Interactions of Nucleotide Release Factor Dss4p with Sec4p in the Post-Golgi Secretory Pathway of Yeast*

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Sec4 is an essential gene encoding a small GTPase that is involved in Golgi to cell surface transport in Saccharomyces cerevisiae and is a paradigm for studies on the mode of action of Rab proteins. We describe here the features of interaction of Sec4p with the accessory protein Dss4p. Dss4p is found both on membranes and in the cytosol; however, it is the membrane fraction that is complexed to Sec4p. Dss4p, like its mammalian counterpart, Mss4, binds zinc, and disruption of the zinc-binding site disrupts the ability of the protein to interact with Sec4p. DSS4 overexpression can rescue the lethal phenotype of two alleles of SEC4, corresponding to dominant mutations of Ras. We demonstrate that this suppression is due to the ability of Dss4p to form a tight complex with the mutant forms of Sec4p and hence sequester the mutant protein from its inhibitory effect. These results imply an in vivo role for Dss4p as a guanine nucleotide dissociation stimulator. In vitro the protein has the ability to stimulate the dissociation rate of both GDP and GTP from Sec4p. We examined the relationship of GDI1 and DSS4 with SEC4 both genetically and biochemically. These results exclude a role for DSS4 in the recruitment of Sec4p/GDI onto membranes.

The Sec4 protein belongs to the Rab subgroup of the Ras superfamily of small prenylated GTPases (1). Although Rab proteins are known to play a crucial role in membrane traffic, their mechanism of action is not well understood. Many Rab proteins have been identified, each being found to have a distinct subcellular localization. This finding has led to the hypothesis that each Rab protein functions at a discrete step of exocytic or endocytic transport although it is not the determinant for docking specificity of transport vesicles (2). Sec4p has been shown to play an essential role at the final stage of the secretory pathway in yeast (1) and has been proposed to function in a cyclical fashion to control forward traffic between the Golgi and plasma membrane (3).

GTP-binding proteins have the ability to assume different nucleotide-dependent conformations, and therefore each can act as a regulatory switch. Generally, by analogy to Ras, the GDP-bound form is thought of as the “resting” or inactive state. Exchange of nucleotide for GTP results in activation of the protein until the GTP is hydrolyzed by the GTPase activator protein-stimulated intrinsic GTPase activity. For Rab proteins, this GDP/GTP cycle is accompanied by a physical cycle of localization between the donor and acceptor compartments (4).

The cycle of nucleotide binding, hydrolysis, and localization is modulated through interaction with other proteins. Guanine nucleotide dissociation inhibitor (GDI) is a general factor that complexes with the GDP-bound forms of Rab proteins, shielding their geranylgeranyl groups from the aqueous environment and hence enabling them to shuttle through the cytoplasm (5). At the donor compartment, a GDI displacement factor receives GDP-bound Rab protein followed by a guanine nucleotide exchange activity that catalyzes the replacement of GDP with GTP. The GTP-bound Rab protein is then incorporated into secretory vesicles.

Current questions in the field have focused on the individual roles played by GDI and exchange factors in promoting the recycling of Rab proteins back onto the donor compartment and preparing them for another round of vesicle attachment.

One such accessory factor involved in SEC4 regulation was identified as a spontaneous dominant suppressor of the thermosensitive allele sec4-8 at 34 °C and termed DSS4-1 (6). The gene encoding Dss4p is non-essential in vivo but shows a synthetic negative effect in combination with sec4-8. Biochemical characterization of Dss4p, a 17-kDa, hydrophilic protein, shows that it acts on Sec4p as a GDP dissociation stimulator. Because the intracellular concentration of GTP is in excess of that of GDP, a protein that can affect the release of GDP may have the in vivo property of an exchange factor. In this paper we examine the biological role played by Dss4 in the cycle of Sec4 function.

EXPERIMENTAL PROCEDURES

Reagents—Oligonucleotides were prepared by Monica Talmor, Department of Pathology, Yale University. Restriction enzymes, T4 DNA ligase, and polymerase were from New England Biolabs (Beverly, MA). Tag polymerase was from Boehringer Mannheim. Zymolyase-100T was from Seikagaku Corp. (Tokyo, Japan). Secondary antibodies were from Jackson ImmunoResearch Labs Inc. (West Grove, PA). 9E10 ascites fluid was prepared by the Pocono Rabbit Farm and Laboratory Inc. (Canadensis, PA). Low range SDS-PAGE standards were from Bio-Rad. All other chemicals were purchased from Sigma except as noted.

Strains, Media, and Microbiological Techniques—Yeast strains used in this study are listed in Table I. Cells were grown in rich medium (YPD) or in minimal medium (SD) supplemented for auxotropic requirements as described (7). General molecular biological methods were as described (8). Transformations were performed by the lithium ace-
For the zinc overlay assay the filters were washed in metal binding buffer (MBB, 100 mM Tris-HCl, pH 7.4, 50 mM NaCl) for 1 h, incubated with 5–10 µCi of 2ZnCl2 (2 Ci/mg, NEN Life Science Products) per lane in 5–20 ml of MBB for 30 min to 1 h. Filters were then washed for 15 min in three changes of MBB, wrapped in Saran Wrap, and exposed to XAR-5 film (Eastman Kodak Co.) with an intensifying screen. Autoradiograph development was done in Kodak NTB-2 emulsion without anti-hydrazide included in the SDS-PAGE standards was used as a positive control.

Antibodies and Electrophoresis—Cellular protein extracts were prepared by lysis of spheroplasts in KHEM (50 mM KCl, 10 mM EGTA, 50 mM Hepes-KOH, pH 7.2, 1.92 mM MgCl2, 1 mM DTT) containing 1% Triton X-100 and protease inhibitors. Solubilized lysates were cleared by centrifugation in a SW50.1 rotor at 36K for 1 h. Protein concentration was determined by the method of Bradford (15), adjusted to 3 mg/ml, and immunoprecipitation carried out using 9E10 ascites fluid. For immunoprecipitation from 35S-labeled lysates, cells in log phase were labeled with 3 h with 100 µCi of Pro-Mix (Amersham Corp.), 1 A260 unit of cells/ml of media. For SDS-PAGE, samples were heated for 5 min at 100 °C in sample buffer containing 2% SDS and run on 15% slab gels. After transfer to nitrocellulose (BA 83, 0.2 µm, Schleicher & Schuell) blots were probed with primary antibody αYpt1p at a dilution of 1/2000, αYpt51p at a dilution of 1/1000, affinity purified αYpt31p at a dilution of 1/400, αGdi1p at a dilution of 1/3000, 9E10 ascites fluid at a dilution of 1/5000.

In Vivo Interaction Assays—Recombinant Gdi1p and Ds4p were produced according to previously published protocols (16–18). To isolate the Sec4-Gdi1p complex, 100 µg of His6-tagged Sec4p containing membranes were incubated with recombinant Gdi1p in buffer A (0.8 M sorbitol, 25 mM Hepes, pH 7.5, 5 mM MgCl2, 1 mM DTT, 500 mM NaCl, 10 mM GDP, and protease inhibitors) for 30 min at 30 °C. The reaction was layered on top of a sorbitol barrier consisting of buffer A containing 1.5 M sorbitol and microcentrifuged for 20 min at 4 °C. Supernatants containing unbound Sec4p were discarded, and Sec4p-purified bead pool was incubated and incubated for 30 min with a 50% slurry of Pro-Bond resin (Invitrogen) that had been prewashed in buffer A containing 100 µg/ml bovine serum albumin. The resin was then subjected to four washes with buffer A containing 100 µg/ml bovine serum albumin and two washes with buffer B (0.8 M sorbitol, 25 mM Hepes, pH 7.5, 5 mM MgCl2, 1 mM DTT, 100 mM NaCl, 10 mM GDP, and 100 µg/ml bovine serum albumin). At this stage, the resin was incubated for 30 min at 30 °C in buffer B either alone or containing 1.5 µM Ds4p. The resin was washed a further three times with buffer B followed by elution of the His6-tagged Sec4p with buffer A containing 50 mM EDTA. Duplicate aliquots of the washes and eluates were prepared for SDS-PAGE and immunoblotted with either αSec4p or αGdi1p.

Nucleotide Association Assays—GTP Association Rate Assays—GTP dissociation rates were determined (16). GTP-5S association was carried out under standard conditions. Sec4p was incubated with either Ds4p or control buffer in the presence of 5 mM [55S]GTP-5S at 2 µCi/ml at 30 °C. The reaction was initiated by the addition of recombinant Ds4p. Aliquots of the reaction mixture were removed at various times as indicated and filtered through Millipore nitrocellulose filters. Protein-associated radioactivity was assessed in a scintillation counter.

RESULTS

Ds4p Associates in Vivo with Sec4p and Ypt1p—To investigate which Rab proteins are complexed to Ds4p in cellular extracts, native immunoprecipitation studies were performed using an epitope-tagged Ds4p. Analysis of the immunoprecipitates by [α-32P]GTP overlay assay showed the presence of at least two GTP-binding proteins (Fig. 1A, lane 2). These two proteins were identified as Sec4p and Ypt1p by a second round of immunoprecipitation using protein-specific antibodies (Fig. 1A, lanes 3 and 4). The immunoprecipitates were also probed for the presence of other candidate Rab proteins as follows: Ypt51p acting along the endocytic route (19) and Ypt31p, the S. cerevisiae homolog of the mammalian Rab protein Rab11 that acts in intra-Golgi transport (20). The presence of either Ypt51p or Ypt31p could not be detected by Western blotting of the immunoprecipitates (Fig. 1B). Ypt31p was not present in Ds4p immunoprecipitates even from cells expressing Ypt31p from a multicopy plasmid although it has the ability to interact with Ds4p in a two-hybrid assay (data not shown). It is possible therefore that Ds4p interacts in vivo with only a small...
subset of Rab proteins or that the affinity of interaction of some Rab proteins is too weak to be revealed biochemically.

The Complex of Dss4p and Sec4p Is Present on Membranes—
Dss4p is a hydrophilic protein, so it was of interest to know whether the complex of Dss4p and Sec4p exists on membranes or in the cytosolic fraction. To examine this question, native Dss4 immunoprecipitations were performed from post-nuclear supernatants containing wild type (single copy) levels of Dss4 that were fractionated into 100,000 × g pellet (HSP) and supernatant (HSS) fractions. Fig. 2A shows the relative levels of Dss4p in the HSP and HSS fractions when present at single copy and when expressed from a multicopy plasmid. At single copy, Dss4p is equally distributed between HSP and HSS fractions. Increasing the gene dosage and protein level by expression of DSS4 from a multicopy 2 μm plasmid results in the same distribution of Dss4p as when DSS4 is expressed at single copy. As previously demonstrated (4), Sec4p is distributed between the membrane fraction and the cytosolic fraction where presumably it is in association with Gdi1p. Fig. 2C shows the relative levels of Sec4p associated with the Dss4p immunoprecipitations. The majority of the Sec4p found in Dss4p immunoprecipitates is from the HSP and not the HSS fraction. Consistent with this, the amount of Sec4c immunoreactivity remaining in the post-immunoprecipitation supernatants is depleted compared with the levels of Sec4p prior to immunoprecipitation (Fig. 2B). In the post-immunoprecipitation supernatants, the level of Sec4c associated with the cytosolic HSS fraction is relatively unchanged, and Sec4p immunoreactivity is depleted from the membrane-associated HSP fraction. These data clearly show that the Sec4p-Dss4p complex is present in the HSP or membrane-associated fraction; presumably Sec4p, which is membrane-associated via its C-terminal prenylation, recruits Dss4p onto the membrane although other factors may also play a role in this process.

DSS4 Expressed at High Copy Suppresses Dominant Negative Alleles of SEC4—Studies on Rab proteins have been greatly facilitated by knowledge of p21ras structure/function obtained through mutational analysis and structural studies. Dominant negative point mutations of Ras superfamily members have been used to disrupt wild type protein function. Expression of the dominant negative alleles of SEC4, with SEC4N34 and SEC4I133 behind the regulatable GAL1 promoter leads to lethality when these cells are grown with galactose as the carbon source (Table I (3)). These point mutations are in the guanine nucleotide-binding site at positions conserved between all Ras superfamily members. SEC4I133 contains an asparagine to isoleucine substitution and results in a protein unable to bind guanine nucleotides. This SEC4 allele is a dominant negative inhibitor of membrane traffic in yeast (3). SEC4N34 contains a substitution of asparagine for serine at a position that is conserved for all small GTPases and is analo-

**Fig. 1. Ypt1p and Sec4p but not Ypt51p or Ypt31p associate with Dss4p.** Co-precipitation analysis was performed on yeast strains containing either myc-tagged Dss4p (DSS4-myc) or untagged Dss4p (DSS4). Immunoprecipitates were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with either [α-32P]GTP to reveal the presence of small GTPases or immunoblotted with polyclonal antisera specific for Ypt1p, Ypt31p, or Ypt51p. Association was monitored by a two-step precipitation protocol. The first step involved nondenaturing immunoprecipitation with the α-myc monoclonal antibody 9E10. After extensive washing, the samples were boiled in sample buffer containing SDS and DTT. The boiled samples were then diluted with immunoprecipitation buffer and subjected to a second round of immunoprecipitation with polyclonal antisera specific to either Ypt1p or Sec4p. A, Dss4p selectively complexes with two GTP binding proteins, Ypt1p and Sec4p. Nitrocellulose blots were probed with [α-32P]GTP to reveal the presence of small GTP-binding proteins. The primary Dss4p immunoprecipitate (lane 2) was subjected to a second round of immunoprecipitation with antibodies specific for Sec4p (lane 3) or Ypt1p (lane 4). For comparison, a sample of yeast total lysate (lane 1, post-nuclear supernatant) is included. B, presence of Ypt1p, Ypt31p, and Ypt51p in the Dss4p immunoprecipitates. The Dss4p immunoprecipitate was probed with antibodies specific to Ypt1p (lane 2, α-Ypt1), Ypt31p (lane 4, α-Ypt31), or Ypt51p (lane 6, α-Ypt51). For comparison, a sample of yeast post-nuclear supernatant is included (lanes 1, 3, and 5).

**Fig. 2. The complex of Sec4p and Dss4p exists on membranes.** A, post-nuclear supernatants (Total) and equivalent HSP and HSS fractions from yeast lysates expressing DSS4-myc as the sole copy of DSS4 and from a multicopy plasmid were analyzed by SDS-PAGE and transferred to nitrocellulose, and the filters were blotted with 9E10 antibody. Each lane represents 0.25 × 10⁶ cell equivalents. B, S1 lysates were fractionated into HSP and HSS fractions as in A to show the distribution of Sec4p immunoreactivity in the different fractions. Samples were boiled in sample buffer before SDS-PAGE followed by Western blotting with affinity purified polyclonal antisera specific for Sec4p. The S1, HSP, and HSS fractions were used to perform Dss4p immunoprecipitations with 9E10 antibody and then analyzed to reveal the amount of Sec4p immunoreactivity remaining in the post-immunoprecipitation supernatants. Each lane can be directly compared with the equivalent lane before immunoprecipitation shown in B. C, native Dss4p immunoprecipitations performed from S1, HSP, and HSS fractions were analyzed to show the relative levels of Sec4p associated with Dss4p. Immunocomplexes collected on Protein A-Sepharose were boiled in sample buffer containing 100 μl DTT; samples were then processed for Western blotting with affinity purified polyclonal antisera to Sec4p.
The vesicles are similar in size and appearance to vesicles present in other late secretory mutants that accumulate post-secretory vesicles when grown in galactose-containing medium to induce expression of the mutant genes (Fig. 3, A and C, respectively) both showed a dramatic accumulation of secretory vesicles that when grown in galactose-containing medium to induce expression of the mutant genes (Fig. 3, A and C (22)). The vesicles are similar in size and appearance to vesicles present in other late secretory mutants that accumulate post-Golgi vesicles. These data directly demonstrate that although expression of Sec4N34 and Sec4A133 induces dominant secretory defects resulting in lethality, the formation of secretory vesicles is not inhibited. Similar analysis of Sec4N34 and Sec4A133 expressing strains harboring a multicopy plasmid containing DSS4 shows a normal cellular morphology, the vesicle accumulation has been alleviated, and the cells are now viable (Fig. 3, B and D). DSS4 can suppress the dominant acting alleles of Sec4, and this provides an in vivo assay for DSS4 function.

Dss4 Forms a Complex with Dominant Negative Alleles of Sec4—In the case of Ras, the Ser to Asn and Asn to Ile cognate mutants disrupt normal protein function by competing for GEF interactions to form a stable, catalytically inactive complex (23). Overexpression of the relevant GEF can alleviate the interactions to form a stable, catalytically inactive complex (23). Overexpression of the relevant GEF can alleviate the lethality of a mutant may accommodate GDP in its binding pocket but probably adopts a conformation corresponding to a transition state blocked by tightly complexing with these mutants, which probably adopt a conformation corresponding to a transition state blocked by tightly complexing with these mutants, which probably adopt a conformation intermediate between the GDP- and GTP-bound states (21). The lethality of Sec4A133 and Sec4N34 can be suppressed with Dss4p strongly interacts with the Asn-34 and Ile-133 alleles of Sec4p, weakly with the wild type Sec4p, and not at all with the Leu-79 allele. The level of interaction was unchanged regardless of whether the Sec4 construct was lacking the C-terminal prenylation motif, indicating that the observed interaction does not require the geranylgeranylation of Sec4p.

Immunoprecipitations were performed in detergent-solubilized lysates from cells expressing the dominant negative Sec4 alleles in the presence of epitope-tagged (lanes 1 and 3) and -untagged DSS4 (lanes 2 and 4) present on multicopy plasmids. Cells were metabolically labeled with 35S followed by 9E10 (a-myc epitope monoclonal) immunoprecipitations of the detergent-solubilized lysates. Lanes 1 and 2 are derived from lysates of cells expressing Sec4N34 and Sec4A133. Sec4 efficiently co-precipitated with the mutant protein forming a complex stable in the presence of 1% Triton X-100 (Fig. 5). These results indicate that Dss4p can tightly associate with nucleotide-free conformations of Sec4p and hence sequester the mutant protein from its inhibitory function.

Interaction of GDI1 with Sec4—Overexpression of GDI1 does not suppress the Sec4A133 mutant (Table II), although such a mutant allele is predicted to be GDP-associated which is favored for interaction with GDI. This result suggests that the Sec4A133 mutant cannot physically interact with Gdi1p, and this conclusion is further strengthened by the result of a two-hybrid assay (Table III). Although Gdi1p interacts with the wild-type Sec4p, no interaction is observed with the Sec4p lacking its C-terminal cysteines, reflecting the Gdi1p requirement for Sec4p prenylation. The results observed with Gdi1p are in contrast to the interaction of Sec4p and Dss4p in such an assay where positive interactions are observed between the
FIG. 4. Growth of two-hybrid pairs on selective media. Y190 reporter strain was co-transformed with plasmid pairs as indicated to monitor protein interactions via the growth on selective media lacking histidine. For each pair, genes fused to the GAL4 DNA binding domain plasmid are shown circled. pSE111 is an irrelevant plasmid used to demonstrate lack of autologous activation of the Gdi1p construct. Note that productive interactions are observed between Dss4p and Sec4p in the case where Dss4p is fused to the GAL4 DNA binding domain but not in the reverse orientation.

wild type Sec4, Sec4ΔC, Sec4N34, and Sec4I133 proteins. No interactions between Dss4p and Gdi1p could be detected in this system (Fig. 4).

Dss4p Is a Zinc-binding Protein—The amino acid sequence of Dss4p revealed the presence of several cysteines in a grouping reminiscent of a zinc cation binding site, and NMR studies of Mss4, the mammalian homolog of Dss4p (29), have revealed the presence of a zinc cation binding site in the region most conserved between the two proteins (30). Zinc binding of Dss4p was directly tested by performing a $^{65}$Zn$^{2+}$ overlay assay (31) on immunoprecipitates of Dss4p (Fig. 6A). 9E10 immunoprecipitates from cells expressing DSS4 myc3 showed $^{65}$Zn$^{2+}$-binding activity at the expected molecular weight (lane 2) that was not observed in untagged controls (lane 1). To explore this further, we expressed Dss4p with a point mutation of Cys-104 $\rightarrow$ Ser, a residue in a structure-based sequence alignment analogous to a residue identified in Mss4 as directly coordinating a zinc cation (30). The mutant Dss4 protein was tested for functionality in vivo and for zinc cation binding in the overlay assay. This point mutation, unlike wild type DSS4, was unable to suppress the dominant lethal effect of overexpression of the SEC4N34 and SEC4I133 alleles (Fig. 6B), indicating that it cannot stably associate with its substrate in vivo. Also, unlike the wild type protein, zinc binding was not detected in immunoprecipitates from cells expressing the DSS4 allele bearing the substitution of serine for cysteine 104 (Fig. 6A, lane 4). Zinc binding proteins that coordinate zinc ions with residues that are closely spaced in their primary amino acid sequences generally perform well in a zinc overlay assay, as such proteins require minimal renaturation to reconstitute a functional zinc-binding site (32). Zinc binding of Dss4p probably represents a small autonomously folded protein domain or zinc finger, with the zinc ion serving to stabilize the protein structure. Although not demonstrating that this cysteine residue directly participates in coordination of a zinc cation, these results indicate that Dss4p is a zinc-binding protein and that this structural feature is required for its biological activity.

DSS4 Does Not Interact with sec19-1, Which Encodes a Transcrted Version of GDI1—Sec4p is found in both the cytosol and membrane fractions (4). Sec4p in the cytosol fraction is in the inactive state (33). One hypothesis is that Sec4p, complexed with GDI in the cytosol, is released from the inhibitory action of GDI and is activated by the exchange factor at the Golgi or donor compartment. We sought to examine the possible interactions of GDI and Dss4p in several ways. The budding yeast S. cerevisiae, possesses only one GDI gene, GDI1, that is essential for every stage of vesicular traffic examined (33). The availability of a temperature-sensitive allele

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of GDI1, sec19-1 offers the possibility of using a molecular genetic approach to examine putative interacting genes. The first question we asked was whether DSS4 could compensate for a lowered rate of GDI function when overexpressed in the cell. The GDI1 allele sec19-1 is a temperature-sensitive allele that results in cessation of membrane traffic at the restrictive temperature (33). To determine the nature of the mutation, plasmid gap repair was performed. GDI1 was subcloned into a centromeric vector containing the selectable marker URA3. The vector was then gapped with various pairs of restriction enzymes (see “Experimental Procedures”) and the resulting plasmid transformed into the sec19-1 mutant strain (NY420). Transformants were stamped and tested for growth at 37 °C. This procedure narrowed down the site of the temperature-sensitive mutation downstream of the EcoRI site (Fig. 7A). Plasmids from these cells were rescued and sequenced, which revealed one base pair mutation of A to T at nucleotide 1526 of the published GDI1 sequence (33). This single base pair mutation resulted in a change of arginine at codon 434 of the open reading frame to an opal stop codon. To confirm this result, sec19-1 cells were transformed with the opal, amber, and ochre tRNA suppressors. Only the opal tRNA suppressor was able to support growth of NY420 at the restrictive temperature supporting the conclusion that the sec19-1 allele is caused by a 17-amino acid C-terminal truncation of the protein. Premature stop codons are also observed to be responsible for the defect in Rab escort protein function leading to choroideremia (34). Examination of the structure of GDI (35) suggests that premature truncation would disrupt the region most conserved between GDI and Rab escort protein family members and lead to protein instability (36).

To assess if Dss4p could overcome the loss of GDI1 function represented by the sec19-1 allele, we transformed NY420 with DSS4 on a multicopy plasmid. The transformants were not able to rescue growth of the sec19-1 mutant at the restrictive temperature (data not shown). Previous work has shown that the combination of DSS4 and sec19-1 shows no synthetic negative effects (2) and together with this result indicates that DSS4 cannot compensate for impaired GDI1 function, suggesting an indirect relationship via Sec4p, with Dss4p acting downstream of GDI1 function. To explore this result further, we examined whether Dss4p could act to disrupt a pre-formed complex of Gdi1p-Sec4p.

Dss4p Cannot Disrupt the Interaction of GDI and Sec4—To examine any possible interactions between Dss4p and Gdi1p biochemically, we used Gdi1p-specific antibodies to look for the presence of Gdi1p in native Dss4p immunoprecipitations (Fig. 7B). These results show that in contrast to Sec4p and Ypt1p, no GDI immunoreactivity was observed in the Dss4p immunoprecipitates. Rab recruitment onto target membranes is mediated via prenylated Rab-GDI complexes and accompanied by nucleotide exchange (37). Although nucleotide exchange lags behind membrane binding (37, 38), it is conceivable that a nucleotide exchange factor may aid this process by acting to relieve the association of GDI with Rab protein by guanine nucleotide exchange. A scheme was set up to assay for possible three-way interactions between Sec4p, Gdi1p, and Dss4p in vitro. Briefly, preformed Sec4p-Gdi1p complexes were incubated with excess amounts of recombinant Dss4p and were then examined to see if the Dss4p protein had the capacity to displace Gdi1p. The results of this analysis are presented in Fig. 7C. Recombinant Gdi1p can be used to release Sec4p from membranes of yeast cells expressing His10-tagged Sec4p as their sole copy of SEC4. The Sec4p-Gdi1p complex can then be stably attached to a solid-phase resin with Gdi1p associated via the His10 tag of Sec4p. The Sec4p-Gdi1p heterodimer was incubated with or without Dss4p, and aliquots of the supernatant were assayed for Sec4p and Gdi1p to determine if Dss4p has caused Gdi1p to dissociate from Sec4p. Under all conditions tested, the Sec4p-Gdi1p complex remained intact and was not perturbed by

### Role of Dss4p in Sec4p Function

#### TABLE III

| Sec4 construct | Amino acid change | Class | Dss4 binding | GDI binding |
|----------------|------------------|-------|--------------|-------------|
| Wild type      |                  |       | -            | +           |
| ΔC             | Cys214 → Δ      | Wild-type protein lacking C-terminal prenylation | + |
| I133            | Asn133 → Ile   | Nucleotide binding deficient | + + + + +  |
| N34            | Ser34 → Asn    | Equivalent to Ras Asn-17 | + + + + +  |
| L79            | Glu79 → Leu   | GTPase-deficient | - |

Fig. 6. A, Dss4p binds 65Zn(II) in an overlay assay. 9E10 immuno-precipitates from 4 μg of lysate expressing either wild type untagged Dss4p (lane 1), DSS4-myc3 (lane 2), or DSS4 Ser104-myc3 (lane 4) from multicopy plasmids were transferred to nitrocellulose filters after SDS-PAGE. The filters were probed with 65Zn(II)Cl2 for 1 h and washed for 15 min. The filters were exposed to XAR film (Kodak) for 18 h at -80 °C with an intensifying screen. Carbonic anhydrase (31 kDa, lane 3) is included as a positive control for zinc-binding activity. B, DSS4 Ser104 no longer has the ability to suppress dominant negative alleles of SEC4. Cells expressing SEC4Ser104 or SEC4G123 behind the GAL1 promoter are plated on galactose-containing media in the presence of high copy plasmids expressing either wild type DSS4 or DSS4Ser104, DSS4G123 no longer has the ability to suppress the lethality of these dominant negative SEC4 alleles.
incubation with Dss4p (Fig. 7C). At the end of the experiment, the Sec4p together with associated Gdi1p could be eluted from the resin by incubation with EDTA which demonstrated that the amount of Sec4p associated with the resin was essentially unchanged.

Dss4p Stimulates Guanine Nucleotide Release from Sec4p—In vitro, Dss4p has the ability to catalyze GDP release on Sec4p. Using recombinant purified proteins the question of the effect of Dss4p on the GTP dissociation/association rate was examined (Fig. 8). An in vitro filter assay was used to examine the effect of Dss4p on the rate of GTP release from Sec4p, similar to the assay used to demonstrate the acceleration of GDP release by Dss4p on Sec4p (6). The results of this assay show that Dss4p can accelerate the rate of GTP release from Sec4p (Fig. 8A). The question of the promotion of GTP association onto Sec4p was also examined in a similar fashion. Dss4p does not have the ability to promote GTP association onto Sec4p over the intrinsic rate (Fig. 8B). The biochemical properties of Dss4p suggest that its role in regard to Sec4p is a factor that has the ability to stimulate the dissociation of both GDP and GTP from Sec4p and not as an exchange protein that can stimulate the rate of GTP association.

DISCUSSION

Rab proteins were once proposed to be molecular “tags” directing the transport of a vesicle to its correct target, but recent
findings indicate rather that they act as switches regulating vesicle fusion (39, 40). A single chimera of the yeast proteins Ypt1p and Sec4p, which function in endoplasmic reticulum to Golgi and post-Golgi transport, respectively, can function at both stages of the pathway without detectable misrouting (2, 41). Rather than being directly involved in membrane fusion events, Rab proteins may regulate the ability of proteins involved in membrane recognition events to function correctly.

Dss4p shows a very tight association to the dominant negative SEC4 point mutations Asn-34 and Ile-133. Strong dominant inhibitory effects of point mutations of Ras superfamily members have been hypothesized to result from the distinct structures of these mutants approximating the nucleotide-free state and so disrupting wild type protein function through competition for GEF interactions (25, 42–44). Although Dss4p can stably complex to and prevent the lethality of dominant SEC4 mutants, DSS4 is not an essential gene for growth so cannot be the only target for the inhibitory action of these SEC4 alleles in vivo.

GDI protein can distinguish between the two nucleotide-bound states of Rab proteins, binding to the GDP-bound and not the GTP-bound Rab (45). However, our data showing that Gdi1p, unlike Dss4p, does not interact with the Asn-34 point mutant of SEC4 suggests the assumption that such a mutant probably adopts a distinct structure intermediate between the GDP- and GTP-bound states. The Ser to Asn cognate mutant may accommodate nucleotides in its binding pocket but remains in the nucleotide-free conformational state and thus is analogous to the Asn to Ile cognate mutant that is unable to bind nucleotide. A point mutant of Ras, RasD57A has an even greater affinity for GDP than RasS17A but is not a dominant inhibitor of Ras function (42). Overexpression of GDI1 does not suppress the dominant lethality of a SEC4N34 mutant, further indicating that this mutant allele is not in the GDP binding conformation that would be favored for a physical interaction with GDI. Our results showing that GDI will not interact with mutations in SEC4 such as ΔC, which removes the site of geranylglyceranyl addition, and Ile-133, which abolishes nucleotide binding, is in good agreement with the known requirements for Rab-GDI interaction.

Dss4p seems to act on Sec4p in a manner analogous to other guanine nucleotide dissociation inhibitor stimulator proteins, by stabilizing the nucleotide-free form of its target (26). Structural analysis of Mss4, the mammalian counterpart of Dss4p, has shown that the protein binds Zn\(^{2+}\) and that this binding site is important in its association with its substrate, Rab3A (30). Mutation of a cysteine in the conserved region between Mss4 and Dss4p abolishes the ability of the molecule both to bind zinc and to block the dominant lethal effects of SEC4N34 and SEC4I133, indicating that zinc cation binding is crucial for the ability of Dss4p to act on Sec4 in vivo.

How does Dss4p contribute to Sec4p function? One possibility is that Dss4p helps to coordinate the bulk exocytic membrane flow. Dss4p is capable of interaction with several Rab proteins. In cellular lysates, interaction with Ypt1p and Sec4p can be detected, both of which are Rab proteins involved in forward pathways of exocytosis. The mammalian counterpart Mss4 also appears to interact in vitro with a distinct subset of Rab proteins involved in exocytic and not endocytic transport (46). It is likely that the role of DSS4 is a positive one, since genetic analyses show that deletion of DSS4 is synthetically lethal with a subset of other late-acting SEC genes (6). It would appear unlikely that Dss4p is a major factor involved in regulating a specific GDP/GTP exchange event. In vivo, Dss4p does not possess all of the activities expected of a bona fide exchange factor; it cannot stimulate the rate of GTP acquisition by Sec4p and only accelerates GDP release significantly at high molar ratios. DSS4 is a nonessential gene and no homologs of DSS4 exist in S. cerevisiae, suggesting the existence of other activities capable of performing nucleotide exchange on Ypt1p and Sec4p. The existence of multiple GEFs for Ras is well known (47). At least two factors with GEF activity toward Rab3A have already been described, (48), and it is possible that a similar situation may exist for other Rab proteins. Perhaps different proteins acting on Sec4p are differently regulated, with DSS4 only being essential under certain specialized conditions such as have been described for the Ras exchange factors encoded by the SCD25 and CDC25 genes (49).

We find no evidence for any role of Dss4p in promoting membrane attachment of the Sec4-GDI complex or for Dss4p affecting GDI displacement from Sec4p. These results support the hypothesis that the nucleotide exchange event is distinct from recruitment of Rab/GDI to the donor compartment (5). Dss4p may therefore act downstream of Sec4p recruitment onto the donor compartment. Our data demonstrate the site of interaction between Dss4p and Sec4p to be on membranes. Perhaps Dss4p helps to play a role in stabilizing Sec4p to back extraction by Gdi1p ensuring that delivery is a one-way process. Thin section electron micrographs from cells expressing the SEC4 dominant negative mutants show a dramatic accumulation of post-Golgi vesicles. These data suggest that activated Rab protein may not be required for vesicle formation. Endoplasmic reticulum-budded vesicles in S. cerevisiae can acquire Ypt1p to become fully functional transport intermediates (51, 52), implying that Ypt1p is not required for vesicle biogenesis and can be activated on the budded vesicle itself. In the case of p21ras, location and membrane association is critical for activation (50), and it appears that this may also apply to Rab proteins. A dominant negative allele of YPT1, Ile-121, that does not bind guanine nucleotides, blocks transport from the endoplasmic reticulum at a stage prior to vesicle consumption and downstream of vesicle production (53). Furthermore, Rab proteins are not thought to participate directly in the final docking and fusion steps of transport but have been implicated to perform their downstream functions on the transport vesicle (54). In mammalian cells, Rab5-GDI can specifically bind to isolated transport vesicles, and these vesicles contain activities which displace GDI and promote GDP/GTP exchange (55). One role for Dss4p is suggested by the high stoichiometry required for Dss4p action and the fact that a substantial pool of membrane-associated Sec4p can be found in a complex with Dss4p. Dss4p can function as a chaperone, prolonging the stability of Sec4p during its transition through the nucleotide-free state. It is conceivable that the site of GDP/GTP exchange on Rab proteins may vary according to the requirements of the particular stage of membrane traffic, differing, for example, between organelles involved in homotypic fusion and vesicles mediating traffic between different organelles. Dss4p may associate transiently or stably with other as yet unidentified proteins that localize its interaction with Sec4 in vivo. We are currently assessing this possibility as well as investigating the location of Sec4p activation to help clarify these issues.

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