Selective membrane recruitment of Rab GTPases

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Mannose 6-phosphate receptors (MPRPs) deliver newly synthesized, soluble lysosomal enzymes from the trans-Golgi network (TGN) to pre-lysosomes (Fig. 1) (for review, see Kornfeld and Mellman 1989; Kornfeld 1992). Two types of MPRs have been identified to date. One is a 300-kD transmembrane glycoprotein that also binds insulin-like growth factor II; the second is a dimer or tetramer of 45-kD subunits and requires divalent cations for ligand binding in vitro. Both types of MPRs release their ligands upon encountering the low pH within pre-lysosomes, and then return to the Golgi complex to reinitiate another cycle of biosynthetic enzyme transport. MPRs are also present at the cell surface. These receptors are in rapid equilibrium with their intracellular counterparts, and at least the 300-kD MPR is capable of endocytosing extracellular lysosomal hydrolases and delivering them to endocytic compartments.

Work from a number of laboratories has suggested that the Rab family of Ras-like GTPases plays a key role in regulating receptor trafficking (for review, see Zerial and Stenmark 1993; Narendra and Balch 1994; Pfeffer 1994). For example, Rab5 has been shown to regulate early endosome fusion both in vivo (Gervel et al. 1991) and in vivo (Bacchi et al. 1991). Rab4 functions in receptor recycling between early endosomes and the cell surface (van der Sluijs et al. 1992). In addition, Rab1 and its yeast homolog, YPT1, play a key role in the transport of proteins between the endoplasmic reticulum (ER) and the Golgi (Plutner et al. 1991; Horvath and Schekman 1991; Seges 1991; Tisdale et al. 1992). Rab proteins are thought to function in transport vesicle targeting and/or fusion events, because siRNA mutant yeast strains accumulate secretory vesicles (Novick et al. 1980), anti-YPT2 antibodies inhibit ER-derived transport vesicle fusion (Plutner et al. 1995; Horvath and Schekman 1991; Seges 1991), and anti-Rab5 antibodies block early endosome fusion (Gervel et al. 1991).

We showed recently that the Rab9 protein is localized primarily to the surface of late endosomes and can facilitate the transport of MPRs from late endosomes to the TGN in vitro (Lombardi et al. 1993). To investigate the physiological significance of our findings, and to explore the significance of MPR recycling in living cells, we generated a dominant inhibitory form of Rab9 protein based on well-characterized mutations of Ras. We showed that a dominant negative form of Rab9, Rab9(21N), strongly inhibited MPR recycling in living cells (Riederer et al. 1994). The block was specific in that the rates of biosynthetic protein transport, fluid-phase endocytosis, and receptor-mediated endocytosis were unchanged. These data showed a role for the Rab9 GTPase in efficient lysosomal enzyme delivery.

To further define the mechanism by which Rab9 facilitates MPR recycling, we reconstituted the selective targeting of prenylated Rab9 protein onto late endosome membranes (Soldati et al. 1994). Rab9 protein occurs in the cytosol as a stoichiometric complex with a protein termed GDP GDI (Soldati et al. 1994; for review, see Pfeffer et al. 1995). Rab9/GDI complexes are functional in terms of their capacity to stimulate MPR recycling to the TGN in vitro (Dirac-Svejcar et al. 1994). These experiments showed that GDI presents Rab9 to the transport machinery in a functional form, thus we employed prenyl Rab9/GDI complexes as substrates for our membrane recruitment studies.

Homogenous complexes of prenylated Rab9 bound to GDI were reconstituted using purified, prenylated Rab9 and bovine brain GDI-α. Three potential membrane targets were tested: MPR-enriched late endosomes, ER membranes, and lysed red blood cell (RBC) ghosts.

Prenylated Rab9, presented to MPR-enriched membranes as a GDI complex, became membrane-associated upon incubation at 37°C, but not at 4°C. ER-derived membranes showed significantly less binding at both temperatures. Binding was both specific and efficient: As much as 30-50% of the added Rab9 protein became membrane-associated after 40 minutes (Soldati et al. 1994).

Careful comparisons of the rates of membrane association and nucleotide exchange revealed an intermediate in Rab membrane recruitment. Rab9-GDP becomes membrane associated, and nucleotide exchange followed, but only after a distinct lag. Similar results were reported by Zerial and coworkers for the membrane recruitment of Rab5 (Ulrich et al. 1994). From this, we proposed that the selective recruitment of prenylated Rab proteins onto membranes may be catalyzed by a GDI or GDI displacement factor (Fig. 2) (Soldati and Pfeffer et al. 1994).

Figure 2 summarizes our current view of GDI function (Pfeffer et al. 1995). Uncoupled GDI retrieves GDP-bearing Rab proteins from target membranes after a vesicular transport event (step 1). Prenylated Rab9/GDI complexes are then recognized by target membrane-specific proteins. Rab proteins are recruited with high specificity (Soldati et al. 1994; Ulrich et al. 1994).
Figure 1. Intracellular transport of M6PRs. Soluble intracellular mannose-6-phosphate-terminating oligosaccharides bind M6PRs in the TGN and are collected into clathrin-coated vesicles. The vesicles deliver the recognized complexes to the plasma membrane, where the low pH triggers enzyme release. M6PRs can then recycle back to the TGN or traffic to the cell surface. Lysosomal enzymes can also exit the TGN via the constitutive secretory pathway which takes them directly from the TGN to the plasma membrane by default. Such enzymes may then be internalized by M6PRs by conventional receptor-mediated endocytosis (for review, see Kronke 1992). Rab9 function is required for M6PR recycling between late endosomes and the TGN.

1994) by a process that displays an apparent $K_{m}$ of about 10-20 nM (Soldati et al. 1994). Although it appears that the membrane interaction site for Rab9/GDI complexes is Rab-specific, it is not yet clear whether the proteolipid protein that couples this site directly to GDI, in addition to that portion of the Rab protein that would be presented by GDI (Fig. 2, step 1), and, during GDI displacement (step 3) and returned to the cytosol; the Rab associates with membrane fragments in its GDP-bound conformation, prior to what is presumed to be a catalytic nucleotide exchange event. A major prediction of this model is that the GDP should be Rab-specific. To explore the specificity of the recruitment of the Rab9, we compared the membrane recruitment of Rab9, Rab9, and Rab9. These experiments also addressed whether two Rab proteins localized to a single organelle use the same entry site for recruitment onto late endosomes.

Figure 2. A model for GDP functions. (1) Unoccupied, cytosolic GDP dissociates prenylated, GDP-bearing Rab proteins from target membranes. (2) GDP dissociates Rab9 from the donor membranes from which ascospore transport vesicles form. Such membranes would include the portions of the TGN from which vesicles form, the Golgi complex, endosomes, and the plasma membrane. (3) According to this model, Rab9 GDI complexes are recognized by a Rab-specific GDP-displacement factor (GDF). Rab-GDP becomes membrane-associated with GDF release into the cytosol (step 3). Rab-GDP would then be converted to Rab-GTP by the action of a GTPase-activating or GTPase-activating GDI exchange (GDF) such as DseI (step 4). The proteins that mediate organelle-specific association of Rab9 are not yet known.

Then a direct colocalization was not straightforward. To circumvent this problem, we examined the distribution of these proteins in CHO cells stably expressing 50-fold higher than normal levels of Rab9 that were subsequently transiently transfected with a Rab7 expression construct. The bulk of the anti-Rab7 staining overlapped with the distribution of Rab7 in perinuclear structures and other punctate compartments throughout the cytoplasm; Rab7 was also detected in structures that stained less strongly for Rab9 and Rab9 overlap with Rab9 in a similar pattern. Rab9 staining was more efficient in their distribution in CHO cells; some endosomes stained more strongly for Rab9 than Rab9, and vice versa. To compare the recruitment properties of Rab9 with those of other Rab proteins, we expressed (Rab9, Rab9, myc-tagged Rab9, and myc-tagged Rab7 proteins individually using recombinant baculoviruses and purified each of the proteins from the ChAPS-activated membranes of baculovirus-infected insect cells (Soldati et al. 1994). Purified Rab proteins were mixed with equimolar amounts of GDI-α and complexed formed spontaneously upon dialysis to remove detergents. Complex formation was verified in each case by gel filtration on Sephacryl S-1000. Under these conditions, the Rab9/GDI complexes contained stoichiometric amounts of GDI and the respective Rab proteins. Presumably, Rab9 associates with GDI-α with an apparent $K_{D}$ of 20 nM (Shapiro and Pfeffer 1994), thus, reconstituted complexes formed under these conditions are stable. Moreover, when prenyl Rab9 is present in molar excess relative to GDI-α during reconstitution, uncomplexed molecules aggregate and thus preclude such aggregation is readily detected by ultracentrifugation or gel filtration chromatography. All of the Rab9/GDI complexes used in this study remained soluble and failed to sediment upon ultracentrifugation.

RAB7 AND Rab9 Display SELECTIVE MEMBRANE RECRUITMENT IN VITRO

Rab9 is 37% identical in sequence to Rab9 protein (Chavrier et al. 1990a; Lombardi et al. 1993) and is also localized to late endosomes (Chavrier et al. 1990b). When complexes of prenyl Rab9 bound to GDI-α were incubated with endosome-enriched membrane fractions, Rab9 was recruited onto the membranes. Like Rab9, this process displayed a saturable initial rate, consistent with an apparent $K_{m}$ of 22 nM for membrane recruitment. For comparison, Rab9 was recruited with an apparent $K_{m}$ of 9 nM (Soldati et al. 1994) as estimated by Lineweaver-Burke plot analysis.

We also examined the recruitment of poyntic Rab1B onto the membrane fractions used in this study. Complexes of poyntic Rab1B bound to GDI-α were delivered to Rab9 by poyntic Rab1B enrichment of ER membranes. 0.76 pmol was recruited onto 3 nM of membrane in 40 minutes, in reactions containing 4 pmol Rab1B protein.

RAB9/GDI COMPLEXES INHIBIT THE RAB9 AND EXTENT OF MYC-TAGGED RAB9 RECRUITMENT

It is now well established that Rab proteins display distinct distributions and thus Rab-specific determinants dictate their localization (Chavrier et al. 1991; Brunaud and Nervi 1995; Donn et al. 1995; Steinmark et al. 1996). Yet certain organelles contain multiple Rab proteins. If two Rab proteins of late endosomes use a single recruitment device for their recruitment onto late endosomes, the two Rab should be competitive inhibitors of each other's recruitment. To establish the validity of this experimental approach, the ability of Rab9 to compete with the recruitment of myc-tagged Rab9 was first examined.

Addition of as little as a fourfold excess of Rab9/GDI complex to test for a significant inhibition of both the initial rate and extent of myc-tagged Rab9 recruitment. By all criteria tested, the recruitment of myc-tagged Rab9 was interfered with by Rab9. Virtually identical results were obtained when myc-Rab9 was tested as an inhibitor of Rab9 recruitment. We next attempted to analyze these data using kinetic methods. Lineweaver-Burke analyses of competitive experiments carried out at different myc-Rab9/GDI concentrations suggested that Rab9/GDI complexes behaved as competitive inhibitors (data not shown). Such analyses were complicated, however, by signal-to-noise comparisons, and by the fact that Rab9 recruitment is difficult to measure with high precision (<10% error), because the assay requires immunoblotting and densitometric quantitation of all samples. In addition, rather than assuming a single purified enzyme in solution, the assay utilizes prenylated Rab9/GDI complexes mixed with enriched, but heterogeneous heterogenetic membrane fractions. Despite these limitations, crude analyses yielded very useful information.

A semi-competitive plot of the data yielded a biphasic curve that could be decomposed into two lines which would correspond to apparent $K_{m}$ of about 10 nM and about 20 nM. The nonlinear results indicated that Rab9/GDI complex preparations contained two inhibitors of different potencies. It is already established that GDIα is a strong inhibitor of Rab9 recruitment (Soldati et al. 1994; Ullrich et al. 1996). The ability of GDIα to inhibit Rab9 recruitment was therefore carefully quantified. In this case, analysis of the inhibitory potential of GDIα as a function of the reciprocal of the initial rate yielded a single line corresponding to an apparent $K_{m}$ of about 20 nM. This value was very close to one of the values obtained when Rab9/GDI complex were analyzed (28 nM).

The equimolar transport of the nucleotide of Rab9 with GDI-α is less than 23 nM (Shapiro and Pfeffer 1993). Thus, concentrations of Rab9/GDI significantly less than 20 nM, the complexes are likely to be unstable. In addition, when Rab9/GDI complexes are added to inhibit myc-Rab9 recruitment, very few of Rab9 and myc-tagged Rab9 are in the same mem-

brane-associated complexes and as a single free mole of GDIα. This free GDIα then works together with remaining Rab9/GDI complexes to inhibit subsequent rounds of myc-Rab9 recruitment. At low Rab9 concentrations, the predominant inhibitor would be free GDIα; at high Rab9 concentrations, the predominant inhibitor would be complexes of Rab9/GDI.

Given this complexity, the simplified explanation of our data is that Rab9/GDI complexes inhibit myc-Rab9 recruitment, and that even a very small fraction of Rab9 is the most important factor for Rab9 recruitment (9 nM under these conditions. The close similarity of these values with an experimental error of ~10% validated this system to investigate possible competition between Rab9 and Rab9 proteins. Moreover, these experiments have not determined the identity of Rab9/GDI complexes; therefore, Rab9/GDI may not be potent inhibitors of Rab9 recruitment.

DO OTHER RAB9/GDI COMPLEXES INHIBIT Rab9 RECRUITMENT?

As discussed earlier, Rab9 and Rab9 are each recruited onto endosomes with apparent
Table 1. Rab8/GDI Complexes Differ in Their Ability to Inhibit Rab9 Recruitment

<table>
<thead>
<tr>
<th>GDI complex</th>
<th>Apparent Kₐ (mM)</th>
</tr>
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<tbody>
<tr>
<td>Rab9</td>
<td>9</td>
</tr>
<tr>
<td>Rab7</td>
<td>112</td>
</tr>
<tr>
<td>Rab1B</td>
<td>40</td>
</tr>
</tbody>
</table>

In each of the preparations by SDS PAGE. As would be expected, RabB1B complexes showed very little inhibition when GDI could not be liberated. The approximate apparent Kᵦ determined from these data was 4.4 µM. Rab8 cross-linked to GDI (Rab8 x GDI) was the strongest inhibitor, with an apparent Kᵦ of about 345 mM. This represents an approximately 2-fold loss in the capacity of Rab9 to inhibit as a cross-linked species. Nevertheless, Rab7 cross-linked to GDI was significantly less potent an inhibitor of Rab9 recruitment than were similarly cross-linked Rab9 complexes, despite the fact that Rab7 inhibitory potential was reduced only about 9-fold in the cross-linked complexes.

The cross-linking conditions used in these experiments were necessarily harsh, because they were designed to yield preparations lacking any unreacted free GDI. In addition, the detectable decreases of the cross-linked complexes were due to the attachment of BS3 at important surface

Although cross-linked Rab1B/GDI complexes (Rab1B x GDI) essentially failed to inhibit Rab9 or Rab7 recruitment, Rab8 x GDI and Rab9 x GDI did poorly inhibit the recruitment of their respective Rab constructs. Rab8 x GDI and Rab9 x GDI also inhibited the recruitment of the other late endosomal Rab proteins. Nevertheless, Rab7 x GDI more potently inhibited Rab9 recruitment than Rab7 recruitment; similarly, Rab7 x GDI more potently inhibited Rab9 recruitment than Rab7 recruitment.

These data confirm that GDI can act as a general inhibitor when released during a parallel recruitment process. When this complication is eliminated, cross-linked GDI is a weaker inhibitor of Rab9 recruitment than Rab7 cross-linked to GDI.

**CROSS-LINKED Rab9/GDI COMPLEXES BIND ENDOSONESOMES AND INHIBIT Rab9 RECRUITMENT**

An acknowledged difficulty with the interpretation of the experiments described thus far was the fact that free GDI was likely to be released during the cross-linking reactions, as a consequence of the parallel recruitment of the added competitor Rab protein or due to complex instability. To attempt to simplify the analysis, we prepared complexes of either prelysed Rab1B, Rab7, or Rab9 that were covalently attached to GDI (cross-linking agents, SDS) and of the preparations (Rab9 x GDI) as monitored by the mobility of the cross-linked complex material on SDS-PAGE.

**DISCUSSION**

In this study, we have compared the membrane recruitment of two late endosomal Rab proteins. Despite the fact that both proteins overlap in terms of their subcellular localization, the proteins were readily distinguished in terms of their ability to inhibit Rab9 recruitment. Rab7 and Rab9 were each recruited with high selectivity onto endosomes membranes with either GDI of values 10–20 mM. Rab9 competed for the recruitment of myr-tagged Rab9 with an apparent Kᵦ of 9.9 mM, but Rab7 was much less potent in inhibiting Rab9 recruitment, displaying an apparent Kᵦ of 112 mM (Table 1). These values suggest strongly that Rab9 and Rab7 interact with Rab1B complexes in a manner similar to that with Rab1B, Rab7, or Rab9, that were covalently attached to GDI using the cross-linking agents, SDS. Each of the preparations (Rab9 x GDI) was essentially unreacted free GDI obtained during Rab9 recruitment, showing very little inhibition when GDI could not be liberated. The approximate apparent Kᵦ determined from these data was 4.4 µM. Rab8 cross-linked to GDI (Rab8 x GDI) was the strongest inhibitor, with an apparent Kᵦ of about 345 mM. This represents an approximately 2-fold loss in the capacity of Rab9 to inhibit as a cross-linked species. Nevertheless, Rab7 cross-linked to GDI was significantly less potent an inhibitor of Rab9 recruitment than were similarly cross-linked Rab9 complexes, despite the fact that Rab7 inhibitory potential was reduced only about 9-fold in the cross-linked complexes.

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**Models for Rab Protein Recruitment**

Kinetic experiments indicate that Rab proteins become membrane-associated in their GDP-bound conformations prior to nucleotide exchange (Soldati et al. 1994; Sollner et al. 1994). This "initiation" association is followed by GDP displacement, and we have postulated that this may be catalyzed by a GDP-displace-
form, by GDI. Rab's are usually found on the pairs of organelles with which they interact; the membranes onto which they are recruited, and the fusion targets of vesicles forming from that initial compartment. Thus, Sec23p is found on both secretory vesicles and the yeast plasma membrane (Goud et al. 1988). RabGDI is found on both the plasma membrane and early endosomes, and Rab8a is present on both late endosomes and the TGN (Lombardi et al. 1993).

The presence of RabGDI on membranes representing both the beginning and end of their functional cycles implies that the distributions of Rab proteins in membrane fractions reported here must be interpreted with care. It is interesting that in proportion to the amount of RabGDI present in the membranes, the dense, ER-enriched membranes were ten times more active in RabGDI recruitment than the Golgi-endosome-enriched fraction. It is not clear whether RabGDI accumulates at the destination site of transport vesicles on which it functions, or whether it is concentrated on the less dense ER-Golgi intermediate compartment, but the recruitment machinery is present on both light and dense membrane-bound compartments. Again, localization of the recruitment machinery itself will resolve these questions.

In summary, Rab recruitment is a selective process that utilizes Rab-specific components. Rab recruitment is mediated by a protein that recognizes Rab proteins bound to GDI. Recruitment is accompanied by GDI release and subsequent exchange of bound GDP for GTP. The next challenge will be to identify the proteins responsible for the organelle- and Rab-specific targeting of this class of Ras-like GTPases.

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