

Phosphorylation of GDI and membrane cycling of rab proteins

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Membrane transport is known to be regulated by protein phosphorylation and by small GTPases of the rab family. Using specific antibodies, we have identified a 55 kDa phosphorylated protein which co-immunoprecipitated with the cytosolic forms of rab5 and other rab proteins. We demonstrate, on the basis of its mobility in two-dimensional electrophoresis gels and its immunological properties, that this protein is rab GDI (p55/GDI). We also found that, a minor fraction of p55/GDI is membrane associated, but, whilst also complexed with rab proteins, it is not phosphorylated. On the basis of these data we suggest that the cycling of rab proteins between membranes and cytosol is regulated by phosphorylation of p55/GDI.

Small GTP-binding protein; Rab5; Rab GDI; Membrane traffic

1. INTRODUCTION

Intracellular membrane traffic events are known to be regulated by small GTPases of the SEC4/YPT1/rab family and also by protein phosphorylation [1]. In this study we have investigated whether there is a point of convergence between these two regulatory mechanisms, by looking for interactions between phosphorylated proteins and rab proteins.

More specifically we have studied the small GTP-binding protein rab5, which localizes to early endosomes and the plasma membrane [2], and has been shown to regulate both fusion between early endosomes in vitro [3] and early endocytic events in vivo [4]. Since early endosome fusion is also regulated by phosphorylation/dephosphorylation events, in both mitosis and interphase [5–7], we investigated the association of phosphorylated proteins with the rab5 protein. For these experiments we have used antibodies raised against the hypervariable carboxyl-terminal domain of the rab5 protein; antibodies raised against this domain are highly specific and have been extensively used in localisation studies [2,8,9].

By analogy with the related p21 ras proto-oncogene and by fluorescence spectroscopic measurements (M.J. Clague, unpublished observations), rab proteins undergo a conformational change upon hydrolysis of GTP. This molecular switching mechanism can be used to impart directionality, and specificity, to the process that they control [10]. Small GTP-binding proteins are

regulated by interactions with other proteins which control the rate of this switching; proteins which inhibit GDP dissociation (GDI), facilitate nucleotide exchange (exchange factor), or enhance GTPase activity (GAP) of rab proteins have all been identified [11–14].

In this study we show that rab5, and other rab proteins, are associated with GDI both in cytosol and on membranes, and that in vivo the cytosolic GDI protein is phosphorylated when complexed to rabs. Previously GDI has been shown to associate with an array of rab proteins and to promote their dissociation from membranes [15–17]. However, it was not clear how this association could be regulated. Our results, together with an analysis of the membrane/cytosol distribution of the different forms, suggest that the cycling of rab proteins on and off membranes is regulated by GDI. We propose a simple model incorporating a universal role for GDI in specifying the directionality of this cycle.

2. MATERIALS AND METHODS

2.1. Cells and reagents

BHK cells were maintained and seeded for experiments as described previously [18]. The polyclonal antibody against rab5 was raised against the C-terminal peptide according to the method described by Louvard et al. [19]. The polyclonal antibodies raised against C-terminal peptides of GDI and rab2 were kind gifts from Oliver Ullrich and Marino Zerial (EMBL, Heidelberg). The affinity purified antibodies against the C-terminal of rab4 were a kind gift from Peter van der Sluijs and Ira Mellman (Yale University School of Medicine, New Haven). The rab5 C-terminal (PKNEPQNPGANSARGR, [2]) and N-terminal (MANRGATRPNGPNTGNK) peptides were synthesized by Dominique Nalis (EMBL, Heidelberg). Guanosine [α - 32 P]triphosphate and [32 P]orthophosphate were obtained from Amersham-Buchler, Germany. [35 S]methionine/cysteine (EXPRES 35 S) was obtained from DuPont/NEN Research Products, Boston, MA. *N*-Octylpolyoxyethylene (Octyl-POE/Rosenbusch-Tenside) was obtained from Bachem-Biochemica GmbH, Heidelberg.

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Abbreviations: GDI, guanine nucleotide dissociation inhibitor; PNS, post-nuclear supernatant.

2.2. Metabolic labeling

For metabolic labeling of phosphorylated proteins, 90% confluent cells (16 h after seeding) were taken and incubated for 4–6 h with 0.5 mCi/ml of [32 P]orthophosphate in phosphate-free Dulbeccos modified Eagles medium (DME), supplemented with 1% fetal calf serum (Gibco BRL, Germany) which had been dialyzed for 15 h against Tris-buffered saline (TBS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4). For [35 S]methionine/cysteine labeling, 40% confluent cells (4 h after seeding) were taken and incubated for 16 h in methionine/cysteine free DME supplemented with 1% fetal calf serum and 0.2 mCi/ml EXPRE 35 S.

2.3. Immunoprecipitation

Post-nuclear supernatant (PNS) was prepared from BHK cells as described previously [18], except that TBS was used in place of phosphate-buffered saline. Membrane and cytosol fractions were prepared by centrifuging PNS at 150,000 $\times$ g for 15 min in a Beckman TL100 ultracentrifuge, and taking the pellet or supernatant, respectively. Membrane pellets were resuspended in homogenization buffer (HB; 3 mM imidazole, 250 mM sucrose, pH 7.4) before solubilisation, so that the protein concentration was equal to that of the cytosol. Samples were then mixed with a 4-fold excess of Rosenbusch-Tenside buffer (RTB; 12.5 mM HEPES, 1.0 mM DTT, 0.5 mM EGTA, 100 mM KOAc, 1 mM MgCl $_2$, 1% *n*-octylpolyoxyethylene, pH 7.4) for 30 min at 4°C. Insoluble material was removed by centrifuging at 150,000 $\times$ g. The supernatant (250 mg) was mixed with antiserum (3 ml) for 1 h at 4°C. In peptide competition experiments, peptide (720 mg) was mixed with antiserum for 30 min at 4°C and the mixture then added to the supernatant. Protein A-Sepharose beads (5 ml/ml of antiserum), which were preblocked with cold solubilized PNS when immunoprecipitating metabolically labeled proteins, were added and mixed for 30 min at 4°C. The immune complexes were washed three times with RTB and three times with TBS. Finally the immunoprecipitated proteins were solubilized in sample buffer (2% SDS, 100 mM

DTT, 60 mM Tris, pH 6.8, 0.001% Bromophenol blue, 15% glycerol) and SDS-PAGE carried out.

For immunoprecipitation of phosphorylated proteins all buffers were supplemented with 0.1 mM VO $_3^-$ and 10 mM KF, and the final concentration of MgCl $_2$ was brought to 10 mM immediately after homogenization. Solubilized samples (60 mg protein) were added to antibodies (2 ml antiserum), which had been pre-coupled to Protein A-Sepharose beads (10 ml), and mixed at 4°C for one hour. When immunoprecipitating phosphorylated proteins from PNS a 10:1 volume ratio of immunoprecipitation buffer (IB; 50 mM Tris, 150 mM KCl, 1% NP40, 10 mM MgCl $_2$, 0.1 mM VO $_3^-$, 10 mM KF, pH 7.4) was added directly to the PNS and mixed for 30 min at 4°C. IB was then also used, instead of RTB, for washing immunoprecipitates. Insoluble material was removed from 32 P-labeled samples by centrifuging at high speed in a microfuge for 2 min.

2.4. Electrophoresis and [32 P]GTP-overlay

SDS-PAGE was carried out according to the system of Laemmli [20], using 12.5% gels. Labeled proteins were visualised by fluorography; gels with 35 S-labeled proteins were treated with Enhance (NEN) and intensifying screens were used for 32 P-labeled proteins. Gels were exposed for 24 h to 1 week. For two-dimensional gel electrophoresis, samples were run on IEF tube gels, with a linear pH gradient between pH 4.5 and 7.4, and then on 15% acrylamide second dimension resolving gels as previously described [21].

GTP-overlay was as previously described [4]. Briefly, after SDS-PAGE, the gels were equilibrated in 20% glycerol, 50 mM Tris-HCl, pH 7.5 and the proteins then transferred to nitrocellulose at 50 mA for 16 h (Transfer buffer; 3 mM Na $_2$ CO $_3$, 10 mM NaHCO $_3$, pH 9.9). The nitrocellulose was then rinsed in binding buffer (BB; 50 mM NaH $_2$ PO $_4$, 10 mM MgCl $_2$, 2 mM DTT, 0.3% Tween 20, 4 mM ATP, pH 7.5) and incubated for 2 h with [α - 32 P]GTP (2 mCi/ml) in BB. The nitrocellulose was then washed extensively with BB, dried and exposed to film with an intensifying screen.

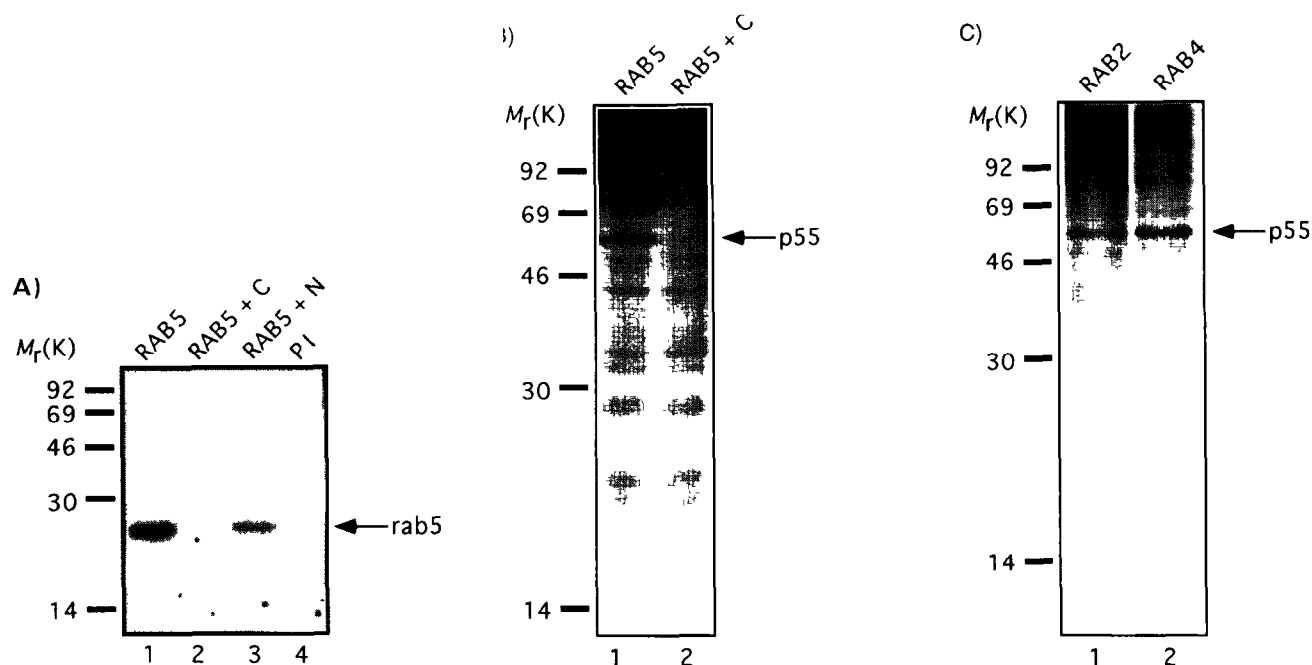


Fig. 1. Rab proteins are complexed with a 55 kDa phosphorylated protein. (A) The Rab5 C-terminal antiserum is specific, as shown by [32 P]GTP overlay after immunoprecipitation from membranes. Lane 1, rab5 antiserum; lane 2, rab5 antiserum with C-terminal rab5 peptide; lane 3, rab5 antiserum with N-terminal rab5 peptide; lane 4, preimmune sera. (B) and (C) Immunoprecipitation from post-nuclear supernatants (PNS), prepared from [32 P]orthophosphate labeled cells, with anti-rab antibodies. (B) lane 1, rab5 antiserum; lane 2, rab5 antiserum with C-terminal rab5 peptide. (C) lane 1, rab2 antiserum; lane 2, affinity purified rab4 antibodies. Molecular weight markers are indicated.

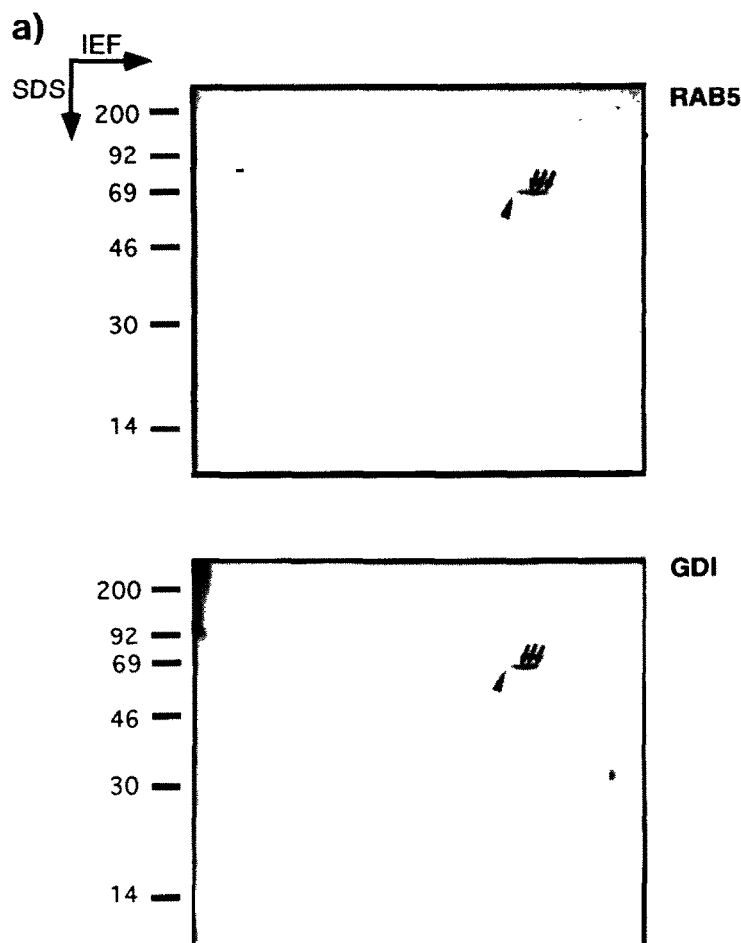


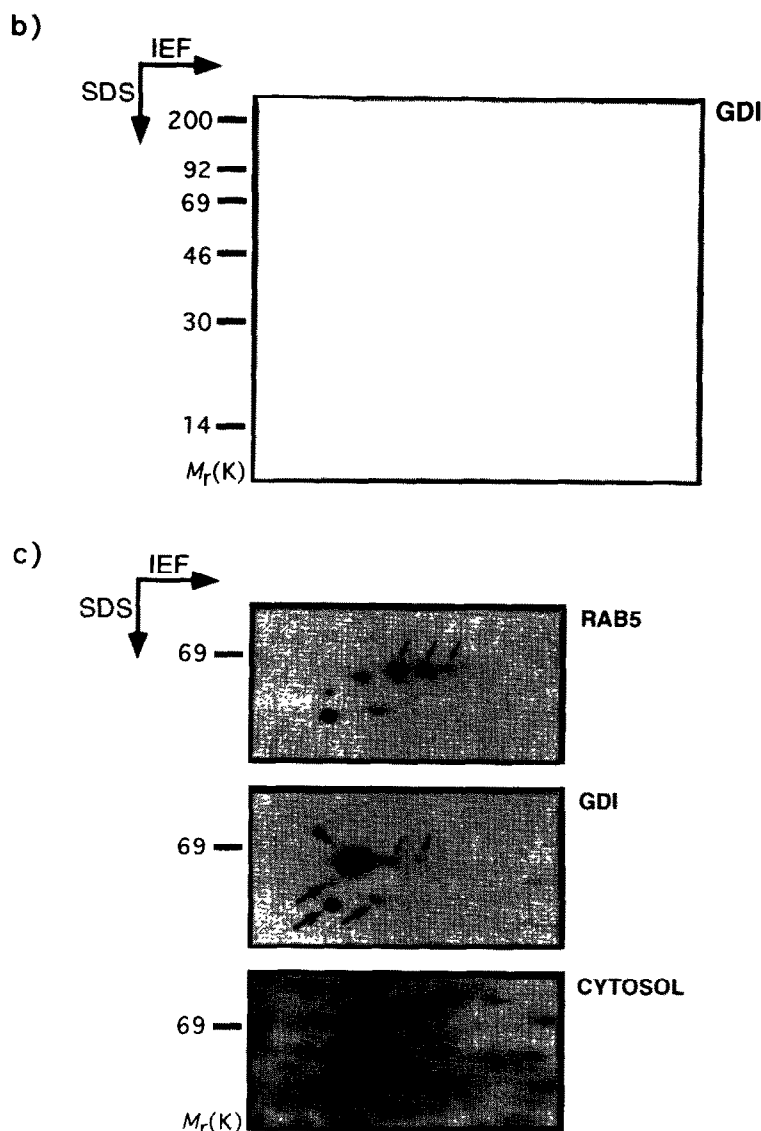
Fig. 2. 2D gel analysis of immunoprecipitates from cytosol of [32 P]orthophosphate (a) and [35 S]methionine (b and c) labeled BHK cells. (a) Immunoprecipitation with antibodies against rab5 (upper panel) or GDI (lower panel). (b) Immunoprecipitation with antibodies against GDI. (c) Sections of 2D gels, showing the area where GDI and its isoforms migrate, obtained after immunoprecipitation with antibodies against rab5 (upper panel) or after longer exposure of the gel shown in (b, middle panel). The lower panel shows the pattern of total cytosolic proteins seen in the same area of our 2D gels. The major isoform recognized by anti-GDI antibodies is indicated with an arrowhead and the other isoforms indicated with small arrows. The large arrows indicate major contaminants seen in all of the gels. The apparent molecular weight of p55/GDI is higher on our 2D gels (approx. 69 kDa) than on our 1D gels (approx. 55 kDa), we refer to it throughout this paper by its later apparent molecular weight.

3. RESULTS AND DISCUSSION

Fig. 1A shows that rab5 is specifically immunoprecipitated from BHK extracts by our C-terminal antiserum, and that immune complex formation is competed by the peptide against which the serum was raised. We then show that, when the same antiserum was used to immunoprecipitate from PNS prepared from 32 P-labelled cells, a phosphorylated protein of 55 kDa (p55) was specifically co-immunoprecipitated, although rab5 itself was not detectably phosphorylated (Fig. 1B). By virtue of its mobility in 1D gels, we suspected that p55 may be GDI. Since it has been proposed that essentially all cytosolic rab proteins are complexed to this protein [15,17], we tested antibodies specific for other rab proteins. Antibodies against rab2 and rab4 both immunoprecipitated phosphorylated p55 (Fig. 1B). Al-

though rab4 co-localises with rab5 on endosomes [8], it is significant that rab2 specifically localises to organelles of the early biosynthetic pathway [2,22].

Next we used high resolution 2D gel electrophoresis to compare the physical characteristics of p55 with those of GDI. Cytosol was prepared from 32 P-labeled cells and immunoprecipitations carried out using either the anti-rab5 antibodies or an antibody raised against a peptide from the C-terminal region of GDI. Autoradiographs of the two immunoprecipitates were then compared (Fig. 2a). The isoelectric focusing of p55 indicates the existence of several phosphorylation states (indicated by arrowheads in Fig. 2a and 2b), and, significantly, the anti-GDI antibody recognizes a protein with the same physical characteristics. Phosphoamino acid analysis, after acid hydrolysis of p55, showed that serine residues were phosphorylated (not shown).



In order to investigate whether non-phosphorylated p55/GDI is also complexed with rab5 in cytosol, we repeated the immunoprecipitation experiments using cytosol prepared from cells metabolically-labeled with [35 S]methionine/cysteine. After immunoprecipitation with the anti-GDI antibody, a single polypeptide was revealed in 2D gels, which must be an unphosphorylated form of GDI, as its mobility is slightly more alkaline than any of the phosphorylated forms immunoprecipitated by the same antibody (compare with Fig. 2a). Upon longer exposure of this gel the phosphorylated forms of GDI become evident (Fig. 2c, middle panel). In contrast the anti-rab5 and anti-rab4 antibodies almost exclusively co-immunoprecipitated these phosphorylated forms of GDI (shown for rab5, Fig. 2c, upper panel). When we treated the 35 S-labelled anti-rab5 immunoprecipitate with alkaline phosphatase

prior to electrophoresis, these more acidic forms were shifted back to the position of unphosphorylated GDI (not shown). These experiments establish that cytosolic rab5, and other rab proteins, predominantly interact with the phosphorylated forms of cytosolic GDI.

One surprising result of the above experiments was the observation that phosphorylated forms of p55/GDI were more effectively precipitated by anti-rab protein antibodies than by the anti-GDI antibody. In fact two-dimensional gel analysis of the complete cytosol (Fig. 2c, bottom panel), shows that the phosphorylated forms of GDI are much more abundant than the unphosphorylated form. We presume that the GDI antibody does not efficiently immunoprecipitate the phosphorylated GDI because the C-terminal epitope recognized by the antibody is masked by phosphorylation or by association with rab proteins.

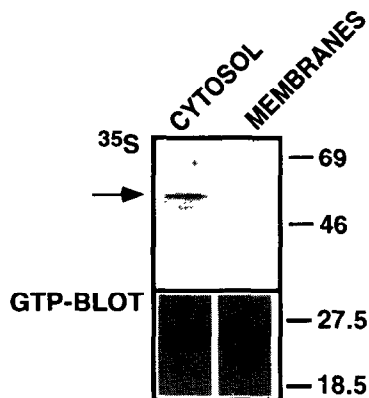


Fig. 3. GDI is associated with GTP-binding proteins in cytosol and on membranes. (a) Immunoprecipitation of ³⁵S-labeled proteins from cytosol or membranes by GDI antibodies. The position of GDI is indicated. (b) Visualization of small GTP-binding proteins by [³²P]GTP overlay following immunoprecipitation with GDI antibodies from membranes and cytosol of unlabeled cells. Only the 18–30 kDa range is shown.

When we repeated these immunoprecipitation experiments using membrane fractions in place of cytosol, we were unable to detect any phosphorylated p55/GDI (data not shown), although $\approx 10\%$ of the total immunoprecipitable [³⁵S]methionine/cysteine-labeled GDI is membrane associated (Fig. 3). In contrast, approximately 80% of the rab5 protein is membrane associated [3]. We found that a significant fraction of the membrane associated form of GDI is complexed to rab proteins, as evidenced by [³²P]GTP overlay of small GTP-binding proteins after co-immunoprecipitation with anti-GDI antibodies (Fig. 3). Altogether, our data confirm that the cytosol contains a small proportion of rab proteins but the bulk of p55/GDI [3,17], and shows that these cytosolic rab proteins interact with a phosphorylated form of GDI. In contrast the fraction of membrane associated rab proteins which are complexed with p55/GDI interact with its unphosphorylated form (Fig. 4a).

Since its discovery it has been apparent that GDI function must be regulated. The involvement of post-translational modifications has been postulated, but remained obscure [16,23]. Phosphorylation/dephosphorylation as reported here represents a strong candidate for fulfilling this role and, in Fig. 4, we depict a simple scheme by which this mechanism may regulate the association of rab proteins with membranes. We propose that the specific association of rab proteins with membranes is initiated by interaction of the cytosolic, phosphorylated GDI–rab complex with a membrane associated protein, presumably a nucleotide exchange factor. Exchange of GDP for GTP would promote dissociation of the GDI–rab complex, due to the vastly reduced affinity of GDI for the GTP bound form of the rab protein [24], thus the lipoyl moiety of the rab

protein would be freed to insert into the adjacent membrane. In this way the rab protein would be delivered to the membrane in its active conformation. Following GTP hydrolysis, the GDP-bound rab protein, by associating with free GDI, would be extracted from the membrane. Phosphorylation of GDI would prevent reinsertion of the rab protein into the membrane, except by interaction with the specific exchange factor.

This model is consistent with properties of the unphosphorylated form of GDI reported by other groups; namely, inhibition of GDP dissociation and promotion of rab dissociation from the membrane [17,25]. Our model proposes that rab proteins are presented to the appropriate membrane in a complex with GDI, this makes comprehensible a study by Araki et al. [24], who have shown that purified rab3a will non-specifically associate with membranes (e.g. mitochondria, erythrocytes). A cycle of membrane association and dissociation is implicit in rab protein involvement in vesicular membrane transport [10], and we predict that GDI will not be associated with membranes of vesicular intermediates of membrane transport, but will be found on both donor and acceptor organelles. Although we have not so far localised the kinase and the phosphatase activities, we have supposed them to be cytosolic; in this configuration the homeostatic potential of GDI is most

a)

	CYTOSOL	MEMBRANES
GDI	80-90% RAB-GDP[P] > GDI	10-20% RAB-GDI > (GDI)
RAB 5	10-20% RAB5-GDP[P] > RAB5	80-90% RAB5 > RAB5-GDI

b)

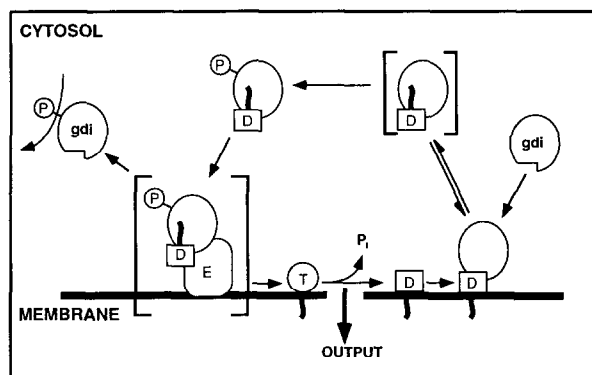


Fig. 4. (a) Summary of the distribution of GDI and rab5 complexes in membranes and cytosol (see text for refs.). (b) Model for the regulation of rab protein association with membranes (see text for explanation). Complexes of GDI with rab protein which have not been detected, and correspond to the putative intermediates, are shown in parenthesis. D, GDP-bound rab protein; E, exchange protein; GDI, guanine nucleotide dissociation inhibitor; P, phosphate; P_i, inorganic phosphate; T, GTP-bound rab protein.

simply realised. In conclusion, our studies suggest that GDI serves as universal regulator of membrane transport, perhaps controlling both the specificity and the directionality of the rab protein cycling pathway.

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