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Interaction of Sec4 with GDI proteins from bovine brain, *Drosophila melanogaster* and *Saccharomyces cerevisiae*

Conservation of GDI membrane dissociation activity

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Rab GDP dissociation inhibitor (Rab GDI), will induce the dissociation of GDP-bound rab3A from synaptic membranes and will inhibit GDP dissociation from Sec4, a member of the Rab subgroup of the Ras GTPase superfamily which is required for exocytosis in *Saccharomyces cerevisiae*. We report that Rab GDI releases GDP-bound Sec4 from yeast membranes. dGDI, a *Drosophila* homologue can similarly inhibit GDP dissociation from Sec4 and release GDP-bound Sec4 from yeast membranes. An activity partially purified from yeast cytosol dissociates GDP-bound Sec4 from yeast membranes, suggesting that yeast also possess a GDI protein that functions to recycle Sec4 from its target membrane.

Sec4; GDP dissociation inhibitor (GDI); Secretion; GTP-binding protein; Exchange protein; Yeast

1. INTRODUCTION

Sec4 is a 23.5 kDa GTP binding protein required for the transport of secretory proteins from the Golgi to the cell surface in the yeast *Saccharomyces cerevisiae* [1,2,3]. It is a member of the rab subgroup of the ras superfamily of small GTP binding proteins, comprising amongst others Ypt1 and Ypt7 which have both been implicated at distinct stages of vesicle transport in *S. cerevisiae*, and the rab proteins which participate in specific stages of endocytosis and exocytosis in mammalian cells [3–16]. Members of the ras superfamily of small GTP binding proteins cycle between two forms, the GTP bound form and the GDP bound form [17,18]. This cycle is mediated by GTP hydrolysis (GTPase activity) and the exchange of GDP for GTP onto the protein. Additionally, many small GTP binding proteins are either fully membrane associated or distributed between membrane and cytosolic pools. Membrane association of members of the rab subfamily is dependent on the covalent addition of one or two geranylgeranyl (prenyl) groups. These are attached via thioether linkages to cysteine residues at the C-terminus of the protein (for review see [19]).

For the rab proteins it has been put forward that the cycle of GTP binding and hydrolysis is coupled to a cycle of subcellular localization [20,21,22]. Consistent with the proposal, Sec4 is made as a soluble protein in yeast which rapidly associates with secretory vesicles that are then transported to the plasma membrane [3,21]. Sec4 can then recycle back through a soluble pool onto newly formed vesicles [3,21]. Furthermore, it has been shown that hydrolysis of GTP by Sec4 plays an important role in vesicular transport in *S. cerevisiae* [22], and that membrane localization of Sec4 is essential for its function [21]. An analogous cycle of subcellular localization has been shown for the small GTP binding protein rab3A [23].

Since the initial discovery that a cytoplasmic protein (GAP) could stimulate the rate of GTP hydrolysis on the mammalian ras proteins [24], the role of regulatory factors in modulating the biological activity of members of the ras superfamily has rapidly come to the fore. Indeed, two regulatory proteins have recently been reported for Sec4 [22,25]. The first was a GTPase activating protein (Sec4-GAP) which specifically stimulates the rate of GTP hydrolysis on Sec4 [22]. The second, Dss4, was identified through a genetic screen for dominant suppressors of *sec4–8* and has been shown to act as an exchange factor for Sec4 [25]. In this paper we describe the interaction of Sec4 with a third type of regulatory

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Abbreviations: Sec4, the protein product of the *SEC4* gene; KP_i, potassium phosphate; DTT, dithiothreitol; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; GTPγS, guanosine 5′-*O*-thiotriphosphate.

factor known as GDI. The first GDI protein was isolated by its ability to inhibit the rate of GDP dissociation from rab3A, hence the name rab3A GDI (rab3A *G*DP *D*issociation *I*nhibitor [26]). Rab3A GDI will also stimulate the release of prenylated rab3A in the GDP-bound form from membranes, thus linking the cycle of rab3A membrane localization to the cycle of guanine nucleotide binding and hydrolysis [27]. By forming a stable soluble complex with prenylated, GDP-bound rab3A, rab3A GDI will prevent attachment of rab3A to membranes [27]. Rab3A GDI can also slow the dissociation of GDP from Sec4 [28] and rab11 [29] and will regulate the membrane association of numerous rab proteins [30,31]. Because rab3A GDI has now been shown to be active on many members of the rab family, the protein has been renamed rab GDI [31]. Interestingly, a second GDI protein has been isolated which is closely related in sequence to rab GDI. This protein will inhibit dissociation of GDP from rab11 but not from rab3A and has been given the name rab11 GDI [29]. Neither rab GDI nor rab11 GDI will interact with members of the ras or rho subgroups of the ras superfamily, suggesting that there maybe a family of sequence related GDI proteins specific for the rab proteins [27,29]. Taken together, these observations suggest that rab GDI proteins may play an important role in the recycling of rab proteins from their target membranes through a soluble pool onto newly synthesized transport vesicles. A similar mechanism may also regulate members of the rho subgroup of the ras superfamily since a GDI protein for rho has been cloned and sequenced [32]. This protein appears to be distinct from the rab GDI proteins since it has a molecular mass of 27 kDa compared to around 54 kDa for the rab GDI proteins and shares no homology with the complete rab GDI sequence or with the partial sequence known for rab11 GDI at the amino acid level.

Recently a homologue of rab GDI known as dGDI has been cloned and sequenced from *Drosophila melanogaster* [33]. dGDI shares 68% identity and 81% homology with rab GDI which suggests that rab GDI proteins may be conserved throughout evolution. In this paper we report that rab GDI and dGDI will both cause the dissociation of Sec4 from membranes in a cell free assay and that dGDI will inhibit the dissociation of GDP from Sec4. We also describe here a GDI activity for Sec4 that we have partially purified from the cytosol of *S. cerevisiae*

2. EXPERIMENTAL

2.1. *Materials and chemicals*

Sec4 protein was purified from an overproducing yeast strain [34]. Rab3A GDI and DGDI were purified from bovine brain cytosol and *Drosophila* embryos, respectively [26,33].

2.2. *Preparation of membranes from yeast*

NY13 (*MATa, ura3–52*) cells were grown overnight at 25°C with aeration in YP medium (1% Bacto yeast extract and 2% Bacto-Peptone; Difco Laboratories) supplemented with 2% glucose. One thousand OD_{599} units of cells were pelleted at room temperature, washed with ice-cold 10 mM NaN_3 and resuspended in spheroplast medium (1.4 M sorbitol, 50 mM KP_i (pH 7.5), 10 mM NaN_3, 27.5 mM β-mercapthoethanol and 10 mg of zymolyase-100T (ICN Biomedicals Inc.)) to a final volume of 30 ml and incubated at 37°C for 45 min. The resulting spheroplasts were pelleted by centrifugation at 3,000 × *g* for 5 min at 4°C, resuspended in 20 ml of ice-cold lysis buffer ([0.8 M sorbitol, 10 mM triethanolamine, 1 mM EDTA] pH 7.2, containing 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of leupeptin, pepstatin, chymostatin aprotinin and antipain [this protease inhibtor cocktail is known as 1 × PIC]), dounced 20 times with a Wheaton 40 ml tissue grinder and centrifuged at 450 × *g* for 3 min at 4°C and the supernatant collected. The pellet was resuspended in 10 ml of ice-cold lysis buffer, dounced 20 times, centrifuged at 450 × *g* for 3 min at 4°C, the supernatant removed and pooled with the first supernatant. The pooled supernatants (S1 fraction) were then centrifuged at 10,000 × *g* for 10 min in a J20 fixed angle rotor (Beckman) at 4°C. The supernatant was removed, the pellets were washed with buffer A (0.8 M sorbitol, 25 mM HEPES, pH 7.6, 5 mM $MgCl_2$, 1 mM DTT, 1 × PIC) and resuspended in a total of 5 ml of buffer A using a 7 ml Wheaton tissue grinder. The resultant membrane preparation was rapidly frozen in liquid nitrogen and stored at −80°C.

2.3. *Sec4 membrane dissociation assay*

For each individual assay 100 μg of the yeast membrane preparation was incubated in buffer A in the presence or absence of the sample to be tested, in a total volume of 150 μl for 30 min at 30°C. Following this incubation, each sample was layered onto a 200 μl sorbitol barrier (1.5 M sorbitol, 25 mM HEPES, pH 7.6, 5 mM $MgCl_2$, 1 mM DTT, 1 × PIC) in a new 1.5 ml microcentrifuge tube and centrifuged for 20 min at top-speed in an Eppendorf Micro Centrifuge at 4°C. This allowed pelleting of the membranes, while soluble material remained above the sorbitol barrier. 120 μl of supernatant was removed, heated in sample buffer containing 2% SDS and 1% β-mercaptoethanol for 5 min at 100°C and stored at −20°C until analysed. The sorbitol barrier and remaining supernatant were removed from the pellet fraction, which was then resuspended in buffer A to a final volume of 150 μl (the original assay volume). The resuspended pellet was then heated in sample buffer and stored as described for the supernatant fraction. Equal volumes (60 μl) of supernatant and pellet samples were analysed for Sec4 content by SDS-PAGE on 14% gels according to Laemlli [35] in parallel with molecular weight markers (prestained low molecular weight, Gibco BRL). Proteins were electrophoretically transferred to nitrocellulose, probed with rabbit anti-Sec4 antiserum (1:1000) and radioiodinated staphylococcal protein A (Amersham) and the amount of Sec4 on the nitrocellulose quantitated according to Goud et al. [3]. To study the effect of exchanging GDP or GTPγS onto the membrane-bound Sec4 protein in the presence or absence of GDI protein, yeast membranes were preincubated in buffer A containing 7 mM EDTA and either 0.4 mM GDP or 0.4 mM GTPγS for 4 min at 30°C. This was followed by the addition of 7 mM $MgCl_2$ and a further incubation of 15 min at 30°C. The Sec4 membrane dissociation assay was then performed as described above, but the total assay volume was 175 μl.

2.4. *GDP-off rate assay*

Measurement of the Sec4 GDP-off rate in the presence and absence of GDI was done by a modification of the GDP-off rate assay described by Kabcenell et al. [33]. Sec4 protein (133 nM) was incubated for 60 min at 30°C in the presence of 2 mM [8,5′-^{3}H]GDP (25–50 Ci/mmol, Dupont-New England Nuclear, specific activity 10,000 cpm/pmol) in buffer B (50 mM HEPES, pH 8.0, 200 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.1% Lubrol). The [8,5′-^{3}H]GDP loaded Sec4 protein was placed on ice and diluted two-fold with buffer B containing 100 μM GDP alone or along with the sample to be tested for its effect on the Sec4 GDP-off rate, and incubated at 30°C. Aliquots were removed over time and filtered through 25 mm Type HA filters (Millipore) which were then washed, dried and subjected to

scintillation counting. The computer program 'Enzfitter' was then used to fit the data to an exponential decay equation, utilizing non-linear regression analysis [36].

2.5. *Preparation of 100,000 × g supernatant from sec4–8 cells*

The yeast strain NY405 (*MATa, ura3–52, sec4–8*) was grown overnight at 25°C with aeration in YP medium supplemented with 2% glucose. One thousand OD_{599} units of cells were washed, spheroplasted, lysed and S1 fraction prepared as described earlier for the preparation of yeast membranes. The S1 fraction was subjected to centrifugation at 100,000 × *g* for 70 min at 4°C using a Beckman Type 50 Ti rotor. The top layer of lipid was removed and the remaining supernatant was rapidly frozen in liquid nitrogen and stored at −80°C until use.

2.6. *Miscellaneous procedures*

Protein content was analysed according to Bradford [37] using bovine immunoglobulin (Sigma) as the standard.

3. RESULTS

3.1. *Nucleotide dependent dissociation of Sec4 from yeast membranes by Rab3A GDI*

When rab GDI was incubated with yeast membranes for 30 min at 30°C, most Sec4 dissociated from the pellet and entered the supernatant fraction (Fig. 1). Subsequently we found that if the membranes were preincubated with GDP to allow its exchange onto the membrane-bound Sec4, rab GDI dependent dissociation of Sec4 from membranes was again nearly complete (Fig. 1). However, if the membranes were preincubated in the presence of GTPγS to allow exchange of this non-hydrolyzable analogue of GTP onto Sec4, rab GDI dependent dissociation of Sec4 from the pellet into the supernatant was inhibited (Fig. 1). The fact that the inhibition was not total may be due to incomplete exchange of GTPγS onto membrane-bound Sec4. These results suggest that rab GDI causes dissociation of GDP-bound, but not GTPγS-bound Sec4 from yeast membranes.

3.2. *Nucleotide dependent dissociation of Sec4 from yeast membranes by dGDI*

Since rab GDI and dGDI share 81% sequence homology [33], we examined whether dGDI could also solublize membrane-bound Sec4. When yeast membranes were preincubated in the presence of GDP, followed by addition of dGDI for 30 min at 30°C, 80% of Sec4 dissociated from the membranes and moved into the supernatant fraction (Fig. 2A). However, following a preincubation of yeast membranes in the presence of GTPγS, addition of dGDI released only 15% of Sec4 into the supernatant fraction (Fig. 2A). These results indicate that, like rab GDI, dGDI can cause the dissociation of GDP-bound but not GTPγS-bound Sec4 from yeast membranes.

3.3. *dGDI inhibits the dissociation of GDP from Sec4*

We next determined if dGDI could inhibit the dissociation of GDP from Sec4. Using the GDP-off rate assay described in section 2, we measured the intrinsic rate of GDP dissociation from Sec4 as being 0.24 min^{-1} at 30°C (Fig. 2B) which was similar to the value previously reported by Kabcenell et al. [34]. In the presence of 0.58 mM dGDI the rate of GDP dissociation from Sec4 was inhibited approximately 10-fold (Fig. 2B). In addition, we found that the inhibition of GDP dissociation from Sec4 by dGDI was dose dependent when analyzed using 0.16 mM to 0.66 mM dGDI protein (data not shown).

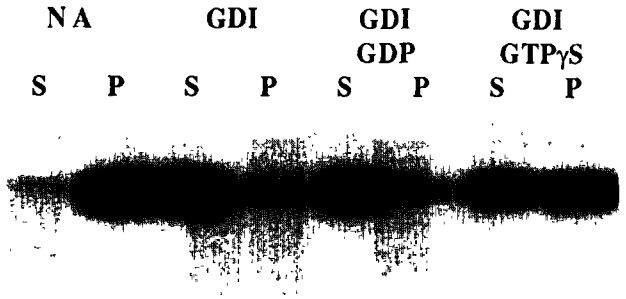

Fig. 1. Effect of rab GDI on membrane-associated Sec4. Yeast membranes were incubated in the presence or absence of 0.45 μM rab GDI at 30°C for 30 min. The membranes were then pelleted by centrifugation and the supernatant (S) and pellet (P) fractions analyzed for their Sec4 content by SDS-PAGE and immunoblotting using rabbit anti-Sec4 antiserum (1:1000). NA, no preincubation of yeast membranes and no addition of rab GDI; GDI, no preincubation of yeast membranes, addition of rab GDI; GDI GDP, preincubation of yeast membranes with 0.4 mM GDP followed by addition of rab GDI; GDI GTPγS, preincubation of yeast membranes with 0.4 mM GTPγS followed by addition of rab GDI.

3.4. *Identification and characterization of a GDI membrane dissociation activity for Sec4 from the yeast* Saccharomyces cerevisiae

Since rab GDI and dGDI can both interact with Sec4, we examined whether the yeast *Saccharomyces cerevisiae* contained a cognate GDI activity. As a potential source of this activity we used cytosol prepared from *sec4–8* cells (strain NY405) since by SDS-PAGE the Sec4–8 protein runs at a higher molecular weight and is expressed at a lower level than wild-type Sec4. Thus, by Western blot it is possible to distinguish between the Sec4–8 protein already present in a 100,000 × *g* supernatant and the wild-type Sec4 released from the membrane preparation in the Sec4 membrane dissociation assay. We found that a 100,000 × *g* supernatant prepared from *sec4–8* cells did contain an activity which caused dissociation of Sec4 from yeast membranes. We then subjected this 100,000 × *g* supernatant (1.7 ml, 9.44 mg) to Sephacryl S-100 gel-filtration chromatography (80 ml; Pharmacia) in buffer C (20 mM HEPES, pH 7.6, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT). 2.2 ml fractions were collected and tested using the Sec4 membrane dissociation assay as described in the legend to Fig. 3A. Two peaks of activity were detected (Fig. 3A), the minor peak A which eluted at the void volume of the column (fractions 26 and 27) and the more prominent peak B

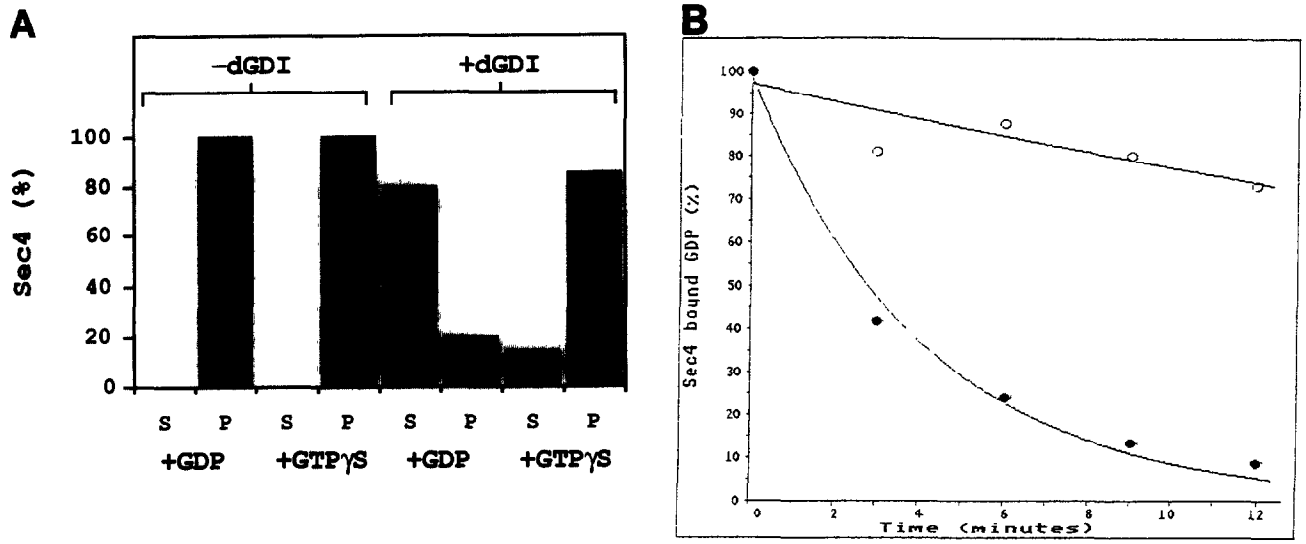

Fig. 2. Effects of dGDI on membrane-associated and soluble Sec4. (A) dGDI will cause the dissociation of GDP-bound but not GTPγS-bound Sec4 from yeast membranes. Yeast membranes (100 μg) were incubated in the presence (+dGDI) or absence (−dGDI) of 0.46 μM dGDI. The membranes were separated from the soluble fraction by centrifugation and the supernatants (S) and pellets (P) analyzed by SDS-PAGE followed by immuoblotting for their Sec4 content. Each assay is represented as the percentage distribution of Sec4 (^{125}I cpm) between the supernatant and pellet fractions. Yeast membranes for the assays were preincubated in the presence of 0.4 mM GDP (+GDP) or 0.4 mM GTPγS (+GTPγS) before addition of dGDI. (B) dGDI inhibits the dissociation of GDP from Sec4. The GDP-off rate assays were performed as described in section 2. Assays were performed in the absence (●) or presence (○) of 0.58 μM DGDI. This figure is representitive of three independent experiments. Each time point was done in duplicate.

that eluted between fractions 41 and 45 with an estimated molecular weight of between 50 and 70 kDa. Fractions 41 to 45 (peak B) were then pooled and diluted five-fold with buffer D (20 mM HEPES, pH 7.6, 1 mM $MgCl_2$, 1 mM DTT) and loaded onto a DEAE-Sephacel column (5.3 ml; Pharmacia). After washing the column with 16 ml of buffer E (buffer D containing 10 mM NaCl) the column was eluted with a linear salt gradient (10–300 mM NaCl, in buffer D). 2.2 ml fractions were collected and tested in the Sec4 membrane dissociation assay. One peak of activity was detected which eluted between 150 and 220 mM NaCl. Fractions 8 and 9 (containing the peak of activity) were pooled and concentrated ten-fold using a Centricon 10 microconcentrator (Amicom Corp.). This concentrated sample was tested for nucleotide-dependent dissociation of Sec4 from yeast membranes as described in section 2 and was found to cause the dissociation of Sec4 from membranes that had been preincubated in the presence of GDP. However, this activity was inhibited if the membranes had been preincubated in the presence of GTPγS (Fig. 3B). Thus, we have identified and partially purified a GDI activity from yeast cytosol that will cause the nucleotide dependent dissociation of Sec4 from membranes.

4. DISCUSSION

In this paper we report the interaction of rab GDI proteins from three different species with Sec4. Previously it has been reported that rab GDI will inhibit dissociation of GDP from rab3A, rab11 and Sec4 [26,28,29]. Rab GDI can also regulate the subcellular localization of rab3A by causing the dissociation of GDP-bound rab3A from synaptic membranes and vesicles [27]. Here, we have shown that rab GDI will also cause the dissociation of GDP-bound Sec4 from yeast membranes (Fig. 1). This result reinforces the suggestion previously made [28] that there may be a homologue of rab GDI for Sec4 in *S. cerevisiae*.

dGDI is the third rab GDI protein to be identified and was isolated by its association with the *Drosophila* developmental mutation *quartet* [33]. *Quartet* causes late larval lethality, small imaginal discs and low mitotic activity in larval brains and a basic shift in the isoelectric point of three abundant proteins [38,39,40]. One of these proteins was purified and an antibody raised that allowed cloning and sequencing of the cDNA. This sequence was found to encode a protein with 68% identity and 81% similarity to the rab GDI sequence and was named *Drosophila* GDI (dGDI) [32]. However, the interaction of dGDI with one or more small GTP binding proteins of *D. melanogaster* has yet to be shown. Here we report that dGDI can cause the release of GDP-bound Sec4 from yeast membranes (Fig. 2A) and will inhibit the dissociation of GDP from Sec4 (Fig. 2B), confirming that dGDI does exhibit the biochemical properties of a GDI protein. Since dGDI has a more basic isoelectric point in *quartet* animals, it has been suggested that it undergoes some form of post-transla-

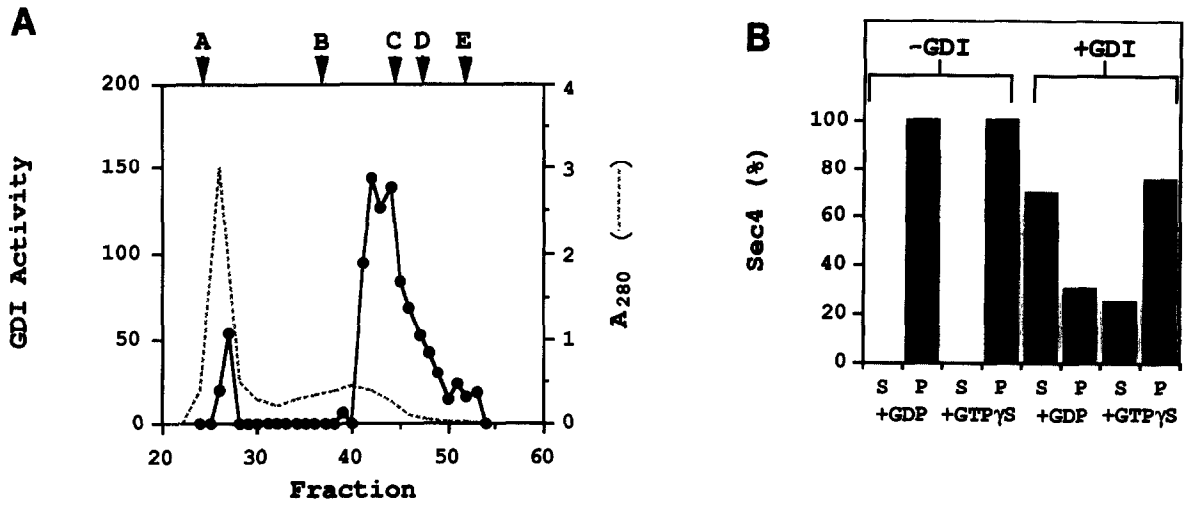

Fig. 3. Characterization of a yeast GDI activity for Sec4. (A) Sephacryl S-300 gel-filtration column chromatography of a 100,000 × *g* supernatant from *sec4–8* cells. The 100,000 × *g* supernatant from *sec4–8* yeast cells was applied to a S-300 Sephacryl gel-filtration column (80 ml), eluted with buffer B and 2.2 ml fractions collected. GDI activity (●) is given as the amount of Sec4 (^{125}I cpm) released from 100 μg of yeast membranes when incubated with 57 μl of each fraction at 30°C for 30 min and analyzed by SDS-PAGE and immunoblot as described in section 2. The column was calibrated with molecular weight markers (Sigma): A, dextran (2,000 kDa); B, alcohol dehydrogenase (150 kDa); C, ovalbumin (45 kDa); D, carbonic anhydrase (29 kDa); D, cytochrome *c* (12.4 kDa). (B) A yeast GDI protein for Sec4 will cause release of GDP-bound but not GTPγS-bound Sec4 from membranes. After DEAE-Sephacel column chromatography, fractions 8 and 9 were pooled and concentrated 10-fold using an Amicom micrococentrator. Sec4 membrane dissociation assays were then performed in the presence (+GDI) and absence (−GDI) of 50 μl of the pooled, concentrated fractions as described in section 2. Each assay is represented as the percentage distribution of Sec4 (^{125}I) between the supernatant (S) and pellet (P) fractions. Yeast membranes were preincubated in the presence of 0.4 mM GDP (+GDP) or 0.4 mM GTPγS (+GTPγS) before addition of the pooled concentrated DEAE fractions.

tional modification which regulates its activity in *D. melanogaster* [33]. However, the nature of this modification has yet to be understood. Future purification of both isoforms of dGDI and comparison of their activities using the assays described in this paper may help us to understand how modification of dGDI regulates its biochemical activity.

Since we had shown that GDI proteins from bovine brain and *D. melanogaster* will interact with Sec4, we examined the question of whether *S. cerevisiae* contains a comparable GDI activity for Sec4. Using the Sec4 membrane dissociation assay we have shown that yeast cytosol does indeed contain a GDI activity which elutes between 50 and 70 kDa when subjected to S-300 gel-filtration column chromatography. Furthermore, after consecutive S-300 gel-filtration chromatography and DEAE-Sephacel ion-exchange chromatography this enriched activity will cause the dissociation of the GDP-bound form of Sec4 from yeast membranes, but is inhibited when Sec4 is in the GTPγS-bound form. Therefore, we have identified an activity in yeast cytosol that behaves as a GDI protein for Sec4. Since both rab GDI and dGDI can interact with Sec4 we predict that the GDI protein whose actvity we have detected in yeast cytosol will share significant homology at the amino acid level with these two proteins. Cloning and sequencing of the gene encoding this protein and comparison with the rab GDI and dGDI amino acid sequences may help us to predict regions of GDI proteins that interact with Sec4.

The identification of a GDI protein for Sec4 suggests a possible mechanism for the observed recycling of Sec4 from the plasma membrane onto a new round of secretory vesicles. Expanding on the original model of Sec4 function [21,22], which suggests that GTP hydrolysis is coupled to the release of Sec4 from the plasma membrane, we propose that after GTP hydrolysis, Sec4 in the GDP-bound form is released from the plasma membrane by GDI. However, there still remains the question of how Sec4 becomes available for attachment to a new round of secretory vesicles if it is complexed to GDI in the cytosol, as seems to be the case for rab3A and rab GDI. One possibility is that an intracellular signal causes the dissociation of Sec4 from GDI. This signal could be in the form of a modification of GDI or Sec4, i.e. phosphorylation/dephosphorylation, or it could be an interaction with a protein which can compete with GDI for Sec4. One possible candidate for this competing protein is Dss4, the exchange factor identified for Sec4 [25]. In this scenario, Dss4 would compete with GDI for Sec4 causing dissociation of the Sec4/GDI complex, so that Sec4 would be available for attachment to a new round of secretory vesicles, possibly aided by Dss4. Purification, cloning and sequencing of this yeast GDI protein should help us to understand in more detail how the cycles of Sec4 localization and GTP binding

and hydrolysis are coupled, and how this relates to the role of Sec4 in regulating post-Golgi vesicular transport in *S. cerevisiae* .

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