Mitotic phosphorylation of rab4 prevents binding to a specific receptor on endosome membranes

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Phosphorylation of the monomeric GTPase rab4 in mitotic cells leads to its relocalization from endosome membranes to the cytosol. To determine the mechanism underlying this change in distribution, we established an *in vitro* assay that reconstituted specific binding of rab4 when endosome-containing membranes were incubated with rab4 complexed with its cytosolic chaperone, GDP dissociation inhibitor (GDI). rab4 was found to bind to a saturable receptor associated with highly purified endosomes. Membrane binding and nucleotide exchange were physically distinct, since an active soluble fragment of the rab4 receptor, but not rab4 nucleotide exchange activity, could be released from membranes by elastase cleavage. Interestingly, the soluble fragment could be used to fully reconstitute rab4 membrane binding. *In vitro* phosphorylation of rab4 by cdc2/cyclin B kinase did not affect formation of rab4–GDI complexes, but did completely inhibit rab4 binding to its receptor. In contrast, *in vitro* phosphorylation of membranes did not result in the dissociation of bound rab4, nor were mitotic membranes deficient with respect to binding non-phosphorylated rab4. Thus, mitotic cells appear to accumulate rab4 in the cytosol by phosphorylating rab4 during the soluble phase of its normal activity cycle, thereby preventing membrane attachment.

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Introduction

In most eukaryotic cells, the onset of mitotic prophase is marked by significant changes in organelle structure and function (Warren, 1993). The endoplasmic reticulum (ER) fragments, the Golgi complex vesiculates and the nuclear envelope disassembles, allowing a dividing cell to partition its membrane components equally to each daughter cell. These events are accompanied by the cessation of transport through the endocytic and secretory pathways. The mechanism underlying the block in vesicular transport and organelle fragmentation is thought to involve a transient inhibition of a constitutively active fusion machinery in the face of continued budding of transport vesicles, resulting in the progressive vesiculation of vacuolar organelles (Warren, 1993). The fusion inhibition is lifted at the onset of telophase, at which time organelles begin to reassemble

(Sager *et al.*, 1984; Featherstone *et al.*, 1985; Souter *et al.*, 1993).

The protein components that serve as targets for mitotic inhibition of membrane traffic are unknown, although some candidates have been identified (Warren, 1993; Rabouille *et al.*, 1995). Among these are at least two members of the rab family of monomeric GTPases. Although both rab1 and rab4 are membrane bound during interphase, they are largely found as soluble, cytosolic proteins during mitosis (Bailly *et al.*, 1991; van der Sluijs *et al.*, 1992a). rab1 has been shown to function at one or more steps in ER to Golgi transport (Peter *et al.*, 1994), while rab4 has been localized to early endosomes and functions in the recycling of receptors to the plasma membrane (van der Sluijs *et al.*, 1992b). Thus, redistribution of rab proteins to the cytosol may affect both endocytic and secretory transport. In the case of rab4, it is likely that phosphorylation is mediated by the mitotically active kinase cdc2/cyclin B (Bailly *et al.*, 1991; van der Sluijs *et al.*, 1992a). rab4 has been shown to be a substrate for cdc2 kinase *in vitro*. Moreover, alteration of the single consensus cdc2 recognition site in the rab4 sequence (Ser196, found in the COOH-terminal hypervariable region of rab4) produced a mutant protein that remained membrane associated during mitosis and failed to be phosphorylated in intact cells or *in vitro* (van der Sluijs *et al.*, 1992a).

Although the phosphorylation of rab4 on Ser196 is responsible for its accumulation in the cytosol, the mechanism of relocalization has not been determined. *In vitro* membrane binding experiments with the small GTP-binding proteins rab5 and rab9 have indicated that rab membrane association is a multistep process (Novick and Garrett, 1994; Pfeffer, 1994). Accordingly, inhibition of rab4 endosome association may occur at one of several steps, the identification of which would help define the features of membrane binding itself. One well-known component of the rab membrane attachment cycle is the cytosolic protein GDP dissociation inhibitor (GDI). GDI serves the dual function of extracting GDP-bound rab proteins from membranes as well as delivering them specifically to their target organelles (Novick *et al.*, 1994). When purified rab9–GDI or rab5–GDI complexes were isolated and incubated with membranes or SLO-permeabilized cells, respectively, each rab protein was delivered to its appropriate target membrane: early endosomes for rab5 and late endosomes for rab9 (Soldati *et al.*, 1994; Ullrich *et al.*, 1994). Little membrane-bound GDI was detected, suggesting that GDI was dissociated at or shortly after membrane binding. These studies also showed that nucleotide exchange occurs just after GDI release. The initial recognition event may thus be coupled to a GDI-displacement factor (GDF), which releases GDI and enables rab membrane attachment. Following membrane insertion,

GDP-bound rab is GTP bound through the actions of a membrane bound guanine nucleotide exchange factor (GEF). The identities of the receptor, GDF and GEF activities remain unknown, although proteins with GEF activity have been isolated (Burton *et al.*, 1994).

To investigate the mechanism of membrane attachment of rab4 and its regulation during the cell cycle, we developed an *in vitro* assay that reconstitutes rab4 binding to endosome membranes. Our results demonstrate that phosphorylation of rab4 by cdc2 kinase inhibits the initial interaction of rab4-GDI with its receptor.

Results

GDI mediates rab4 saturable binding to CHO cell membranes

To determine why rab4 phosphorylation inhibited the association of rab4 with endosome membranes, it was first necessary to establish and characterize an assay to measure rab4 recruitment to membranes *in vitro*. Such assays have recently been established for rab9 and for rab5, and have elucidated some of the basic features of rab protein binding to target membranes (Soldati *et al.*, 1994; Ullrich *et al.*, 1994). In general, soluble rab proteins are found in an 80 kDa complex with GDI. In addition to masking the geranylgeranyl moiety, GDI also appears to play a role in ensuring the specificity of membrane recruitment (Dirac-Svejstrup *et al.*, 1995). However, the saturation kinetics of rab binding and the precise relationship between binding, GDI dissociation and nucleotide exchange remain unresolved at the protein level. Of particular interest is whether rab proteins bind to a limited number of specific receptor sites or are directed to an unlimited number of sites by transient interactions with a specific receptor element or GDF (Pfeffer, 1994).

We isolated rab4-GDI complexes on a Sephacryl S100 column following incubation of purified, recombinant histidine-tagged rab4 (H6-rab4) and GDI produced in *Sf9* cells or *Escherichia coli*, respectively (Soldati *et al.*, 1994). Based on elution profiles, complex formation was judged to be 30–40% efficient when recombinant rab4 was purified from total baculovirus-infected *Sf9* cell lysates; in contrast, complex formation was >95% efficient when rab4 was isolated from insect cell membranes (see Materials and methods). This correlated with the percentage of prenylated versus total rab4 that was recovered from total cell lysates or membrane pellets as judged by Triton X-114 phase separation (data not shown). Prenylation (geranylgeranylation) is required for association with GDI (Soldati *et al.*, 1993; Ullrich *et al.*, 1993).

To monitor whether rab4 can be recruited to membranes by GDI, we incubated the isolated purified GDI-rab4 complex with an endosome-enriched membrane fraction for increasing lengths of time at 37°C. Incubations were terminated by shifting the reaction mixture to 0°C, conditions which greatly inhibit rab4 binding to membranes (N.Ayad and I.Mellman, unpublished). Bound GDI or rab4 was detected by quantitative Western blot of membranes collected by ultracentrifugation. Recruited H6-rab4 was distinguished from endogenous rab4 on the basis of molecular weight. As shown in Figure 1A, GDI-mediated delivery of rab4 increased with time, reaching a plateau at 30 min ($t_{1/2}$ = ~6 min). Membrane-associated GDI was

Fig. 1. Characterization of rab4 binding to membranes *in vitro*. (A) Kinetic analysis of rab4 membrane binding. Purified rab4-GDI complexes (25 nM) were incubated with an endosome-containing membrane fraction isolated from CHO cells (see Materials and methods) for the indicated times (0, 10, 30 and 60 min). The reaction mixture was then shifted to 0°C and membrane-bound rab4 or GDI separated from soluble rab4-GDI by ultracentrifugation (100 000 g, 10 min). Membrane pellets were separated by SDS-PAGE and a Western blot performed with anti-GDI and anti-rab4 antibodies. The extent of rab4 and GDI recruitment was assessed by image digitization relative to a rab4 or GDI standard curve. A representative assay is shown with individual time points assayed in duplicate and varying with <10% standard error. (B) Concentration-dependent binding of rab4 but not GDI to endosomes. Endosome containing membranes were incubated with increasing concentrations of rab4-GDI (0, 10, 25, 50, 100 and 150 nM rab4). The reaction mixture was shifted to 0°C and the membrane-bound rab4 or GDI detected by quantitative Western analysis relative to a rab4 or GDI standard curve, as above. A representative assay is shown with each concentration assayed in duplicate and varying with <10% standard error. (C) Detection of recruited histidine tagged by Western blot. The Western blot of rab4 from experiment quantified in (B) is shown. An increasing amount of rab4-GDI was incubated with endosome-enriched membranes and the membrane-bound rab4 detected by Western analysis using a rabbit anti-human rab4 antibody. Histidine-tagged rab4 (H6-rab4) had an expectedly lower mobility on SDS-PAGE than endogenous rab4, allowing endogenous and recruited rab4 to be easily distinguished.

not detected at any time point, although the antibody used for detecting GDI was at least as sensitive as that used for rab4 (not shown). Since the free GDI generated as a

result of rab4 binding could in principle extract rab4 after delivery to membranes, it was possible that the assay reflected an equilibrium between GDI-mediated rab4 binding and re-extraction. This seemed unlikely, however, since all assays were performed in the presence of GTP γ S, under which conditions membrane-bound rab4, like other rab proteins, undergoes rapid nucleotide exchange and thus has a markedly reduced affinity for GDI (Garrett *et al.*, 1994; N.Ayad and I.Mellman, unpublished). Indeed, free GDI was able to extract >90% of the newly recruited membrane-bound rab4, but only when GDI was added in 50-fold molar excess (not shown).

rab4 membrane recruitment was also concentration-dependent, with half-maximal binding occurring at ~40–50 nM, similar to values obtained for other rab proteins (Soldati *et al.*, 1994; Ullrich *et al.*, 1994) (Figure 1B and C). Moreover, rab4 binding was saturable, suggesting that it reflected an interaction with a limited number of binding sites. We did not observe a concentration at which membrane-bound GDI could be detected, further supporting the idea that GDI dissociation may be tightly coupled to rab4 binding.

rab4 receptor activity is associated with endosome membranes

Since rab4 has been localized by cell fractionation and immunocytochemistry to early endosomes (van der Sluijs *et al.*, 1991, 1992b; Daro *et al.*, 1996), we next investigated whether the rab4 receptor activity measured *in vitro* was similarly distributed. To this end, different subcellular fractions isolated from CHO cells were probed for rab4 binding activity. To provide a marker for early endosomes, cells were labeled by incubation in HRP for 10 min at 37°C prior to homogenization.

CHO homogenates were first separated by flotation using a discontinuous sucrose gradient which permitted the isolation of fractions that were relatively endosome-enriched and endosome-depleted (Schmid *et al.*, 1988, 1989; Whitney *et al.*, 1995). As found previously, the endosome-enriched low density membranes (0.86/1.1 M interface) contained >75% of the total HRP, with the HRP activity having been enriched 6- to 8-fold relative to total protein. Approximately 35% of the activity of the lysosomal marker β -hexosaminidase was also found in the low density membrane fraction. These membranes were largely depleted in the resident ER marker calnexin as judged by Western blot; in contrast, high density membranes at the 1.1/1.3 M interface contained most of the calnexin but <20% of the HRP applied to the gradient. Approximately 40% of the lysosomal marker was found at this interface. Thus, in agreement with previous results, the high density membranes were judged as being endosome-depleted.

The endosome-containing and -depleted fractions were assayed for rab4 binding by incubation with rab4–GDI complexes as described above. As shown in Figure 2A, rab4 recruitment to the endosome-containing membranes was 7- to 8-fold greater than that to the endosome-depleted membranes, in agreement with the degree of enrichment of endosomes in this gradient fraction. Recruitment to the endosome-depleted membranes was barely greater than background, defined by incubations in which membranes were not added ('no membrane control'). No appreciable

amounts of membrane-associated GDI were detected as having been recruited to any membrane fraction (not shown).

To increase the purity of the endosomes used for these experiments, endosome-enriched membranes from sucrose gradients were further fractionated by free-flow electrophoresis (FFE), a technique that allows the separation of endosomes and lysosomes from Golgi, ER and plasma membrane on the basis of surface charge (Schmid *et al.*, 1988; Amigorena *et al.*, 1994; Whitney *et al.*, 1995). As shown in Figure 2B, membranes containing markers for endosomes (HRP, 10 min pulse) and lysosomes (β -hexosaminidase) were deflected towards the anode relative to the major peak of protein, previously defined as containing markers for remaining ER, Golgi and plasma membranes. Relative to the sucrose gradient fraction used for FFE, the endosomes were enriched an additional 6-fold (HRP activity/ μ g protein), in agreement with previous results. Endosome/lysosome-containing fractions were pooled (Pool 2) as were the non-shifted fractions (Pool 1) (Figure 2B). As shown previously, plasma membrane, ER and Golgi membranes were confirmed as being depleted from the anodally shifted membranes in Pool 2 (van der Sluijs *et al.*, 1991; Whitney *et al.*, 1995).

The two pools were assayed for rab4 binding activity by incubation with rab4–GDI complexes. The binding of rab4 to the highly purified endosome membrane fraction (Pool 2) was several-fold greater than binding to the endosome-depleted membrane fraction (Pool 1) (Figure 2C). Indeed, binding to the endosome-depleted membranes was indistinguishable from background (no membrane control). Since the endosome fraction contained some lysosomal membranes, we cannot completely exclude the possibility that these organelles also possessed rab4 binding activity. However, in intact cells, rab4 never co-localizes with lysosomal or late endosomal markers (van der Sluijs *et al.*, 1992; Daro *et al.*, 1996), and *in vitro*, fractions from dense regions of the sucrose gradients that were relatively enriched in lysosomes and depleted in endosomes did not bind rab4.

The rab4 receptor is an elastase-releasable membrane protein that is distinct from the receptor for rab5

We next investigated the physical properties of the endosomal rab4 receptor activity using a standard assay containing 3 μ g endosome-enriched membranes treated as indicated in Table I. Stripping membranes with high salt (1 M KCl) or sodium carbonate (pH 11) only slightly reduced rab4 binding, demonstrating that rab4 recruitment was not due to a protein that was peripherally associated with endosome membranes. Although binding of rab4 to endosomes was also not inhibited by trypsin treatment, it was partially inactivated by bromelain and completely (>98%) inactivated by elastase treatment (1 mg/ml, 1 h, 37°C). Since elastase was previously found to release a functional domain of the signal recognition particle (SRP) receptor from the ER, we investigated whether elastase had an analogous effect on the endosomal rab4 receptor. By incubating elastase-stripped membranes with the elastase supernatant released from an equivalent amount of membrane (following inactivation of the elastase with 1 mM elastatinal), rab4 binding activity could be completely

Fig. 2. Specific recruitment of rab4 to endosome membranes from CHO cells. **(A)** rab4 binds to endosome-enriched and not endosome-depleted membrane fractions isolated by sucrose density gradient centrifugation. rab4-GDI complexes were incubated with endosome-enriched and endosome-depleted membrane fractions isolated from a discontinuous sucrose gradient, as described (see Materials and methods). Membranes were isolated from cells that had been incubated with HRP for 10 min prior to homogenization, to provide a functional marker for early endosomes. Equal amounts of membrane protein were added to reaction mixtures containing rab4-GDI complexes (25 nM rab4). The reaction was stopped after 40 min and the extent of rab4 or GDI membrane association assessed by quantitative Western blot.

(B) Isolation of purified endosome fractions by free-flow electrophoresis. 10^6 CHO cells were labeled with HRP and pooled with 10^9 carrier cells. The resulting total cell pool was subsequently homogenized. Endosome-enriched membranes were isolated from a discontinuous sucrose gradient and injected into a free-flow electrophoresis apparatus. Fractions were collected and marker assays performed. The fluorescamine protein assay (filled squares) was used to detect total protein, the major peak of which was found (as documented previously) to largely coincide with enzyme markers for plasma membrane, ER and Golgi membranes. A β -hexosaminidase assay (filled circles) was performed to detect lysosomes and an HRP assay (open circles) performed to detect endosomes. Fractions 47–53 were combined into Pool 1 and fractions 56–63 combined into Pool 2. **(C)** rab4 receptor activity is selectively present on endosomes purified by free flow electrophoresis. A membrane binding experiment was performed using membranes concentrated from fractions 57–65 or 47–53 from the FFE fractionation shown in (B). rab4-GDI complexes (25 nM) were incubated with membranes enriched in unshifted, plasma membrane-containing fractions (Pool 1) or with anodally shifted fractions containing endosomes and lysosomes (Pool 2). The extent of rab4 membrane binding was assessed as described. Data shown are averages of representative assays performed in triplicate.

reconstituted (Table I). Taken together, these results indicate that the rab4 receptor of endosome membranes is a trypsin-resistant, elastase-releasable integral membrane protein. Identical results were obtained using membranes prepared from CHO cells, rat liver and bovine liver.

rab5 is another well-characterized endosome-associated rab protein which has also been shown to bind to early endosome membranes in intact cells as well as *in vitro* (Ullrich *et al.*, 1994). To determine whether rab4 and rab5 were recruited by common or distinct receptor elements, we next investigated if complexes of rab5-GDI could compete with the binding of rab4 from rab4-GDI complexes. Endosome-enriched membranes from CHO cells were incubated with subsaturating concentrations (25 nM) of rab4-GDI together with increasing concentrations of rab5-GDI (0–250 nM). As shown in Figure 3A, the

amount of rab4 recruited was not affected by the presence of even 10-fold excess rab5-GDI. The rab5-GDI complexes used were themselves fully functional since they mediated the simultaneous recruitment of significant amounts of rab5. Thus, rab4 and rab5 did not compete with each other for binding, suggesting that rab4 binds to a receptor element distinct from that for rab5.

The possibility that rab4 and rab5 bound to distinct receptor elements was more directly demonstrated by their differential sensitivities to elastase. Endosome-enriched membranes were isolated from rat liver (Fuchs *et al.*, 1994) and treated with 1 mg/ml of elastase. rab5-GDI and rab4-GDI complexes were incubated with control membranes or elastase-treated membranes and a quantitative Western blot using anti-rab4 or -rab5 antibodies was performed. As shown in Figure 3B, rab4 binding was

Table I. Characteristics of the rab4 receptor activity on endosomes

rab4 membrane binding (%)	
No treatment	100 ± 3
KCl	94 ± 12
Carbonate	92 ± 15
Trypsin	106 ± 9
Bromelain	71 ± 9
Elastase	1 ± 1
Elastase + elastase supernatant	112 ± 4

To determine the basic features of the rab4 receptor, endosome-enriched membranes from CHO cells were treated with either 1 M KCl, 0.1 M Na₂CO₃ pH 11.0 or with 1 mg/ml trypsin, bromelain or elastase, and then assayed for their ability to recruit rab4 from soluble rab4-GDI complexes, as described. To reconstitute rab4 membrane binding to elastase treated membranes, endosome-enriched membranes were treated with 1 mg/ml of elastase, washed and collected by ultracentrifugation. The resulting supernatants were treated with elastatinal and then added back to elastase-treated membranes; rab4 binding activity was determined using the standard recruitment assay.

almost completely abolished by elastase treatment, while rab5 binding to the same membranes was unaffected. Thus, unlike the presumptive receptor for rab4, the rab5 receptor activity was elastase resistant, strongly suggesting that the two receptors are, at least in part, physically distinct.

rab4 receptor is distinct from rab4 guanine nucleotide exchange activity

Exchange of GDP for GTP on rab5 and rab9 has been found to occur after only a brief lag following the recruitment of both rab proteins to endosome membranes (Soldati *et al.*, 1994; Ullrich *et al.*, 1994). Although these findings indicate that nucleotide exchange and receptor activities are kinetically distinct, they might still be due to the same protein or protein complex. Having demonstrated that the presumptive receptor for rab4 is elastase-sensitive, we next investigated whether rab4 GEF was similarly inactivated by elastase. Using the assay developed for measuring rab9 GEF activity (Soldati *et al.*, 1994), various concentrations of recombinant, prenylated rab4 were incubated in the presence of endosome-enriched membranes for increasing periods of time in the presence of [³⁵S]GTPγS. The addition of 3 μg of endosome-enriched membranes from bovine liver stimulated rab4 GEF activity several fold, relative to the intrinsic non-enzymatic exchange seen in the absence of added membranes or to the amount of [³⁵S]GTPγS that bound to membranes in the absence of added rab4 over a 1 h time course (Figure 4A). Although modest, this degree of stimulation by crude membrane preparations was similar to that observed for rab5 (Horiuchi *et al.*, 1995), and was also observed over a range of rab4 concentrations (0.1–2.5 μM) (not shown). Importantly, when elastase-treated membranes were used, no significant decrease in stimulated GEF activity was observed; nor did elastase reduce the rab4-independent intrinsic binding of [³⁵S]GTPγS to membranes (Figure 4A). The failure of elastase to inactivate rab4 GEF activity was emphasized when exchange activity was normalized for membrane protein added to the assay; as expected, elastase and KCl stripping of the endosome-enriched membranes removed protein from the starting membranes,

Fig. 3. rab4 and rab5 are recruited to membranes through distinct mechanisms. (A) Membrane binding of rab4 is not affected with increasing concentrations of rab5. 25 nM GDI-rab4 were incubated with endosome-enriched CHO cell membranes for 40 min at 37°C in the presence of 0 nM, 5 nM, 50 nM or 250 nM GDI-rab5. Membrane-bound rab4 and rab5 were collected by ultracentrifugation, separated by SDS-PAGE and transferred to nitrocellulose. Membrane binding of histidine-tagged rab4 and rab5 could be easily differentiated as indicated by the migration of the recombinant H6-rab4 and rab5 standards. (B) The rab5 receptor is elastase insensitive. 25 nM GDI-rab4 or GDI-rab5 were incubated with endosome-enriched membranes isolated from rat liver for 40 min at 37°C. 3 μg of control (C), elastase pellet (EP) or elastase pellet and supernatant (EP+S) membranes were used. Membrane-bound rab4 and rab5 were collected by ultracentrifugation, separated by SDS-PAGE and transferred to nitrocellulose.

thus increasing the apparent specific activity of the stimulated exchange reaction (Figure 4B).

Since elastase abolished rab4 binding without affecting rab4 nucleotide exchange activity, it seems likely that the presumptive receptor and GEF for rab4 represent physically distinct entities. However, we should note that rab4 binding was determined using rab4-GDI complexes, while exchange assays were performed using free rab4. Although unlikely, it is possible that there is a distinct GEF activity specific for rab4-GDI, and that this may be an elastase-sensitive component of the receptor. Given the elastase sensitivity of the receptor activity, it was impossible to separately assess the elastase sensitivity of nucleotide exchange using rab4-GDI.

Cdc2 kinase phosphorylates the cytosolic form of rab4

Having established that endosome membranes contain a protein that provides a specific rab4 receptor activity, we next investigated how rab4 phosphorylation controlled the membrane association of rab4 during the cell cycle. The mitotically active kinase cdc2/cyclin B has been shown to have multiple intracellular targets (Nigg, 1993). While most of its substrates are cytosolic, some (e.g. lamin B) are membrane associated. Thus, it is possible that cdc2 kinase acts in either (or both) of two ways. If membrane-bound rab4 is accessible to phosphorylation in mitotic cells, it might dissociate and accumulate in the cytosol. Alternatively, rab4 might be phosphorylated while in the soluble phase of its normal activity cycle and its rebinding prevented.

To determine if rab4 could be phosphorylated by cdc2 kinase while in the cytosol or while bound to membranes,

Fig. 4. Endosome enriched membranes contain an elastase-insensitive GEF for rab4. **(A)** Kinetics of rab4 GDP/GTP exchange. Endosome-enriched or endosome-depleted membranes were isolated by sucrose gradient centrifugation from bovine liver (see Materials and methods). 3 μ g of KCl treated membranes were either treated or untreated with 1 mg/ml elastase and subsequently incubated with 1 μ g of recombinant rab4 (0.4 μ M) isolated from membranes of baculovirus infected *Sf9* cells in the presence of 0.5 μ Ci [35 S]GTP γ S. The reaction was terminated after 0, 10, 30 or 60 min at 37°C and the protein associated radioactivity determined by a filter binding assay (Soldati *et al.*, 1994). [35 S]GTP γ S binding to endogenous membrane-bound GTPases is shown for rab4 + membrane or rab4 + elastase membrane values for each time point. A representative assay is shown with individual time points assayed in duplicate and varying with <10% standard error. **(B)** Characterization of rab4 exchange activity of endosome membranes. Endosome-enriched or endosome-depleted membranes were isolated by sucrose gradient centrifugation from bovine liver (see Materials and methods). Endosome-enriched membranes were subsequently treated with 1 M KCl (pKCl) alone or 1 M KCl followed by 1 mg/ml elastase. 3 μ g of membrane protein, determined after KCl or elastase treatments, were incubated with 1 μ g of recombinant rab4 (0.4 μ M) isolated from membranes of baculovirus infected *Sf9* cells in the presence of 0.5 μ Ci [35 S]GTP γ S. The reaction was terminated after 40 min at 37°C and the protein associated radioactivity determined by a filter binding assay (Soldati *et al.*, 1994). A no-membrane control was included in each assay to determine the extent of intrinsic nucleotide exchange. Nucleotide binding to endogenous membrane GTPases was subtracted from each membrane-containing reaction. Data shown are the averages of three separate experiments, each performed in duplicate.

we compared the ability of recombinant cdc2/cyclin B to phosphorylate rab4 on membranes isolated from CHO cell lines overexpressing rab4 with soluble rab4 in a GDI complex. Membranes containing equivalent amounts of wild-type rab4 or the non-phosphorylatable S196Q rab4 mutant (van der Sluijs *et al.*, 1992a) were incubated with recombinant active cdc2/cyclin B in the presence of [32 P]ATP. To determine whether soluble rab4 could be phosphorylated under the same conditions, the equivalent amount of purified recombinant rab4–GDI complex (concentrations normalized by quantitative Western blot using recombinant rab4 as standard) was also incubated with cdc2/cyclin B. The phosphorylation reactions were terminated (see Materials and methods), the samples lysed in detergent and immunoprecipitated using immune (I) or pre-immune (PI) rabbit serum against rab4. The amount of [32 P]rab4 recovered from each reaction was quantified after SDS–PAGE by phosphorimaging. As shown in Figure 5, cdc2 was found to phosphorylate soluble but not membrane-associated rab4. Immunoprecipitations of reactions containing membranes from wild-type or S196Q rab4-overexpressing cells revealed no detectable phospho-rab4 (lanes 1 and 2 for wild-type rab4; lanes 3 and 4 for S196Q rab4). The equivalent amount of rab4 as a GDI complex, however, was efficiently phosphorylated and specifically immunoprecipitated (lanes 5 and 6). Thus, soluble but not membrane-bound rab4 was accessible to cdc2 kinase, suggesting that phosphorylation might act by preventing the attachment of rab4–GDI to endosomes, as opposed to facilitating the dissociation of membrane-bound rab4.

To determine whether phospho-rab4–GDI complexes are as stable as dephospho-rab4 complexes, we compared the ability of phospho-rab4 and de-phospho-rab4 to complex to

GDI. Purified recombinant rab4 isolated from total cell lysates of *Sf9* cells was incubated with purified cdc2/cyclin B and [γ - 32 P]ATP and subsequently incubated with GDI. The resulting GDI–phospho-rab4 complex was then isolated on a Sephacryl S100 column. As shown in Figure 5B, phospho-rab4–GDI complex formation is ~30% efficient, as was found using 125 I-labeled dephospho-rab4 and GDI.

Phosphorylation of rab4 inhibits interaction with the endosomal rab4 receptor

We next determined directly whether phosphorylation prevented the recruitment of rab4 to endosome membranes. For this purpose, we produced 32 P-labeled rab4–GDI complexes of known specific activity. 3 μ g of purified, recombinant and prenylated rab4 were phosphorylated *in vitro* by activated cdc2 kinase in the presence of [γ - 32 P]ATP. The reaction was stopped and phosphorylation was assessed by immunoprecipitation and scintillation spectroscopy. Given that cdc2 kinase phosphorylates rab4 at a single site (Ser196) and given the specific activity of the [γ - 32 P]ATP used, we calculated that ~20% of the total rab4 added to the reaction had been phosphorylated. The labeled rab4 was then incubated with GDI under standard conditions. That phospho-rab4 was, as expected, capable of forming stable GDI complexes was confirmed by gel filtration on Sephacryl S100 (Figure 5B). This material was incubated with endosome-containing membranes as described above (in the presence of 10 μ M GDP or GTP γ S) and the amounts of total rab4 and 32 P-labeled rab4 were determined. Total rab4 was assessed by quantitative Western blot using blots of recombinant rab4 to generate a standard curve. The molar amount of membrane-bound phosphorylated rab4 was determined by SDS–PAGE and PhosphorImager analysis relative to a

Fig. 5. Cdc2 phosphorylates the cytosolic form of rab4. (A) cdc2 kinase phosphorylates rab4 in a complex with GDI. The ability of cdc2 kinase to phosphorylate membrane bound or soluble rab4 was determined using an *in vitro* phosphorylation reaction. rab4 present on endosome-enriched membranes isolated from CHO cells expressing wild-type human rab4 (WT-rab4) or a mutant rab4 lacking the single cdc2 kinase phosphorylation site (S196Q rab4) was incubated with recombinant cdc2 kinase/cyclin B and [^{32}P]ATP. Wild type rab4-GDI complexes were also incubated under identical conditions, the amount of rab4 (0.1 μg) added having been normalized to the amount of rab4 present on the membrane fractions used. The phosphorylation reactions were stopped and the extent of phosphorylation determined by SDS-PAGE and PhosphorImager analysis of immunoprecipitation reactions using either a rabbit anti-human rab4 antibody (I) or pre-immune serum (PI). ^{32}P -labeled rab4 was only detected when rab4-GDI complexes were used as the substrate. (B) GDI complexes to phospho-rab4. Recombinant rab4 was incubated with cdc2/cyclin and subsequently allowed to complex GDI by overnight dialysis. The resulting incubation mixture was separated on a Sephacryl S100 column. The relative elution times of phospho-rab4-GDI, phospho-rab4 and free [^{32}P]ATP are shown.

standard curve generated using known quantities of [^{32}P]rab4.

As shown in Figure 6, we were unable to detect any binding of [^{32}P]rab4 above background. Using endosome-enriched membranes incubated with 2.5 pmol total rab4-GDI (25 nM in a 0.1 ml reaction volume), 0.75–0.90 pmol of total rab4 bound per 3 μg of membranes, accounting for ~30% of the input rab4. Similar results were obtained in the presence of GDP or GTP γS . Since 20% of the input rab4 was phosphorylated, we would have expected to recover 0.15–0.18 pmol of [^{32}P]rab4 from the membrane fraction if phosphorylation had no effect on recruitment. Yet only 0.06–0.07 pmol of the phosphorylated rab4 was found to have bound, an amount indistinguishable from the amount of [^{32}P]rab4 that bound in the endosome-depleted or no membrane controls. Again, similar results were obtained in the presence of GDP or GTP γS , suggesting that the inhibition of binding by phosphorylation was not secondary to an inhibition of nucleotide exchange. Moreover, at the rab4-GDI or [^{32}P]rab4-GDI concentrations used for these experiments, rab4 binding was linear with respect to the amount of rab4 added (Figure 1B). Thus, cdc2 kinase-mediated phosphorylation of rab4 appeared to inhibit rab4 recruitment to endosome membranes at a very early stage, and at a step other than nucleotide exchange.

rab4 receptor activity remains active on mitotic membranes

Although rab4 phosphorylation appeared to inhibit the binding of rab4 to interphase endosome membranes, it remained possible that an additional modification of the membranes occurs in mitotic cells that further regulates their ability to interact with rab4. To test this possibility directly, membranes were isolated from mitotically arrested cells and compared with interphase membranes for their ability to recruit non-phosphorylated rab4 from

Fig. 6. Phosphorylated rab4 does not bind endosomes in the presence of either GDP or GTP γS . ^{32}P -labeled rab4 in a complex with GDI (25 nM GDI-rab4 complex) was incubated with endosome-enriched and endosome-depleted membranes in the presence of either 10 μM GDP or 10 μM GTP γS . Since the extent of rab4 phosphorylation was determined to be ~20% of total rab4, membrane binding of both total rab4 and phosphorylated rab4 could be assayed simultaneously. After the reaction was stopped, half the sample was analyzed by SDS-PAGE and quantitative Western blot using an anti-human rab4 polyclonal antibody. The remainder of the sample was analyzed by SDS-PAGE and PhosphorImager analysis to quantify ^{32}P . Data shown are averages of representative assays performed in triplicate.

rab4-GDI complexes. As indicated in Figure 7, rab4 binding to mitotic membranes (1 pmol/3 μg membrane protein) was indistinguishable from rab4 binding to interphase membrane (0.975 pmol/3 μg membrane protein). In addition, it is apparent that any GDF activity

Fig. 7. Mitotic membranes contain rab4 receptor activity. Endosome-enriched membranes were isolated by sucrose gradient centrifugation from interphase cells or cells arrested in mitotic prophase in the presence of a phosphatase inhibitor cocktail (10mM NaF, 25 mM sodium β -glycerophosphate, 50 mM sodium vanadate). 3 μ g membrane protein were incubated with rab4-GDI complexes (25 nM) and assayed for recruitment of his-tagged rab4. The reaction was stopped after 40 min of incubation at 37°C. Membranes were collected by ultracentrifugation and quantitative Western blot performed as described. Data shown are averages of a representative assay performed in triplicate.

normally found in interphase membranes was also present on mitotic membranes, since they did not accumulate GDI significantly above background, or more than did interphase membranes.

Discussion

Although their precise activities remain uncertain, members of the rab family are well known to play critical roles in vesicular traffic, most likely by regulating vesicle-vesicle fusion (Pfeffer, 1994). One of the hallmarks of rab proteins is their organelle specificity, with individual members of the family exhibiting characteristic intracellular distributions. rab proteins might thus represent effective targets for the regulation of specific events on the endocytic or secretory pathways. The observation that mitotic cells phosphorylate at least two rab proteins suggests that this modification may be partly responsible for the arrest of membrane traffic that occurs during mitosis. In the case of rab4, phosphorylation also occurs in insulin-stimulated adipocytes concomitant with the plasma membrane insertion of Glut4 transporters which have been sequestered intracellularly within specialized endosomes (Cormont *et al.*, 1993). The phosphatase inhibitor okadaic acid also enhances rab4 phosphorylation and cytosol accumulation in adipocytes (Cormont *et al.*, 1993).

Although the functional significance of rab4 phosphorylation remains unclear, the fact that it accumulates almost quantitatively in the cytosol of mitotic cells presented an excellent opportunity to analyze the steps involved in the recruitment of rab4 to membranes. The organelle

specificity of rab proteins is intimately associated with their functions, yet little is known about how the specificity of organelle binding is achieved. Recent work has demonstrated that early and late endosomes have receptor activities capable of selectively recruiting rab5 and rab9, respectively (Soldati *et al.*, 1994; Ullrich *et al.*, 1994). These *in vitro* binding studies have suggested that recruitment involves a series of steps beginning with the binding of rab-GDI complexes to a cognate rab receptor, the dissociation of GDI by an as yet unidentified GDF, stable association of the rab with the membrane by insertion of the rab prenyl group into the lipid bilayer and the exchange of rab-bound GDP for GTP, an event catalyzed by one or more GEFs. Since it has been shown that GDI dissociation occurs directly following rab binding, it is possible that the receptor and GDF activities are provided by the same protein. Nucleotide exchange, on the other hand, must occur after GDI release, suggesting that GEF is distinct from the receptor activity that initially specifies rab binding (Novick and Garrett, 1994). One broad specificity mammalian GEF has been cloned thus far (Mss4) (Burton *et al.*, 1993, 1994), but it is not clear whether this soluble protein is linked to specific rab binding events.

Information for targeting to a particular organelle is contained within the amino acid sequence of individual rab proteins (Chavrier *et al.*, 1991; Brennwald and Novick, 1993). At least in part, the targeting signal is localized to the COOH-terminal hypervariable region. GDI also plays a role in controlling specificity, however, despite the fact that only two GDI genes are known in animal cells (Pfeffer *et al.*, 1995). Free rab9 will insert into any lipid bilayer, presumably due to its geranylgeranyl moiety (Soldati *et al.*, 1994). Conceivably, GDI acts to control specificity simply by masking a rab protein's hydrophobic prenyl group, preventing promiscuous bilayer insertion and allowing sequence-specific targeting information to be used to initiate the binding process by interaction with a receptor for a particular rab protein.

Our results demonstrate that like rab5 and rab9, rab4 has an endosome-associated receptor activity. The receptor is saturable at increasing concentrations of rab4-GDI, suggesting there are a finite number of binding sites for rab4 or rab4-GDI on endosomes. Like the previously identified rab receptors, the putative rab4 receptor is specific. Of particular interest in this respect was the fact that even rab5-GDI was unable to compete for rab4 binding. This is despite the fact that the two rab proteins share an early endosomal localization (Bucci *et al.*, 1992; van der Sluijs *et al.*, 1992b). Thus, a single organelle may have multiple receptors for multiple rab proteins as has been shown by competition assays for the late endosome proteins rab7 and rab9 (Soldati *et al.*, 1995a). Such a situation might explain how two rab proteins can localize to the same organelle while having highly divergent hypervariable domains (Chavrier *et al.*, 1991).

Our characterization of rab4 binding to endosome membranes also revealed several new features of at least one potential rab receptor. First, the fact that rab4 binding activity was not eluted by high salt or alkaline pH washes, but could be partially or totally inactivated by proteolytic enzymes, strongly suggested that the putative rab4 receptor is an integral membrane protein. These results also demonstrated directly that the receptor is biochemically distinct

from endosome-associated rab4 GEF activity. Although the receptor activity was eliminated by elastase, it was unaffected by trypsin treatment. Trypsin, however, completely inactivated rab4 GEF on endosomes (I.Northwood and I.Mellman, unpublished). Also, endosome-enriched membrane GEF activity was completely elastase-insensitive. It remains possible that receptor and GEF activities are part of a single protein complex, but it is unlikely that the two functions are provided by a single protein. GDF activity, on the other hand, appeared to exhibit the same sensitivities as rab4 receptor activity, consistent with the possibility that the receptor may indeed be identical to GDF (N.Ayad and I.Mellman, unpublished). Also consistent was our observation that little if any GDI could be found as being membrane associated, even at short times of incubation of rab4-GDI with endosome membranes. Thus, GDI dissociation and rab binding may occur simultaneously.

An unexpected but potentially very useful finding was that elastase cleaved off functional rab4 receptor activity. By adding back an elastase supernatant to salt-washed and elastase-treated membranes, full rab4 binding activity could be recovered. This characteristic is reminiscent of early studies of the SRP receptor on the ER, and facilitated the purification of SRP receptor from protease supernatants (Walter *et al.*, 1979; Meyer and Dobberstein, 1980). Our preliminary results suggest that an analogous approach is effective for the isolation of the endosome rab4 receptor. Although also sensitive to elastase, the endosomal receptor for rab5-GDI from rat liver endosomes could not be reconstituted in this manner, further suggesting that receptor elements for rab4 and rab5 are distinct proteins.

How does rab4 phosphorylation lead to an accumulation of rab4 in the cytosol? While we had previously demonstrated that cytosol accumulation was dependent on phosphorylation at a single cdc2 kinase phosphorylation site (Ser196) in the rab4 hypervariable domain (van der Sluijs *et al.*, 1992a), the mechanism of this dramatic redistribution remained unknown. Conceivably, phosphorylation of endosome-associated rab4 might result in its dissociation from membranes, perhaps by increasing its affinity for GDI. Alternatively, phosphorylation might inhibit GEF-mediated GTP-GDP exchange following membrane attachment, possibly reducing the stability of membrane insertion by maintaining bound rab4 in the GDP state (and thus remaining susceptible to extraction by GDI). Our results suggest that neither of these possibilities is true, but rather that phosphorylation prevents the initial recruitment of rab4 from rab4-GDI complexes to endosome membranes even from non-mitotic cells. Ser196 may thus define a component of the endosome targeting sequence contained within the rab4 hypervariable domain. However, substituting alanine or glutamine residues at position 196 did not interfere with endosome localization of the mutant rab4 (van der Sluijs *et al.*, 1992b). Nor did these substitutions interfere with the ability of either mutant to be extracted by GDI *in vitro* (N.Ayad and I.Mellman, unpublished).

The most likely conclusion is that in mitotic cells, rab4 is phosphorylated by active cdc2/cyclin B kinase while in the soluble phase of its activity cycle, and thus prevented from rebinding. In all cases studied so far, membrane-bound rab proteins are largely in the GTP-bound state,

and thus have a relatively low affinity for GDI. Upon discharging their function, the GTP is hydrolyzed, yielding membrane-bound GDP-rabs which are then extracted into the cytosol by GDI. rab4 is a substrate for cdc2 kinase either as a free protein which can then go on to complex with GDI, or as a component of a pre-formed rab4-GDI complex. Whether rab4 phosphorylation occurs prior to or following complexing with GDI is thus immaterial, since the phosphorylated rab4-GDI that accumulates in the cytosol of mitotic cells contains a rab protein that cannot be recognized by the endosomal rab4 receptor until a cell progresses out of mitosis, cdc2 kinase becomes inactivated and rab4 is dephosphorylated.

Materials and methods

Isolation of endosome-enriched and endosome-depleted membranes by sucrose gradient centrifugation

Suspension CHO cells were grown to a concentration of 5×10^5 cells/ml in spinner flasks in α MEM/5% FBS. The cells were harvested by centrifugation (200 g, 10 min, 4°C), washed with PBS and TEAS 250 (10 mM triethanolamine acetate, 1 mM EDTA, 250 mM sucrose) and homogenized using a ballbearing homogenizer. A post-nuclear supernatant (p.n.s.) was obtained by centrifugation (2500 g, 15 min, 4°C), adjusted to 1.3 M sucrose and loaded under a sucrose step gradient (1.3 M sucrose, 1.1 M sucrose, 0.86 M sucrose, 0.25 M sucrose) which was then centrifuged for 90 min at 33 000 r.p.m. in a SW41 rotor. Membranes that collected at the 0.86/1.1 M interface and 1.1/1.3 M interface corresponded to low density endosome-enriched and high density endosome-depleted membranes, respectively. They were isolated, frozen in liquid N₂ and stored at -70°C until use.

Isolation of low density membranes from mitotic cells

Mitotically arrested cells were prepared from synchronized CHO cells followed by mitotic shake-off following a nocodazole block (van der Sluijs *et al.*, 1992a). Briefly, cell synchrony was achieved through nutrient deprivation by culturing confluent monolayers of cells for 24 h in α MEM + 0% FBS. The cells were released from a quiescent state through the addition of fresh serum containing medium (α MEM + 10% FBS) and subsequently arrested in prometaphase by the addition of nocodazole at a concentration of 0.25 μ M. Mitotically arrested cells were then isolated through mechanical shake off 4 h after the addition of the nocodazole. Released cells were concentrated by centrifugation (250 g, 15 min) and washed in ice-cold PBS. As judged by staining with DAPI, >90% of the cells were in metaphase. Membranes from mitotic cells were isolated as described for membranes from interphase cells in the presence of a phosphatase inhibitor cocktail to maintain them in a 'mitotic' state of phosphorylation (10 mM NaF, 25 mM sodium β -glycerophosphate, 50 mM sodium vanadate).

Isolation of low density and high density membranes from bovine liver

1.6 kg of bovine liver was homogenized in buffer C (0.25 M sucrose, 25 mM HEPES, pH 7.4, 25 mM potassium acetate, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM PMSF, 5 mM β -mercaptoethanol, 1 $\times$ TLCK and 1 mM each of chymostatin, leupeptin, antipain and pepstatin) using a Waring blender. The homogenate was passed over a rotating pestle and a p.n.s. was attained (10 min, 3500 g, 4°C). The pellet was discarded and the resulting supernatant was concentrated by ultracentrifugation (100 000 g, 30 min). The cytosol (supernatant) was discarded and the pellet fraction was resuspended in 1.3 M TEAS. The resuspended pellet fraction (30 ml) was then placed at the bottom of a Ti-45 ultracentrifuge tube, upon which 15 ml of 0.86 M and 0.25 M TEAS were layered. Membranes were isolated from the sucrose interfaces and frozen in liquid N₂. β -hexosaminidase assays indicated that endosome/lysosome-enriched membranes were present at the 0.86/0.25 M TEAS interface and that ER-enriched (endosome-depleted) membranes were present below the 1.3 M sucrose interface.

Isolation of endosomes by free flow electrophoresis

CHO cells were fractionated by free-flow electrophoresis using a Weber-Octopus model FFE apparatus (G.Weber GmbH, Munich) (Schmid *et al.*,

1988; van der Sluijs *et al.*, 1991). To provide a label for early endosomes, 1×10^6 CHO cells were labeled in suspension by incubation for 10 min at 37°C in the presence of 10 mg/ml HRP and then combined with 10^9 unlabeled carrier cells. The cells were washed extensively with PBS, homogenized and a p.n.s. obtained. The p.n.s. was loaded onto a discontinuous sucrose gradient and centrifuged in a discontinuous sucrose gradient for 90 min at 34 000 r.p.m. in a SW41 rotor. The 0.86/1.1 M sucrose interface was isolated, treated with 2 µg of trypsin per mg of protein for 5 min at 37°C, trypsin quenched by excess soybean trypsin inhibitor and injected into the FFE chamber exactly as described (Whitney *et al.*, 1995). Marker assays were also performed as previously described (Whitney *et al.*, 1995). Pooled fractions were concentrated by centrifugation for 30 min at 34 000 r.p.m. in a SW41 rotor, collected onto a 1 M sucrose cushion. The membranes and cushions were harvested and stored at -70°C until use.

Purification of recombinant rab4, rab5 and GDI

Recombinant, prenylated rab4 was produced in *Sf9* cells using a baculovirus vector and purified as per the manufacturer's instructions (Invitrogen). Briefly, the complete human rab4a coding sequence was inserted into the pBlueBacHis expression vector. The construct was transfected into *Sf9* cells and the viral titer determined. The virus stock was then used to infect *Sf9* cells grown in suspension at 27°C at a concentration of 10^6 cells/ml. Cell pellets were isolated by centrifugation (250 g, 10 min, 4°C). Cells were gently lysed by freeze-thaw, and total membranes collected by centrifugation (100 000 g, 15 min, 4°C). The resulting cytosol fraction was found by Western blot to contain soluble rab4 which did not partition into the detergent phase of Triton X-114, indicating that the soluble rab4 had not been modified by COOH-terminal geranylgeranylation (Bordier, 1981). The membranes were solubilized in 20 mM sodium phosphate, 500 mM NaCl, pH 7.8 containing 1% CHAPS (Pierce) and 1 mg/ml each of chymostatin, leupeptin, aprotinin and pepstatin (Sigma). The solubilized histidine-tagged rab4 bound a Ni^{2+} affinity resin (Pharmacia/LKB) and was isolated by imidazole elution as per the manufacturer instructions. Purified rab4 was dialyzed against buffer A (64 mM HEPES-NaOH, pH 8.0, 100 mM NaCl, 8 mM MgCl_2 , 2 mM EDTA, 0.2 mM DTT, 10 M µM GDP) containing 0.5% CHAPS. Glycerol was added to a concentration of 50% (v/v). The purified rab4 was then flash frozen in liquid N_2 and stored at -70°C for up to 6 months.

Untagged human rab5 was purified from baculovirus infected *Sf9* cells. *Sf9* cells were grown to 1×10^6 cells/ml and infected with a baculovirus construct, devoid of sequences coding for the NH_2 -terminal histidine tag. Infected cells were harvested, solubilized by freeze-thawing and the cytosol separated from the membranes by centrifugation (100 000 g, 1 h). The membranes were solubilized in buffer B (25 mM Tris, pH 7.4, 5 mM MgCl_2 , 1 mM EDTA) with 0.6% CHAPS and the solubilized material injected onto a Mono Q FPLC column (Pharmacia/LKB) and eluted using a linear gradient (0–1 M NaCl). Fractions were analyzed by SDS-PAGE and Western analysis using anti-rab5 antibody and the purified protein estimated to be ~95% pure by silver staining of the gels.

Bovine GDI-1 (Sasaki *et al.*, 1990) was generously provided by the laboratory of Marino Zerial and purified according to the manufacturer's instructions (Invitrogen). Purified GDI was dialyzed against buffer A and flash frozen in liquid N_2 .

Purification of cdc2 kinase/cyclin B

Baculovirus constructs containing cdc2 and glutathione-S-transferase (GST)-cyclin B cDNAs were generously provided by Dr Helen Piwnicka-Worms. The viruses co-infected *Sf9* cells grown in spinner flasks to a concentration of 10^6 cells/ml. The proteins were purified as described (Harper *et al.*, 1993) and stored in liquid N_2 .

In vitro phosphorylation assays

Phosphorylation assays were performed as described (van der Sluijs *et al.*, 1992a). Briefly, purified cdc2/cyclin B complex was incubated with either recombinant rab4, membranes isolated from cells overexpressing WT-rab4 or S196Q-rab4 for 30 min at 37°C in phosphorylation buffer A (10 mM MgCl_2 , 50 mM Tris-HCl pH 7.4, 80 mM Na β-glycerolphosphate, 6 mM EGTA) supplemented with 5 µCi [γ - ^{32}P]ATP. Kinase reactions were stopped by adding wash buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 10 mM NaF, 25 mM sodium β-glycerolphosphate, 50 mM sodium vanadate and 1% CHAPS). The proteins were immunoprecipitated using an anti-GST rab4 antibody,

separated by SDS-PAGE and phosphorylation quantified using a PhosphorImager (Molecular Dynamics).

Production of GDI-rab4 complexes

Purified GDI and rab4 were allowed to form a complex according to procedures previously used to form rab9-GDI complexes (Soldati *et al.*, 1994). Equimolar amounts (~0.5 µM) of rab4 and GDI were dialyzed extensively against buffer A to remove the CHAPS (Soldati *et al.*, 1995b). Any aggregated rab4 was removed by ultracentrifugation (100 000 g, 30 min, 4°C). GDI-rab4 complexes were then purified by gel filtration using a Sephacryl S100 column. For production of GDI complexes containing phosphorylated rab4, the complete phosphorylation reaction mix containing rab4 was added to purified GDI. The complex was then isolated as above. By SDS-PAGE and autoradiography, only the rab4 was phosphorylated under these conditions. The extent of rab4 phosphorylation was determined relative to a minus rab4 (cdc2 kinase only) control as well as by immunoprecipitation using the anti-GST-rab4 antibody.

Protease treatment of membranes

CHO low density membranes (0.86/1.1 M interface) were incubated with a variety of proteases for 20 min at 37°C. Excess amounts of the appropriate protease inhibitors (trypsin inhibitor, bromelain inhibitor and elastatinal; Sigma) were added and the reaction incubated at 0°C for 10 min. Protease-treated membranes were then isolated by ultracentrifugation (100 000 g, 15 min, 4°C) and tested for rab4 binding activity using rab4-GDI complexes (see below). Elastase treatment of bovine liver low density membranes was performed in an identical fashion to protease treatment of CHO low density membranes.

rab4 membrane binding assay

Membrane binding assays were performed by a modification of the method developed for rab9 (Soldati *et al.*, 1994). Optimized conditions included incubations of membranes and purified rab4-GDI complexes at 37°C for 40 min in the presence of an ATP regenerating system (250 µl reaction volume; 100 µl for assays involving [^{32}P]rab4). After incubation, 500 µl of ice-cold buffer A was added to the reaction and the total volume (750 µl) placed in an ultracentrifuge tube. Membrane-bound rab4 and GDI were separated from unbound GDI and rab4 by ultracentrifugation (10 min, 98 000 r.p.m., TLA100.2 rotor) and the membrane pellets washed with 500 µl buffer A to remove any loosely associated rab4 or GDI. Membrane pellets were resuspended in Laemmli sample buffer and the amount of membrane-associated rab4 or GDI determined by quantitative Western blot using monospecific rabbit polyclonal antibodies raised against *E.coli*-produced recombinant GDI-1 or a GST-human rab4 fusion protein. The amount of phospho-rab4 associated with membranes was determined by performing SDS-PAGE followed by PhosphorImager analysis, as above. Phospho-rab4 membrane binding reactions typically contained 7000 c.p.m. of phospho-rab4 (25 nM, 0.15 µg) isolated as a GDI-rab4 complex.

rab4 guanine nucleotide exchange assay

Standard GTP exchange assays consisted of purified recombinant rab4 (0–2.5 µM) purified from baculovirus infected *Sf9* cell membranes, 2 µM GTPγS, 0.5 µCi [^{35}S]GTPγS (Amersham), ATP regenerating system and 3 µg of endosome-enriched membranes pre-treated with 1 M KCl, or endosome-enriched membranes pre-treated with 1 mg/ml elastase; 0.4 µM (1 µg) rab4 was used in the standard assay. A no-membrane control (incubation of recombinant rab4 in the absence of membranes) was used in each assay to determine the extent of intrinsic, non-enzymatic rab4 exchange; the amount of intrinsic [^{35}S]GTPγS binding to each membrane preparation was determined in the absence of added rab4. Reactions (0.5 µCi [^{35}S]GTPγS, 0.25% CHAPS, 1 mM elastatinal, 100 mM $(\text{NH}_4)_2\text{SO}_4$, 0.2 mM DTT, 25 mM HEPES-KOH pH 7.2, 115 mM KCl, 3 µg membrane protein, recombinant rab4; 100 µl total volume) were shifted from 0°C to 37°C for 0, 10, 30 or 60 min. The reactions were stopped by the addition of ice-cold buffer A and the protein-bound radioactivity determined by a filter binding assay (Soldati *et al.*, 1995b). Filters were dried under an infrared lamp and counted using a scintillation counter.

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