Rab7 and Rab9 Are Recruited onto Late Endosomes by Biochemically Distinguishable Processes*

(Received for publication, June 6, 1995)

Thierry Soldati‡, Carmen Rancaño§, Heidi Geissler, and Suzanne R. Pfeffer¶

From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305-5307

Rab GTPases are localized to the surfaces of distinct membrane-bound organelles and function in transport vesicle docking and/or fusion. Prenylated Rab9, bound to GDP dissociation inhibitor-α, can be recruited selectively onto a membrane fraction enriched in late endosomes; this process is accompanied by nucleotide exchange. We used this system to address whether each Rab uses a distinct machinery to associate with its cognate organelle. Purified, prenylated Rab1B, Rab7, and Rab9 proteins were each reconstituted as stoichiometric complexes with purified GDP dissociation inhibitor-α, and their recruitment onto endosome- or ER-enriched membrane fractions was quantified. The two late endosomal proteins, Rab9 and Rab7, were each re-recruited onto endosome membranes with approximate apparent Km values of 9 and 22 nm, respectively. However, while control Rab9GDP dissociation inhibitor-α complexes inhibited the initial rate of myc-tagged Rab9 recruitment with an apparent K, of ~9 nm, Rab7 complexes inhibited this process much less effectively (apparent K, ~122 nm). Similarly, complexes of the endoplasmic reticulum-localized Rab1B protein were even less potent than Rab7 complexes (apparent K, ~405 nm). Rab9 complexes inhibited Rab7 recruitment with the same low efficacy as Rab7 complexes inhibited Rab9 recruitment. These experiments distinguish, biochemically, the recruitment of different Rab proteins onto a single class of organelle. Since Rab7 and Rab9 are both localized at least in large part, to late endosomes, this suggests that a single organelle may bear multiple Rab recruitment machines.

Rab proteins are small GTPases that participate in the processes by which transport vesicles identify and fuse with their cognate target membranes (for review, see Zerial and Stenmark (1993); Nudler and Balch (1994), and Pfeffer (1994)). Over 30 different Rab proteins have been identified. Distinct sets of Rab proteins are found on the ER, Golgi, intermediate membrane fractions in complex with GDI-α, and the plasma membrane, and on late endosomes. Some are redundant isoforms that carry out a common function (cf. Singer-Krüger et al. (1994)); most others are unique and essential for a particular step of intracellular transport.

Although Rab proteins are readily identifiable as a subfamily of Ras-like GTPases, the proteins are nevertheless highly diverse. For example, Rab9 is only ~30% identical to Rab1A, 2, 3A, 3B, 4, and 5 (Chavrier et al., 1990a). Rab proteins display the greatest extent of sequence diversity at their carboxyl termini. In domain-swap experiments, the hypervariable domain was shown to contain important targeting information and could relocalize a Rab protein to a different location (Chavrier et al., 1991; Brennwald and Novick, 1993). Other regions of the molecule are also important for Rab protein localization and function, in particular, the regions that correspond to α-helix 3-loop 7 of Ras (Dunn et al., 1993; Brennwald and Novick, 1993; Stenmark et al., 1994).

Given the organelle-specificity of Rab proteins, it seemed likely that organelle-specific receptors would mediate Rab recruitment. However, the recruitment machinery is difficult to saturate, both in vivo (Bucci et al., 1992; Lombardi et al., 1993) and in vitro (Soldati et al., 1994; Ullrich et al., 1994), and thus other scenarios have been proposed. One alternative is that Rab recruitment occurs in two steps. Recognition could be accomplished by a catalytic process in which the recruited Rab does not remain bound to the initial entry site; this initial membrane binding event could be followed by subsequent transfer to a saturable receptor site.

Recent studies in which Rab recruitment has been reconstituted using purified components (Soldati et al., 1994) or permeabilized cells (Ullrich et al., 1994) have provided new information regarding the mechanism of Rab protein recruitment. Prenyl Rab proteins, complexed with GDP dissociation inhibitor (GDI), first associate with the appropriate membranes in their GDP-bound conformations prior to a nucleotide exchange event (Soldati et al., 1994; Ullrich et al., 1994). Rab recruitment displays a saturable initial rate, consistent with a catalytic process. GDI displacement precedes nucleotide exchange, and may represent the catalytic event that underlies Rab recruitment.

We sought to test whether two different Rab proteins that reside on a single class of organelle use a common machinery to achieve their organelle-specific localizations. We focused on Rab7 and Rab9, two proteins of the late endosome (Chavrier et al., 1990b; Lombardi et al., 1993). While Rab9 functions in the transport of proteins between late endosomes and the trans-Golgi network (Lombardi et al., 1993; Riederer et al., 1994), work on the yeast homolog of Rab7, Ypt7p, suggests that this protein functions in late endosome fusion (Wichmann et al., 1992).

In this study we show that like Rab9, prenyl Rab7 can be recruited onto late endosomes when added to a membrane fraction in complex with GDI-α. Prenyl Rab1B shows a compa-
rable capacity for recruitment onto ER-enriched membranes. Competitive inhibition experiments suggest that each of these proteins utilizes a distinct but related machinery to accomplish selective membrane targeting.

MATERIALS AND METHODS

Rab Protein Expression and Immunofluorescence—Rab7 cDNA (Chavrier et al., 1990b) was subcloned into the pCVN vector, under the control of the early SV40 promoter (Riederer et al., 1994). Chinese hamster ovary cells expressing Rab9 50- to over endogenous levels were transfected with the Rab7 construct by electroporation (Riederer et al., 1994). Indirect immunofluorescence analysis was performed using a mouse monoclonal antibody directed against Rab9 and an affinity-purified rabbit polyclonal antibody to Rab7 (Riederer et al., 1994). The anti-Rab7 antiserum was raised in rabbits using native, unprenylated Rab7 protein produced in and purified from Escherichia coli. The anti-Rab7 antibodies were then affinity purified by binding and elution from an Affi-Gel 10 (Bio-Rad) matrix to which purified Rab7 had been attached.

Preparation of Membrane Fractions—Late endosome- and ER-enriched membrane fractions were obtained from a rat liver postnuclear supernatant as described previously (Soldati et al., 1994, 1995). Briefly, the endosomal and ER membranes were collected from a sucrose flotation gradient at the 0.5–0.9 M and the 1.3–1.5 M interfaces, respectively. Membrane fractions were characterized by quantitative Western blotting using the following antibodies: rabbit anti-mannose 6-phosphate receptor (Pfeffer, 1987), anti-Rab1B (generous gift of W. E. Balch, Scripps Research Institute, La Jolla, CA), affinity-purified anti-Rab9 (Soldati et al., 1993) and anti-Rab7, and rabbit anti-protein disulfide isomerase (the generous gift of Richard Roth, Stanford University).

Reconstitution of Cross-linking of Rab-GDI Complexes—Rab9, myc-Rab7, and His-Rab1B cDNAs were expressed in SF9 cells after infection with recombinant baculovirus, and the respective proteins were purified as described elsewhere (Soldati et al., 1995). Rab9 containing a 10-amino acid myc epitope tag at the N terminus was engineered by polymerase chain reaction, sequenced, and subcloned into pBlueBac (Pharmingen) before integration into a recombinant Baculovirus containing a 10-amino acid epitope tag at the N terminus was engineered by polymerase chain reaction, sequenced, and subcloned into pBlueBac (Pharmingen) before integration into a recombinant Baculovirus, according to the manufacturer. Recombinant Baculovirus expressing Rab1B, which had been tagged at its N terminus with 6 histidine residues, was a generous gift of Dr. W. Balch (Scripps, La Jolla, CA). GDI-α was purified from bovine brain cytosol as described (Sasaki et al., 1990). Equimolar Rab-GDI complexes were then reconstituted by dialyzing the mixture to remove CHAPS detergent, followed by ultracentrifugation and gel filtration chromatography on Sephacryl S-100 (Soldati et al., 1995). Cross-linked Rab-GDI complexes were prepared by incubating the reconstituted Rab-GDI complexes with 1 mM BS3 (sulfo-DSS, Pierce) for 30 min on ice. The extent of the cross-linking reaction was usually close to 95–100%, as determined by SDS-polyacrylamide gel electrophoresis followed by anti-Rab9, anti-Rab7, or anti-Rab1B and anti-GDI immunoblotting.

In Vitro Membrane Recruitment of Rab Proteins—Specific membrane recruitment of Rab proteins was reconstituted in vitro using purified membrane fractions and pure Rab-GDI complexes, as described previously for Rab9 (Soldati et al., 1994, 1995). Unless indicated otherwise, 16 μg (100 ng) Rab protein, in a stoichiometric complex with an additional 16 μM GDI, was incubated with 3 μg of the corresponding membrane in a 250-μl reaction for the indicated times. After stopping the reaction by the addition of ice-cold buffer and transfer to ice, membranes were recovered by centrifugation, and the amount of membrane-bound Rab protein was monitored by SDS-polyacrylamide gel electrophoresis followed by anti-Rab9, anti-Rab7, or anti-Rab1B and anti-GDI immunoblotting.

RESULTS

Rab7 and Rab9 are both localized to late endosomes. Rab7 was first shown to be a late endosome constituent based upon immunoelectron microscopic localization with the 300-kDa, cation-independent mannose 6-phosphate receptor (Chavrier et al., 1990b), see also Gorvel et al. (1991)). Rab9 also shows dramatic co-localization with this late endosome marker, as determined by confocal immunofluorescence microscopy (Lombardi et al., 1993). Both Rab7 and Rab9 are present at low abundance, and thus a direct co-localization was not straightforward. To circumvent this problem, we examined the distributions of these proteins in Chinese hamster ovary cells stained for Rab7 and Rab9 expression constructs. As shown in Fig. 1, the proteins showed significant co-localization in these cells. The bulk of the anti-Rab7 staining overlapped with the distribution of Rab9 in perinuclear structures and other punctate compartments throughout the cytoplasm; Rab7 was also detected in structures, which stained less strongly for Rab9. In summary, Rab7 and Rab9 overlap significantly in their distribution in Chinese hamster ovary cells; some endosomes stained more strongly for Rab9 than Rab7, and vice versa.

To compare the recruitment properties of Rab9 with those of other Rab proteins, we expressed Rab7, Rab9, myc-tagged Rab9 and His-tagged Rab1B proteins individually using recombinant Baculoviruses and purified each of the proteins from the CHAPS-solubilized membranes of Baculovirus-infected insect cells (Soldati et al., 1995). Purified Rab proteins were mixed with equimolar amounts of GDI-α, and complexes formed spontaneously upon dialysis to remove detergent (Soldati et al.,
Complexes of prenyl Rab7 bound to GDI-a localized to late endosomes (Chavrier et al., 1990a; Lombardi et al., 1994, 1995). Complex formation was verified in each case by gel filtration on Sephacryl S-100. As shown in Fig. 2, under these conditions, the Rab-GDI complexes contained stoichiometric amounts of GDI-a and the respective Rab proteins.

Prenyl Rab9 associates with GDI-a with an apparent $K_m$ of $\leq 23\text{ nM}$ (Shapiro and Pfeffer, 1995); thus reconstituted complexes formed under these conditions are stable. Moreover, when prenyl Rab9 is present in molar excess relative to GDI-a, Rab9 is not detected by ultracentrifugation or gel filtration chromatography. All of the Rab-GDI complexes used in this study remained soluble and failed to sediment upon ultracentrifugation.

As targets for membrane recruitment, we utilized membranes enriched in late endosomes, as judged by their content of the 300 kDa, cation-independent mannose 6-phosphate receptor, or ER, based on the enrichment of protein disulfide isomerase (Table I). The late endosome fraction also contains Golgi membranes, as determined by the distribution of GlcNAc transferase I (Corthesy-Theulaz et al., 1992). The endosome-enriched membranes were enriched 16-fold in Rab9 protein relative to the ER fraction. Rab7 was twice as abundant as Rab9 protein in the endosome-enriched membranes but was only 3.8-fold enriched relative to ER. Differences in enrichment indicate nonidentical protein distributions. Such differences could be due, for example, to the presence of Rab9 on both endosomes and the trans-Golgi network (Lombardi et al., 1993), which co-fractionates with endosomes, while Rab7 may reside uniquely on late endosomes. Alternatively, Rab7 may be present on an overlapping, but distinctly different population of late endosomes (see Fig. 1).

Rab1B was enriched 13-fold in the endosome-Golgi-enriched fraction relative to ER membranes. Rab1B has been reported to be an excellent marker of the ER-Golgi intermediate compartment (Griffiths et al., 1994), which apparently fractionates with Golgi and endosomes upon buoyant density centrifugation. Nevertheless, the ER fraction contained 20-fold more Rab1B than Rab9.

Rab7 and Rab1B Display Selective Membrane Recruitment In Vitro—Rab7 is 57% 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 Rab7 bound to GDI-a were incubated with an endosome-enriched membrane fraction, Rab7 was recruited onto the membranes. Like Rab9, this process displayed a saturable initial rate (Fig. 3), consistent with an apparent $K_m$ of 22 nM for membrane recruitment. For comparison, Rab9 is recruited with an apparent $K_m$ of 9 nM (Soldati et al., 1994) as estimated by Lineweaver-Burk plot analysis.

As shown in Table II, Rab7 membrane recruitment was a selective process. Under conditions in which Rab9 was recruited 6.8-fold more efficiently onto endosome-enriched membranes than onto lysed red blood cell membranes, Rab7 was recruited with a very similar degree of specificity (6.6-fold). Both Rab proteins were recruited to a small, but significant extent onto membranes of an ER-enriched fraction; this may be due to the presence of contaminating late endosomes, since low levels of 300 kDa mannose 6-phosphate receptors were detected in that fraction (Table I). All of these experiments were carried out using equal amounts of membrane protein; this also represented comparable levels of phospholipid (Table I). Red blood cell ghosts yielded background levels of recruitment in all cases (Table II).

We also examined the recruitment of prenyl Rab1B onto the membrane fractions used in this study. Complexes of prenyl Rab1B bound to GDI-a delivered Rab1B efficiently onto ER membranes; 0.76 pmol were recruited onto 3 $\mu$g of membrane in 40 min in reactions containing 4 pmol of Rab1B protein. Despite the fact that Rab1B was enriched 13-fold in the endosome-Golgi-enriched fraction relative to ER membranes, the protein was not recruited with a correspondingly higher efficiency onto these membranes; recruitment levels were close to those obtained for the ER-enriched fraction (0.72 pmol/3 $\mu$g/40 min). These results are consistent with the notion that Rab proteins are recruited onto a specific membrane but may accumulate elsewhere as part of their functional cycle. In this case, recruitment of Rab1B may be primarily onto ER-derived membranes; accumulation may represent Rab1B bound to early Golgi compartments and/or the ER-Golgi intermediate compartment. This is at first surprising, because the Rabs are present at very different levels. The most reasonable explanation is that the recruitment machinery, which appears to act catalytically, is not limiting for recruitment under the experimental conditions employed.

It is important to note that each of the Rab proteins were recruited with similar efficiencies (Table II). In addition, each of the Rab protein preparations displayed similar capacities for GTP binding and hydrolysis (not shown). Thus, differences observed in the experiments described below cannot be explained by differences in the activities of the purified Rab-GDI complexes.

Rab9-GDI Complexes Inhibit the Rate and Extent of myc-tagged Rab9 Recruitment—It is now well established that Rab proteins display distinct distributions and that Rab-specific determinants dictate their localization (Chavrier et al., 1991; Brennwald and Novick, 1993; Dunn et al., 1993). Yet certain organelles contain multiple Rab proteins. If two Rabs of late endosomes use a single recruitment device for their recruit-

---

**Table I**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Endosome-Golgi fraction</th>
<th>ER</th>
<th>Red blood cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPR (au)*</td>
<td>100</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>PDI (au)</td>
<td>5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Phospholipid (µg/mg protein)</td>
<td>396</td>
<td>336</td>
<td>384</td>
</tr>
<tr>
<td>Rab9 (ng/mg)</td>
<td>145</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Rab7 (ng/mg)</td>
<td>300</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Rab1B (ng/mg)</td>
<td>4000</td>
<td>300</td>
<td></td>
</tr>
</tbody>
</table>

*au, arbitrary units derived from quantitative immunoblots.
Recruitment of Rab7 and Rab9 onto Late Endosomes

The initial rate of Rab7 recruitment onto a late endosome-enriched membrane fraction is saturable. Points represent the average of at least duplicate determinations (S.E. ~ 10%). Rab7 recruitment onto late endosomes was linear for ~15 min; the initial rate was obtained from standard 250-μl reactions carried out for 5-10 min with the indicated amounts of Rab7-GDI complexes. The apparent \(K_m\) (22 nM) was determined by Lineweaver-Burk analysis (B).

Fig. 3. A, the initial rate of Rab7 recruitment onto a late endosome-enriched membrane fraction is saturable. Points represent the average of at least duplicate determinations (S.E. ~ 10%). Rab7 recruitment onto late endosomes was linear for ~15 min; the initial rate was obtained from standard 250-μl reactions carried out for 5-10 min with the indicated amounts of Rab7-GDI complexes. The apparent \(K_m\) (22 nM) was determined by Lineweaver-Burk analysis (B).

As shown in Fig. 4A, addition of as little as a 4-fold excess of Rab9-GDI complexes led to 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 indistinguishable from that of wild-type Rab9 protein. A roughly 10-fold excess of Rab9-GDI complexes led to almost complete inhibition of myc-Rab9 recruitment (Fig. 4B). Virtually identical results were obtained when myc-Rab9 was tested as an inhibitor of Rab9 recruitment (not shown).

We next attempted to analyze this data using kinetic methods. Lineweaver-Burk analysis of competition experiments carried out at different myc-Rab9-GDI concentrations suggested that Rab9-GDI complexes behaved as competitive inhibitors (data not shown). Such analyses are complicated, however, by signal-to-noise considerations. It is important to note 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 assaying a single purified enzyme in solution, the assay utilizes prenylated Rab9-GDI complexes mixed with enriched, but nevertheless, heterogeneous membrane fractions. Despite these limitations, crude analyses yielded very useful information, as will be described below.

A semireciprocal replot of the data presented in Fig. 4B (Fig. 4C) yielded a biphasic curve that could be decomposed into two lines that would correspond to apparent \(K_i\) values of ~9 and ~28 nM. The nonlinear results indicated that Rab9-GDI complex preparations contained two inhibitors of different potencies. It is already well established that GDI-\(\alpha\) is a strong inhibitor of Rab recruitment (Soldati et al., 1994; Ullrich et al., 1994). The ability of GDI to inhibit Rab9 recruitment was therefore carefully quantified (Fig. 5). In this case, analysis of the inhibitory potential of GDI as a function of the reciprocal of the initial rate yielded a single line corresponding to an apparent \(K_i\) of ~26 nM. This value was very close to one of the values obtained when Rab9-GDI complexes were analyzed (28 nM).

The equilibrium binding constant for the association of Rab9 with GDI-\(\alpha\) is less than 23 nM (Shapiro and Pfeffer, 1995). Thus, at 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, every mole of Rab9 and myc-tagged Rab9 that becomes membrane-associated generates an equal mole of free GDI. This free GDI 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 GDI; at higher Rab9 concentrations, the predominant inhibitor would be complexes of Rab9-GDI.

Given these complexities, the simplest explanation of our data is that Rab9-GDI complexes inhibit myc-Rab9 recruitment with an apparent \(K_i\) ~9 nM, essentially identical to the apparent \(K_m\) for Rab9 recruitment (9 nM) under these conditions. The close similarity of these values (given the experimental error of ~10%) validated this system to investigate possible competition between Rab7 and Rab9 proteins. Moreover, these experiments suggest that complexes of Rab9-GDI are more potent inhibitors of Rab9 recruitment than free GDI alone.

Do Other Rab-GDI Complexes Inhibit Rab9 Recruitment?—As discussed earlier, Rab7 and Rab9 are each recruited onto endosome membranes with apparent \(K_m\) values of 10–20 nM. If these proteins use the same machinery for their membrane recruitment, they should be comparably potent as inhibitors of Rab9 recruitment. Fig. 6, left, shows that although Rab7-GDI complexes inhibited Rab9 recruitment, they were far less potent inhibitors than Rab9-GDI complexes or GDI alone. In addition, these complexes altered the initial rate but not the extent of Rab9 recruitment. As observed for Rab9 recruitment, the replotted data could be represented by two slopes corresponding to apparent \(K_i\) values of 35 and 112 nM. It is likely that the 35 nM value represents free GDI released during Rab7 recruitment, since the value was very close to that obtained for free GDI (26 nM). Because of the additional, possible instability of Rab7 complexes at low concentrations, the values obtained at higher Rab7 concentrations are also more likely to represent reliable estimates of the \(K_i\) for these complexes.

The inhibitory contribution of the Rab7-GDI complexes was about 12-fold lower than the apparent \(K_m\) for Rab9-GDI complexes. These data suggest strongly that Rab7 is recruited by a machinery that is distinct from that used by Rab9.

Also shown are the results of experiments in which Rab9-GDI complexes were tested as inhibitors of Rab7 recruitment (Fig. 6B, open symbols). Rab9 inhibited Rab7 recruitment to essentially the same extent as Rab7 inhibited Rab9 recruitment.

An important control for these experiments was Rab1B, which is clearly distinct in its localization from Rab7 and Rab9 proteins. Since Rab1B is localized to ER-Golgi compartments, it would not be expected to inhibit the recruitment of Rab7 or Rab9. However, if Rab1B became membrane-associated during the incubation, or if the complexes were unstable, free GDI would be generated, which could inhibit Rab7 or Rab9 recruitment.

Fig. 6, D–F, shows that Rab1B-GDI complexes were indeed weak inhibitors of Rab9 recruitment. Rab9-GDI complexes inhibited Rab1B recruitment to a very comparable extent (Fig. 6E, open symbols) as Rab1B inhibited Rab9 recruitment (solid symbols). As expected, a replot of the reciprocal of the initial
rate as a function of Rab1B-GDI complex concentration yielded a nonlinear set of data that could be represented by two slopes corresponding to apparent \( K_i \) values of 41 and 405 nM. Again, it seems likely that the 41 nM component corresponded to the released GDI. This would indicate that Rab1B-GDI complexes, as such, were 45-fold less potent than Rab9 complexes in inhibiting Rab9 recruitment.

Together, these data suggest strongly that Rab proteins utilize distinct but homologous machineries to direct their selective membrane recruitment.

**Fig. 5. GDI is a strong competitor of Rab protein recruitment.** A, the initial rates of membrane recruitment of Rab9 (●), Rab7 (▲), and Rab1B (■) proteins were determined as indicated in Fig. 4B in the presence of the indicated amounts of free GDI. B, when the data presented in A were replotted as in Fig. 4C, the data yielded a single slope corresponding to the action of a single competitor with an apparent \( K_i \) of 26 nM.

**FIG. 4. Rab-GDI complexes inhibit the rate and extent of myc-Rab9 recruitment onto late endosomes.** A, kinetics of membrane association of myc-Rab9 was monitored in standard reactions containing 4 pmol of myc-Rab9-GDI complexes in the absence (●) or presence of either 16 pmol (▲) or 32 pmol (■) of Rab9-GDI complexes. B, the initial rates of myc-Rab9 recruitment were extrapolated from the linear phase (up to 15 min) of reactions such as those presented in A, carried out in the presence of the indicated molar excesses of competitor Rab9-GDI complexes. C, the data presented in B were replotted as the inverse of the initial rate versus the concentration of inhibitor. The plot yielded a nonlinear curve consistent with the action of two distinct competitor entities. Decomposition of the biphasic curve into two linear portions permitted an estimate of the apparent \( K_i \) of the two competitors (28 nM and 9 nM).
GDI. Although less efficient than wild-type Rab9 protein, chemically cross-linked Rab9-GDI complexes (Rab9XGDI) displayed significant membrane association. In addition, membrane association of Rab9XGDI was saturable (not shown). In contrast, GDI-α alone showed much lower, but nevertheless, detectable levels of membrane association. This experiment shows that Rab9 can be recognized by membrane components while associated with the much larger GDI molecule. This is consistent with a model in which GDI presents the Rab9 protein to the recruitment machinery and contributes selectivity to this process (Dirac-Svejstrup et al., 1994).

We next examined the relative inhibitory potentials of cross-linked complexes of Rab1B, Rab7, and Rab9 with GDI. As would be expected, Rab1B complexes showed very little inhibition when GDI could not be liberated (Fig. 7B). The approximate apparent Kᵢ determined from these data was 4.4 μM. Rab9 cross-linked to GDI (Rab9XGDI) was the strongest inhibitor, with an apparent Kᵢ of ~345 nM. This represents an approximately 20-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 uncross-linked constituents. It seems reasonable to assume that the decreased potencies of the cross-linked complexes were due to the attachment of BS3 at important surface sites.

While cross-linked Rab1BxGDI complexes essentially failed to inhibit Rab9 or Rab7 recruitment, they were relatively strong inhibitors of Rab1B recruitment (Fig. 7C, right). Rab7xGDI and Rab9xGDI most potently inhibited the recruitment of their cognate Rab constituent. Rab7xGDI and Rab9xGDI also inhibited the recruitment of the other late endosomal Rab protein (Fig. 7C). Nevertheless, Rab7xGDI more potently inhibited Rab7 recruitment than Rab9 recruitment; similarly, Rab9xGDI more potently inhibited Rab9 recruitment than Rab7 recruitment.

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

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
The data from Bapparat apparent to inhibit Rab9 recruitment. Rab7 and Rab9 were each re-
proteins were readily distinguished in terms of their capacities to inhibit Rab9 recruitment. Rab7 and Rab9 were each re-
cruited with high selectivity onto endosome membranes with apparent $K_m$ values of $\sim 10$–20 nm. Rab9 competed for the recruitment of myc-tagged Rab9 with an apparent $K_i$ of 9 nm, but Rab7 was much less potent in inhibiting Rab9 recruitment, displaying an apparent $K_i$ of $\sim 112$ nm. These data suggest strongly that Rab proteins utilize distinct machineries for their membrane recruitment. Because Rab7 can inhibit Rab9 re-
cruitment to some extent, and Rab9 can inhibit Rab7 recruitment to an equivalent extent, it appears that the recruitment devices are distinct but homologous. This is not surprising, given the similarity of Rab7 and Rab9 proteins (57%), and the similarity in function of the two Rab recruitment machineries.

Do these experiments imply that a single organelle contains multiple and distinct Rab recruitment devices? The available data are not yet sufficient to permit this conclusion. Although Rab7 and Rab9 overlap significantly in their distribution with the mannose 6-phosphate receptor, the proteins may initially be recruited onto distinct subsets of late endosomes which then fuse. Resolution of this issue will require the identification of the proteins responsible for Rab protein recruitment and determination of their specific distributions.

While Rab1B-GDI and Rab7-GDI complexes inhibited to varying degrees, the initial rate of Rab9 recruitment, neither complex inhibited the overall extent of this process. Similarly, Rab9-GDI inhibited the initial rate but not the extent of Rab7 recruitment. In contrast, Rab9-GDI did inhibit the overall extent of myc-tagged Rab9 recruitment, and His-tagged Rab1B-GDI complexes also inhibited the overall extent of Rab1B recruitment (data not shown). Thus, these data disting-

Our experiments indicate that the Rab protein entry site interacts directly with Rab-GDI complexes. This conclusion is supported by the rapid and saturable binding of cross-linked Rab9-GDI complexes to membranes and the ability of those complexes to inhibit subsequent Rab protein recruitment (Fig. 7). Late endosome-enriched membranes (3 μg) bound 0.2 pmol of cross-linked Rab9-GDI. This is likely to represent a mini-
mum estimate of the number of entry sites since cross-linked Rab9-GDI complexes were less potent than the native complexes in inhibiting Rab9 recruitment. If the entry site is comprised of a protein of 25–50 kDa, that protein would be present at $\sim 0.1\%$ of the total protein in the endosome-enriched fraction. This level is consistent with the low abundance of late endosomal Rab proteins in the cell.

The ability of myc-Rab9, but not Rab1B or Rab7, to inhibit the overall extent of Rab9 binding suggests that the capacity of endosomes for Rab9 recruitment is likely to reflect the abundance of a downstream, Rab-specific binding site. At saturation, 1 pmol of Rab9 is bound per 3 μg of endosome membranes. This would imply that a Rab9 receptor, of assumed mass of $25–50$ kDa, might represent as much as $\sim 1\%$ of total endosome protein.

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; Ullrich et al., 1994). Initial membrane association is followed by GDI displacement, and we have post-
ulated that this may be catalyzed by a GDI-displacement factor (GDF) (Soldati et al., 1994; Pfeffer, 1994). If the initial rates measured in our experiments reflect this catalytic process, our results would suggest that the GDF has a high affinity for Rab proteins ($\sim 10$–20 nm). These values correspond well with the $K_i$ for Rab-GDI interaction, as would be required for a protein to achieve GDI displacement. In addition, the data also suggest that the GDF is Rab-specific. This conclusion is supported by our finding that Rabs are delivered with equal
efficiency to their cognate membrane targets, yet differ significantly in their abilities to inhibit each other’s initial rates of recruitment.

A GDF could represent the entry site and could also be a part of (or tightly associated with) a nucleotide exchange protein on the membrane surface. This nucleotide exchange protein may or may not be Rab-specific alternatively, it may be of intermediate specificity, acting on a subset of Rab proteins. To date, genetic approaches have led to the identification of a membrane-associated protein capable of enhancing the intrinsic rate of nucleotide exchange by Sec4p (Moya et al., 1993). This protein, termed Dss4p, was most active on Sec4p but also showed significant activity on ypt1p (yeast Rab1), which is 47.5% identical to Sec4p. A related mammalian protein, Mss4, shows preference for Sec4p but can act on other Rab proteins (Burton et al., 1993). If Dss4 and Mss4 act on multiple Rab proteins in vivo, they could not alone be responsible for the organelle-specific localization of Rab proteins.

Prenylation of Sec4p is not required for Dss4 action, and Dss4 appears not to utilize Sec4p-GDI complexes as substrates. This suggests that an additional component may also be required to first displace GDI prior to Dss4 action. A GDF may represent the Rab-specific component of the Rab recruitment machinery that might be responsible for the sequestration of Rab into specific membrane compartments.

After membrane association and nucleotide exchange, Rabs then appear to interact with a downstream target that also recognizes Rab-specific structural determinants. Thus, Rab delivery is controlled by the cooperation of perhaps as many as four factors: GDI, GDF, a nucleotide exchanger, and a downstream membrane receptor. The diversity and abundance of Rab proteins and the apparent complexity of their accurate targeting are likely to reflect the importance of this class of transport factors.

Fates of Rab Proteins—After recruitment onto a given membrane target, Rab proteins, in their GTP-bound conformations, are recruited to nascent transport vesicles and then in some way, facilitate V-SNARE/T-SNARE associations (Lian et al., 1994; Søgaard et al., 1994). Rabs are likely acted upon by Rab-specific GT-Pase-activating proteins (Stroun et al., 1993) at the fusion target followed by their retrieval from the membrane, in GDP-bound form, by GDI. Rabs 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, Sec4p is found both on secretory vesicles and the yeast plasma membrane (Goud et al., 1988); Rab5 is found both on the plasma membrane and early endosomes; and Rab9 is present on both late endosomes and the trans-Golgi network (Lombardi et al., 1993).

The presence of Rab proteins 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 Rab1B present in the membrane, the denser, ER-enriched membranes were 10 times more active in Rab1B recruitment than the Golgi-endosome-enriched fraction. It is not clear whether Rab1B 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 denser 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.

Acknowledgments—We thank Dr. William Balch for providing a His-tagged, Rab1B encoding-Baculovirus and anti-Rab1B antibodies, Dr. Marino Zerial for providing a canine Rab7 cDNA clone, Elva Diaz for constructing a myc-tagged Rab9 construct, and Rebecca Simonette for expert technical assistance.

REFERENCES

2 P. Novick, personal communication.