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Requirement for a GTPase-Activating Protein in Vesicle Budding from the Endoplasmic Reticulum

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The binding and hydrolysis of guanosine triphosphate (GTP) by the small GTP-binding protein Sar1p is required to form transport vesicles from the endoplasmic reticulum (ER) in *Saccharomyces cerevisiae*. Experiments revealed that an interaction between Sar1p and the Sec23p subunit of an oligomeric protein is also required for vesicle budding. The isolated Sec23p subunit and the oligomeric complex stimulated guanosine triphosphatase (GTPase) activity of Sar1p 10- to 15-fold but did not activate two other small GTP-binding proteins involved in vesicle traffic (Ypt1p and ARF). Activation of GTPase was inhibited by an antibody to Sec23p but not by an antibody that inhibits the budding activity of the other subunit of the Sec23p complex. Also, activation was thermolabile in pure samples of Sec23p that were isolated from two independent *sec23* mutant strains. It appears that Sec23p represents a new class of GTPase-activating protein because its sequence shows no similarity to any known member of this family.

Protein transport between membrane-bound organelles is mediated by cytosolic and membrane proteins that facilitate the formation, targeting, and fusion of small carrier vesicles (*1*, *2*). An interacting set of *SEC* genes contribute to vesicle budding early in the secretory pathway of *S. cerevisiae* (*3*). Two of these Sec proteins have been purified by means of an assay that reproduces the vesicle-budding event in vitro (*4*–*7*). The first protein, Sar1p, is a small GTP-binding protein that is representative of a large class of proteins similar to Ras that are associated with intracellular membrane traffic (*8*–*10*). The second, Sec23p, is part of a hetero-oligomeric complex, both subunits of which are required for vesicle budding in vitro (6). A mammalian homolog of Sec23p has been immunolocalized to the vesicles, cytoplasm, and tubular extensions of the ER transitional zone adjacent to the Golgi apparatus (*11*).

We isolated two functional forms of Sec23p by biochemical complementation of a *sec23* mutant lysate in an assay of ER-to-Golgi transport. Extracts of cells in which *SEC23* is overexpressed yielded an active monomeric species and the hetero-oligomeric Sec23p complex, whereas extracts of wild-type cells yielded only the complex (6). In an effort to identify other factors that interact with Sec23p, we examined the effect of excess monomer and complex on transport vesicle formation in a wild-type lysate. Briefly, the vesicle budding reaction measures the transfer of a radioactive secretory glycoprotein, yeast α-factor precursor, from rapidly sedimenting ER membranes to slowly sedimenting transport vesicles (*4*). The amount of Sec23p in a wild-type lysate was sufficient for vesicle formation. However, addition of excess Sec23p monomer inhibited budding (Fig. 1A) (*12*) whereas

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excess Sec23p complex had virtually no effect. Hence, Sec23p monomer titrates a limiting component in this reaction that is either not recognized by or not available to the complex.

Addition of pure Sar1p overcame much of the inhibitory effect of the Sec23p monomer. Although the rate of vesicle budding in unsupplemented wild-type reactions was not limited by the amount of Sar1p (Fig. 1B), the rate of budding, which was reduced to 29% of the normal rate by Sec23p monomer (1.2 μM), was restored to 63% of normal with a supplement of Sar1p (0.62 μM) (Fig. 1B, hatched bars). Another small GTP-binding protein, Ypt1p, which is required for the targeting of ER-derived transport vesicles (*4*, *13*), did not compensate for dose-dependent inhibition by the Sec23p monomer (*14*). These results suggest an interaction between Sec23p and Sar1p.

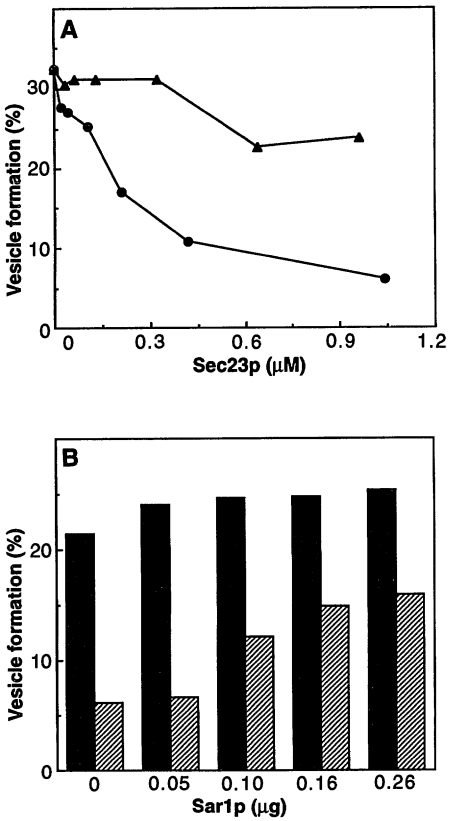

Fig. 1. Effects of excess Sec23p and Sar1p on vesicle formation. (**A**) Excess Sec23p monomer inhibits vesicle formation. Vesicle formation (measured in percent of normal rate) from the ER in permeabilized yeast spheroplasts (25-μl reaction volume) was monitored in the presence of increasing amounts of Sec23p monomer (circles) or Sec23p complex (triangles) for 30 min at 20°C (*4*). (**B**) Inhibition by Sec23p monomer is suppressed by Sar1p. Vesicle formation reactions were conducted with (hatched bars) or without (closed bars) 2 μg of Sec23p monomer (final 1.2 μM) in the presence of increasing amounts of Sar1p.

The sequence of *SEC23* gave no indication of its role in transport vesicle formation and in particular shows no relation to any of the many ancillary factors that influence GTP hydrolysis or nucleotide exchange by small GTP-binding proteins that are related to Ras (*14–17*). In spite of its lack of sequence similarity to GTPase activation proteins (GAPs), Sec23p monomer stimulated GTP hydrolysis by Sar1p more than tenfold (Fig. 2A). Stimulation of Sar1p GTPase was approximately stoichiometric with Sec23p, suggesting a single turnover rate enhancement. The monomer form of Sec23p had no intrinsic GTPase activity and did not activate any such activity in association with human myristoylated ARF (*18*) or yeast Ypt1p (*19*). The GTPase-activation activity and biochemical complementation of *sec23*, which was measured by a mutant transport assay, cofractionated throughout the purification of Sec23p (*14*). The enhancement of GTP hydrolysis by Sar1p may be attributed to the activation of a hydrolytic step, such as is achieved by GAPs, or to accelerated nucleotide dissociation, such as is exhibited by exchange factors (*20*). However, dissociation of [^{3}H]GDP (guanosine diphosphate) bound to Sar1p was not influenced by Sec23p (Fig. 2B); hence, Sec23p must be a member of the GAP family.

The GAP activities of the Sec23p monomer and Sec23p complex were comparable (Fig. 3A). The concentrations of Sec23p required for half-maximal activation of Sar1p were 0.16 μM for monomer and 0.08 μM for complex. These values are

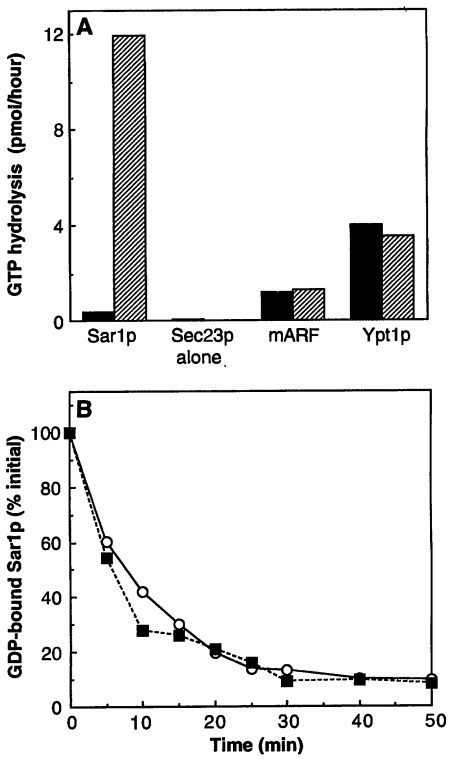

Fig. 2. (**A**) Stimulation by Sec23p of GTPase activity caused by Sar1p. GTPase activities of three GTP-binding proteins (10 pmol each of Sar1p, human myristoylated ARF, and yeast Ypt1p) were assayed in the absence (closed bars) or presence (hatched bars) of Sec23p (10 pmol) monomer at 30°C (*30*). Serving as an additional control, GTP hydrolysis by Sec23p alone (70 pmol). (**B**) The guanidine diphosphate (GDP)–GTP exchange on Sar1p is not stimulated by Sec23p. After incubation with [^{3}H]GDP (2 μM) in 0.5 ml of binding mixture, Sar1p (0.5 μM) was diluted twofold into a reaction that contained GTP (0.2 mM) with (closed squares) or without (open circles) Sec23p monomer (2 μM); time-dependent release of [^{3}H]GDP was monitored (*7*). The time required for dissociation of half of the GTP from Sar1p was 8.5 min both in the presence and in the absence of Sec23p.

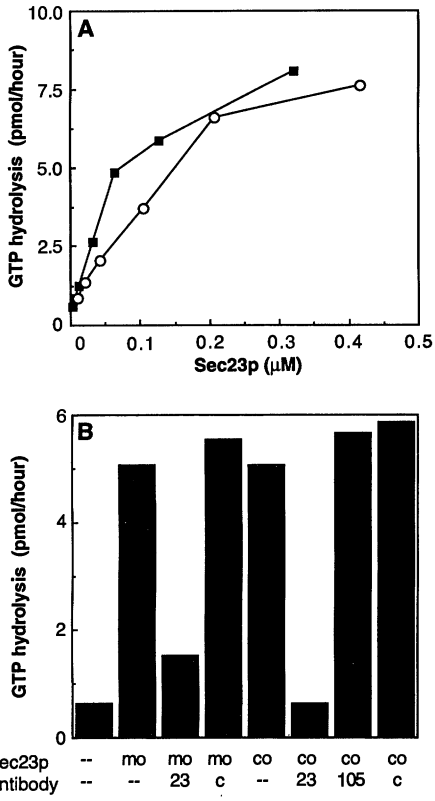

Fig. 3. The Sec23p subunit of the Sec23p-p105 complex exhibits GAP activity. (**A**) The Sar1p GTPase activation by Sec23p monomer and complex. The GAP activity of the Sec23p monomer (open circles) and complex (closed squares) was assayed in a 20-μl GTPase assay mixture that contained 0.5 μM Sar1p in the presence of increasing amounts of Sec23p for 120 min at 30°C. (**B**) Activation of GTPase in the presence of Sec23p and p105 antibodies. The Sec23p monomer or complex (0.5 μM) was incubated with Sar1p (0.5 μM) and with or without various antibodies. Abbreviations: mo, Sec23p monomer; co, Sec23p complex; 23, affinity-purified antibody to native Sec23p (10 μg) (*6*); 105, affinity-purified antibody to p105 (10 μg) (*6*); and c, preimmune immunoglobulin G (IgG) (15 μg) (*6*).

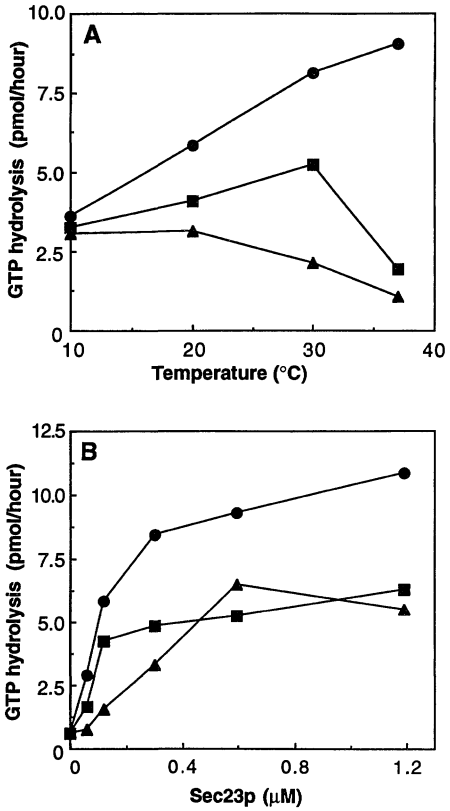

Fig. 4. Activation of GTPase by Sec23p monomer purified from *sec23* mutants. (**A**) The *sec23-1* and *sec23-3* mutant alleles were overexpressed and mutant monomeric forms of Sec23p were purified. Assays of GTPase were conducted with Sar1p (0.5 μM) and monomeric Sec23p (0.5 μM) for 120 min at various temperatures. (**B**) Activity of Sar1p GTPase measured in the presence of various concentrations of wild-type or mutant Sec23p for 120 min at 20°C. Wild-type Sec23p, circles; Sec23-1p, triangles; and Sec23-3p, squares.

close to the half-maximal concentration of the Sec23p complex required to restore transport in a *sec23* mutant lysate (0.05 μM), which suggests a quantitative correlation between the GAP and vesicle budding activities of Sec23p complex. This correlation is supported by the selective effect of inhibitory antibodies on the GAP activity of the Sec23p complex. A polyclonal antibody to Sec23p that inhibits vesicle budding (6) similarly reduced GAP activity (Fig. 3B), whereas antibody to the other subunit of the Sec23p complex (p105), although a potent inhibitor of budding (6), had no effect on the GAP activity (Fig. 3B).

A direct connection between the GAP and vesicle-budding activities of Sec23p was addressed by purification and assay of two thermosensitive mutant forms of the protein. Four independent alleles of *sec23* were cloned; determination of their sequences revealed single missense mutations throughout the gene: S382L (*sec23-1*), G158R (*sec23-2*), R705M (*sec23-3*), and P53S (*sec23-4*) (*21*). Two cloned mutant genes (*sec23-1* and *sec23-3*) were introduced under the control of a regulatable promoter (*GAL1*) to allow overexpression and purification of mutant monomeric proteins. Both proteins displayed reduced GAP activity at temperatures greater than 30°C, which is in contrast to wild-type Sec23p, which was optimally active at 37°C (Fig. 4A). At 20°C, the concentration of Sec23-3p that was required for half-maximal activation (EC_{50}) of Sar1p GTPase was close to the value for wild-type monomer, but the EC_{50} for Sec23-1p was higher (Fig. 4B). This result suggests that Sec23-1p may be impaired in its interaction with Sar1p.

Our results demonstrate that the Sec23p is a GAP that operates on the Sar1p GTPase and not on at least two other small GTP-binding proteins. The Sec23p shows no sequence similarity to Ras-GAP (*22–24*), K-Rev–GAP (*25*), or Bcr-Rac–GAP (*26*). This may be a reflection of Sec23p selectivity. The direct connection between GAP activity and vesicle budding confirms and extends our observation that a nonhydrolyzable analog of GTP, GTPγS, delays vesicle budding from the ER (*4*, *7*).

The other subunit (p105) associated with Sec23p is required for vesicle budding (6) and blocks the toxic effect of excess Sec23p in the budding reaction (Fig. 1), but it has no impact on the GAP activity of Sec23p (Fig. 3A). Perhaps p105 targets Sec23p to a membrane binding site that prevents unregulated interaction with Sar1p. Thus, the Sec23p complex may become available to Sar1p when its function in the promotion of GTP hydrolysis is needed. Without p105 to direct Sec23p to a membrane docking site, the intrinsic GAP activity of Sec23p may be unregulated. Alternatively, the action of Sec23p upon Sar1p may influence the effect p105 has in vesicle budding. Excess Sec23p monomer might compete with the potentially more productive interaction between Sar1p and the Sec23p complex, whereas excess Sec23p complex would merely exchange with endogenous complex and would still play the same role in vesicle budding. Reconstruction of the roles of these purified proteins in the context of a well-defined vesicle-budding reaction offers the unique opportunity to explore the mechanism of GTP-regulated membrane biogenesis.

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12. Vesicle formation was assayed with permeabilized spheroplasts from strain RSY255 [membranes were derived from 3.8 optical density units (at 600 nm) of cells per assay] (*4*). Reactions (25 μl) were supplemented with wild-type cytosol (110 μg) and an adenosine triphosphate (ATP) regeneration system. The Sec23p monomer and complex were prepared as described in (*6*). Pure Sar1p was prepared as described in (*7*).
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21. Mutant alleles of *sec23* were cloned by the gap-repair method (*29*). The mutant alleles and *SEC23* were subcloned with a unique Nco I site located in the ATG of the reading frame (*15*). A 2.9-kb Nco I–Hind III fragment containing the entire reading frame was introduced between a *GAL1* promoter and a *CMK1* terminator on a *CEN* plasmid (YCpGLNT78T). Mutant Sec23p monomer forms were overproduced in each mutant strain grown on galactose medium at 23°C for 12 hours and were purified as before (*6*). S, Ser; L, Leu; G, Gly; R, Arg; M, Met; and P, Pro.
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30. GTPase assays contained 25 mM Hepes-KOH (pH 6.8), 5 mM magnesium acetate, 0.1% Triton X-100, 1 mM ATP, 0.5 mM dithiothreitol (DTT), 2 μM [α-^{32}P-]GTP (10,000 dpm/pmol, Amersham), and protein additions for a final volume of 20 μl (*7*). Yeast Ypt1p was expressed in *Escherichia coli* (*19*). Hydrolysis products were evaluated by thin-layer chromatography (*19*, *27*) and quantified with a PhosphorImager (Molecular Dynamics). Nucleotide exchange was measured at 30°C with a filter binding assay (*7*, *28*).

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