the surface of membranes, it does not seem likely that p40 acts to block Rab9 GTPase in vivo, since it is present in insufficient levels to do so. It is quite possible, however, that local membrane concentration effects are significant. In any event, it is essential to note that the ability of a protein to block GTPase activity may simply be an indirect consequence of tight binding to the GTP-bound form. A secondary outcome of this interaction would be to retain the Rab in its active conformation. Whether or not p40 blocks Rab9 GTPase, it is satisfying to note that a minimal estimate for membrane-associated Rab9 is also ~50 nM, a value quite close to the level of p40.

Other Rab-interacting proteins have been identified that may also slow GTP hydrolysis. For example, Rabphilin binds Rab3A-GTP and blocks its GAP-stimulated GTPase (Kishida et al., 1993). At the nerve terminal where vesicles may wait days before exocytosis, such a factor could be important to retain synaptic vesicles in an exocytosis-competent state. Whether this feature is also critical for a constitutive process such as endosome-to-TGN transport is not yet at all clear.

We and others have shown that Rabs are delivered to distinct membrane-bound compartments in their GDP-bound conformations and only subsequently are these GTPases converted to their active, GTP-bound forms (Soldati et al., 1994; Ullrich et al., 1994). Walch-Solimena et al. (1997) have now reported that Sec2p has the capacity to stimulate nucleotide exchange on Sec4p, converting it to the GTP-bound, active form. Satisfyingly, Sec2p interacts directly with the vesicle-associated, Sec4 GTPase and is necessary for subsequent secretory vesicle localization to the bud tips in yeast (Walch-Solimena et al., 1997). If this paradigm holds true for other transport steps, it would seem that Sec2p-like nucleotide exchange factors would act before p40 or Rabaptin, to convert the respective GTPase to its active conformation. Whether or not p40 blocks Rab9 GTPase activity may simply be an indirect consequence of providing the proteins to block Rab9-GDI presents functional rab9 to the intracellular transport machinery and contributes selectivity to rab9 membrane recruitment. J. Biol. Chem., 269:15427–15430.


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