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Hydrolysis of GTP by Sec4 Protein Plays an Important Role in Vesicular Transport and Is Stimulated by a GTPase-Activating Protein in *Saccharomyces cerevisiae*

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Sec4, a GTP-binding protein of the *ras* superfamily, is required for exocytosis in the budding yeast *Saccharomyces cerevisiae*. To test the role of GTP hydrolysis in Sec4 function, we constructed a mutation, Q-79→L, analogous to the oncogenic mutation of Q-61→L in Ras, in a region of Sec4 predicted to interact with the phosphoryl group of GTP. The *sec4-leu79* mutation lowers the intrinsic hydrolysis rate to unmeasurable levels. A component of a yeast lysate specifically stimulates the hydrolysis of GTP by Sec4, while the rate of hydrolysis of GTP by Sec4-Leu79 can be stimulated by this GAP activity to only 30% of the stimulated hydrolysis rate of the wild-type protein. The decreased rate of hydrolysis results in the accumulation of the Sec4-Leu79 protein in its GTP-bound form in an overproducing yeast strain. The *sec4-leu79* allele can function as the sole copy of *sec4* in yeast cells. However, it causes recessive, cold-sensitive growth, a slowing of invertase secretion, and accumulation of secretory vesicles and displays synthetic lethality with a subset of other secretory mutants, indicative of a partial loss of Sec4 function. While the level of Ras function reflects the absolute level of GTP-bound protein, our results suggest that the ability of Sec4 to cycle between its GTP and GDP bound forms is important for its function in vesicular transport, supporting a mechanism for Sec4 function which is distinct from that of the Ras protein.

Numerous GTP-binding proteins have been identified which play a variety of roles in diverse intracellular processes (for a recent review, see reference 8). These proteins bind both GDP and GTP and possess an intrinsic GTPase activity. They can be thought to function in a cycle in which GTP is first bound and then hydrolyzed. Following hydrolysis, the resulting GDP dissociates from the protein, allowing the association of a new GTP. The conformational state of the protein depends upon the nucleotide bound and can thus be regulated by the exchange of GDP for GTP and the hydrolysis of GTP to GDP.

Various lines of evidence have implicated a subfamily of Ras-like GTP-binding proteins in the regulation of vesicular traffic in eukaryotic cells. Sec4, identified by its role in protein transport from the Golgi apparatus to the cell surface in yeast cells (33), was found to share significant sequence similarity with the Ras protein in the domains required for interaction with guanine nucleotides (36). More extensive similarity was seen between Sec4 and the Ypt1 protein of *Saccharomyces cerevisiae*, which has since been shown to play a role in protein transport at an earlier stage of the secretory pathway (2, 3, 37–39). A large number of mammalian homologs of *SEC4* and *YPT1*, known principally as the *rab* genes, have now been identified (11, 12, 20, 27, 45, 52). Many of the proteins encoded by these genes have been found to localize to a unique stage of the exocytotic or endocytic pathways (11–13, 18, 19, 30, 47). For example, Sec4 is associated with post-Golgi secretory vesicles and the plasma membrane (18), Ypt1 is associated with the Golgi (39) as well as endoplasmic reticulum-Golgi carrier vesicles (38), and Rab5, which has been implicated in endosome-endosome fusion (17), has been localized to early endosomes and the plasma membrane. Together, these findings suggest that the Sec4/Ypt1 homologs play analogous roles, each regulating a distinct vesicular transport event.

Bourne (7) has proposed a model for the role of GTP-binding proteins in secretion in which the cycle of GTP binding and hydrolysis serves to ensure the vectorial flow of the vesicular transport process. In this model, a GTP-binding protein on the surface of a carrier vesicle directs delivery of the vesicle to the appropriate acceptor compartment by virtue of an interaction of the protein in its GTP-bound form with an effector protein on the target membrane. Once fusion occurs, release of the GTP-binding protein from the acceptor compartment is coupled to a change in conformation resulting from hydrolysis of the bound GTP. Release allows recycling of the GTP-binding protein to mediate another round of vesicle delivery and fusion. A feature of this model is that the cycle of nucleotide binding and hydrolysis is coupled to a cycle of subcellular localization. Sec4 appears to undergo such a cycle of localization: Sec4 is synthesized as a soluble protein which rapidly associates with secretory vesicles; it is transported to the plasma membrane (presumably by exocytotic fusion) and can then recycle back onto newly formed secretory vesicles (18). A similar cycle has been shown in the case of the Rab3A protein (14).

A key prediction of the model is that a block in the hydrolysis reaction would ultimately block vesicular transport by interfering with the recycling pathway of the GTP-binding protein. This prediction for Sec4 and related proteins is in striking contrast to observations regarding Ras function.

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The general model is that the Ras protein functions as a signal transducer. The higher the level of GTP-bound Ras, the greater the signal for cell proliferation (5). Blocking hydrolysis results in an increase in the GTP-bound pool of Ras, which causes an increase in the stimulation of the downstream effector, leading to oncogenesis. Thus, hydrolysis of GTP is not directly required for Ras function but is required for appropriate down regulation of its function.

In this study, we have determined the consequence of slowing the rate of GTP hydrolysis on the function of Sec4 in vesicular transport. If Sec4 operates by a mechanism similar to that of Ras, slowing hydrolysis should lead to an increase in function due to the accumulation of the protein in its GTP-bound form. However, if Sec4 functions by the mechanism suggested above, blocking hydrolysis will lead to a loss of Sec4 function despite the accumulation of the protein in its GTP-bound form. To block hydrolysis of GTP by Sec4, we have introduced a mutation in Sec4 which is analogous to the Q-61→L oncogenic mutation of Ras. From the crystal structure of Ras, this glutamine residue is thought to play a role in GTP hydrolysis by forming a hydrogen bond with an H_2O molecule which is thus positioned to attack the phosphoryl bond of GTP (24, 35). Since leucine cannot participate in such an interaction, the rate of hydrolysis is inhibited in the mutant.

A consideration in these studies is that in the case of Ras and several other members of the Ras superfamily, the intrinsic GTP hydrolysis rate is greatly stimulated by interaction with one or more GTPase-activating proteins (GAPs) (4, 15, 16, 23, 26, 43, 46, 51). In evaluating the consequence of blocking the hydrolysis of GTP by Sec4, we must consider not only the intrinsic hydrolysis rate but also the stimulated rate resulting from interaction of Sec4 with a GAP. The existence of such an activity is likely, since GAP activities have been described which stimulate the rate of GTP hydrolysis on the Ypt1 protein (6) or on the *rab*3A protein (10). We have identified a GAP activity specific for Sec4 in a yeast cell lysate. The activity exists in both the soluble and membrane fractions of lysed cells but is enriched in specific activity in the membrane fraction. Most importantly, we have found that in vivo, a slowing of GTP hydrolysis by Sec4 leads to a loss of Sec4 function. This finding supports a model of Sec4 function in which the cycle of nucleotide binding and hydrolysis is critical for continued vesicular transport.

MATERIALS AND METHODS

Expression, purification, and characterization of Sec4-Leu79. For overexpression of the *sec4-leu79* gene, the 2.1-kb *Aat*II-*Sal*I fragment containing *GAL1-sec4-leu79* was isolated from pNB288 (49) and subcloned into YEp13, a multicopy yeast vector, which had been digested with *Aat*II and *Xho*I. This plasmid, designated pNB333, was introduced into the yeast strain NY603 (Table 1) by alkali cation treatment (21), and transformants were selected at 25°C on minimal medium supplemented with 2% glucose. Sec4-Leu79 was purified from the soluble fraction of NY802 cells grown on galactose, and wild-type protein was similarly isolated from NY671 cells as previously described (22). The binding of GTPγS to Sec4-Leu79 and the intrinsic rate of GTP hydrolysis of the protein were determined as described previously for the wild-type protein (22).

Genetic analysis of *sec4-leu79*. A *URA3*-disrupting allele of *SEC4* was prepared in vitro by subcloning a 1.1-kb *Sma*I-*Hin*dIII fragment (from pNB114) containing the *URA3* gene into the *Eco*RV and *Hin*dIII sites of *SEC4*, thus replacing the central portion of the *SEC4* coding sequence. This construction was digested with *Bam*HI and *Eco*RI before transforming a *ura3-52 leu2-3,112* diploid strain to Ura$^+$. The disrupted diploid (NY810) was transformed by integration at *LEU2* with plasmids containing either *SEC4* or *sec4-leu79* and sporulated, and tetrads were analyzed. Progeny which were Leu$^+$ Ura$^+$ have the integrated copy of *SEC4* or *sec4-leu79* (at *LEU2*) as the sole source of Sec4 in the cell. A similar approach was used to construct NY943 (Table 1) and NY945 (Table 1).

TABLE 1. Yeast strains

Strain	Genotype
NY768	*MAT***a** *ura3-52 sec1-1 leu2-3,112*
NY770	*MAT***a** *ura3-52 sec2-41 leu2-3,112*
NY776	*MAT***a** *ura3-52 sec5-24 leu2-3,112*
NY778	*MATα ura3-52 sec6-4 leu2-3,112*
NY780	*MATα ura3-52 sec8-9 leu2-3,112*
NY782	*MAT***a** *ura3-52 sec9-4 leu2-3,112*
NY784	*MAT***a** *ura3-52 sec10-2 leu2-3,112*
NY786	*MAT***a** *ura3-52 sec15-2 leu2-3,112*
NY810	*MAT***a**/*MATα ura3-52/ura3-52 sec4Δ::URA3/SEC4 leu2-3,112/leu2-3,112*
NY814	*MAT***a** *ura3-52 sec4Δ::URA3 leu2-3,112::(LEU2 SEC4)*
NY815	*MAT***a** *ura3-52 sec4Δ::URA3 leu2-3,112::(LEU2 sec4-leu79)*
NY835	*MAT***a** *ura3-52 his4-619 sec4Δ::URA3 leu2-3,112::(LEU2 sec4-leu79)*
NY842	*MATα ura3-52 his4-619 sec4Δ::URA3 leu2-3,112::(LEU2 SEC4)*
NY843	*MAT***a** *ura3-52 his4-619 sec4Δ::URA3 leu2-3,112::(LEU2 SEC4)*
NY867	*MAT***a** *ura3-52 his4-619 sec4Δ::URA3 leu2-3,112::(LEU2 sec4-leu79)*
NY943	*MAT***a** *ura3-52 his3-Δ200 leu2-3,112::(SEC4 LEU2) sec4Δ::HIS3*
NY945	*MAT***a** *ura3-52 his3-Δ200 leu2-3,112::sec4-leu79 LEU2 sec4Δ::HIS3*

Invertase secretion. *SEC4* (NY943) and *sec4-leu79* (NY945) strains were transformed to Ura$^+$ with pRB58, a high-copy-number plasmid carrying the *SUC2* gene encoding invertase. The resulting invertase-overproducing strains were grown overnight at 25°C in minimal medium containing 2% glucose. Cells (25 A_{600} units) were harvested by centrifugation and resuspended in minimal medium containing 0.2% glucose. After 30 min of incubation at 25°C, the cells were pelleted and resuspended in 25 ml of minimal medium containing 0.1% glucose, 1 M sorbitol, 50 mM KPO_4 (pH 7.5), 50 mM β-mercaptoethanol, and 1 mg of Zymolyase 100T. Spheroplasts, formed during a 45-min incubation at 37°C, were pelleted and resuspended in 50 ml of minimal medium containing 0.1% glucose, 1 M sorbitol, and 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.6). Following a 4-h incubation at 13°C, the regenerated spheroplasts were pelleted and resuspended in 750 μl of the same medium. After 15 min of incubation, 300 μCi of ^{35}S-Trans label (New England Nuclear) was added; after a 15-min period of labeling, the chase period was initiated by addition of 50 mM cysteine and 50 mM methionine. At time points during the chase at 13°C, 80-μl aliquots were removed and placed on ice. The spheroplasts were pelleted by a 15-s centrifugation in a Microfuge, and the supernatants were separated from the cells. The cells were resuspended in 100 μl of 1% sodium dodecyl sulfate (SDS), the supernatants were brought to 1% SDS, and all samples were heated to

100°C for 3 min and then diluted with 900 μl of phosphate-buffered saline containing 2% Triton X-100. The samples were cleared by a 10-min centrifugation in a Microfuge, and the supernatants were added to tubes containing 2 μl of anti-invertase antibody (the generous gift of S. Ferro-Novick) and incubated for 15 h at 4°C. The immune complexes were recovered on protein A-Sepharose beads (50 μl of a 3% suspension per tube), washed twice with 2 M urea–200 mM NaCl–100 mM Tris (pH 7.2)–1% Triton X-100, washed twice with 0.5% β-mercaptoethanol, and solubilized by boiling in 50 μl of sample buffer. Aliquots were counted by liquid scintillation.

GAP assays. (i) γ-P_i release assays. To measure GTPase activity, a modification of the rapid filtration assay described by Northup et al. (32) was used (16). Sec4 was preincubated with [γ-^{32}P]GTP at 37°C for 30 to 40 min in buffer A (40 mM sodium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 8.0], 5 mM $MgCl_2$, 1 mM dithiothreitol [DTT]) containing 0.1% Lubrol. For most experiments, the preincubation mix contained 300 nM GTP and 300 nM Sec4 (as defined by its [^{35}S]GTPγS binding capacity). The Sec4/[γ-^{32}P]GTP complex was then diluted 10-fold into assay mixes, prepared in duplicate, containing either 1 mg of bovine serum albumin (BSA) per ml or an amount of cell lysate (stated in figure legends). The assay mix contained buffer A and 1 mM GppNHp. Incubation was carried out for 30 to 60 min, and at least three time points were taken unless noted otherwise. Aliquots of the mixes were diluted into 2 ml of ice-cold TNMg buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 25 mM $MgCl_2$) and rapidly filtered through 25-mm-diameter, 0.45-μm-pore-size type HA filters (Millipore), and the filters were washed and counted as described by Kabcenell et al. (22). To account for nonspecific binding of radiolabel to the filter, assay mixes were included to which [γ-^{32}P]GTP (in 1 mg of BSA per ml) was added in the absence of Sec4. These values were subtracted from the datum points obtained in the presence of Sec4. The rate of loss of GTP bound to Sec4 relative to time was determined by plotting the percentage of initial binding (moles of GTP per mole of Sec4) versus time. Using Cricket Graph software, an exponential curve fit was obtained, and the first-order rate constants were used to compare activities. The rate of GTP hydrolysis by Sec4 in the presence of 1 mg of BSA per ml (no added lysate) was measured in each experiment to determine the intrinsic hydrolysis rate. This rate was subtracted from that measured in the presence of lysate to give the stimulated rate.

For the charcoal assay, Sec4 (160 pmol) was preincubated with 320 pmol of [γ-^{32}P]GTP (125 μCi/μmol) in a 100-μl final volume of buffer A containing 0.1% Lubrol. Unbound GTP was removed by gel filtration on a 1.2-ml G-25 column equilibrated in buffer A containing 0.5 mg of BSA per ml. Column fractions of 100 μl were collected, 5 μl of each was counted, and the first peak of radioactivity, corresponding to protein-bound nucleotide, was pooled and recounted. A free-nucleotide control mixture was prepared by substituting 1 mg of BSA per ml for Sec4 in the binding mix. This mixture was diluted with column buffer to give the same concentration of [γ-^{32}P]GTP (counts per minute per microliter) as in the pooled Sec4-GTP column fractions.

Hydrolysis assays were set up in duplicate as described above except that 20 mM unlabeled GTP was added to reduce the effects of contaminating phosphatases. A 100-μl sample of sucrose gradient fraction 9 or 17 (see Fig. 2) or BSA (2 mg/ml in 40% sucrose) was tested in a total volume of 200 μl. Parallel reactions were done with GTP-bound Sec4 or equivalent amounts of free GTP prepared as described above. Samples (50 μl) were removed at 0, 15, and 30 min and placed into 750 μl of a 5% suspension of Norit A charcoal (Fisher Scientific) in 50 mM NaH_2PO_4 on ice. Samples were mixed and spun at 1,400 × *g* for 10 min at 4°C to pellet the charcoal. A 500-μl sample of the supernatant was removed and quantitated by liquid scintillation counting.

(ii) Guanine nucleotide product analysis. To determine the product of the GTPase reaction, Sec4 (1.6 μM) was preincubated with [α-^{32}P]GTP (2.4 μM) for 30 to 40 min at 37°C. The Sec4/[α-^{32}P]GTP complex was separated from free nucleotide by column chromatography as described above. Sec4/[α-^{32}P]GTP was then added to assay mixes at a final concentration of 30 nM Sec4. The assay mixes were incubated at 37°C in buffer A containing 10 mM GTP. From the assay mixes, aliquots of 10 μl were removed over time and added to 10 μl of 2% SDS–40 mM EDTA prewarmed to 65°C and incubated at this temperature for 4 min. To resolve the nucleotides, 2-μl aliquots were spotted on polyethyleneimine-cellulose thin-layer chromatography plates and developed in 1 M LiCl (46). After drying, the plates were exposed to X-ray film. Cold standards were used to determine the migrations of GTP, GDP, and GMP. To determine the distribution of radioactivity between each nucleotide, the plates were cut and quantitated by Cerenkov counting.

Preparation of lysates and cell fractionation. Lysates were prepared essentially as described elsewhere (18, 50). Wild-type yeast cells (700 A_{600} units) were converted to spheroplasts in the presence of 1.4 M sorbitol for osmotic support. The spheroplasts were gently lysed by resuspension in 0.8 M sorbitol–10 mM triethanolamine (TEA) (pH 7.2)–1 mM EDTA. To remove unlysed cells, the lysate was centrifuged at 450 × *g*. The supernatant from this spin, S1, is considered the starting material for calculating recoveries. For differential centrifugation experiments, S1 was centrifuged at 10,000 × *g* for 10 min to give S2 and P2, the low-speed pellet. S2 was centrifuged at 100,000 × *g* for 1 h to yield S3, the cytosolic fraction, and P3, the high-speed pellet.

For sucrose density gradient fractionation, yeast cells were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) with 10 mM potassium phosphate (pH 6.1) to reduce the expression of phosphatases which are induced by phosphate starvation. S1 was spun at 30,000 × *g* for 30 min to prepare P30, which was resuspended in 2.5 ml of 55% (wt/wt) sucrose in 10 mM TEA (pH 7.2)–1 mM EDTA, and 2 ml was loaded at the bottom of a Beckman SW41 tube. Layers of sucrose solutions in the same buffer were loaded on top of one another as follows: 1 ml of 45%, 1 ml of 42.5%, 1 ml of 40%, 1.5 ml of 37.5%, 1.5 ml of 35%, 1.5 ml of 32.5%, 1 ml of 30%, and 1 ml of 25%. The gradients were spun overnight at 4°C for 16 h at 36,000 rpm (170,000 × *g*) in a Beckman SW41 rotor. The rapid filtration assay was performed as described above except that only single tubes with and without Sec4 were tested for each fraction and only 0- and 30-min time points were taken.

Competition experiments. (i) With Sec4/GTPγS. To study the effect of Sec4/GTPγS on Sec4-GAP activity, the rapid filtration assay was set up as described above except that the preincubation buffer contained only 0.005% Lubrol. To obtain the large amounts of protein needed for the competition experiments, Sec4 was expressed and purified from *Escherichia coli* (34). To avoid interference of Tris-HCl (pH 8.0) and NaCl in the GAP competition experiments, Sec4 was diluted 10-fold with 40 mM HEPES (pH 8.0)–5 mM $MgCl_2$–1 mM DTT and concentrated 10-fold by using a

Centricon 10 microconcentrator (Amicon), and the GTPγS capacity was assayed. The Sec4/GTPγS complex was prepared by incubating 19.3 μM Sec4 with 19.3 μM GTPγS for 30 min at 37°C as described for the rapid filtration assay except that the preincubation buffer contained only 0.0078% Lubrol. Lower concentrations of Lubrol were used in the assay, as high concentrations were found to inhibit Sec4-GAP activity (data not shown) and so mask the competition effect of Sec4/GTPγS. The tube containing the complex was then transferred to ice, and aliquots were added to GAP assay mixes. Inhibition of Sec4-GAP activity was determined by comparison with the rate of stimulated GTP hydrolysis in the absence of competing Sec4/GTPγS.

(ii) With Ypt1/GTPγS. To obtain the necessary Ypt1 protein, the *YPT1* gene was expressed in *E. coli* and the resulting Ypt1 protein was purified. The *YPT1* gene was inserted into the pET11d vector, which uses T7 RNA polymerase to direct expression of cloned genes (40). This was achieved by using the polymerase chain reaction to incorporate a *Bsp*HI restriction site at the initiating AUG of *YPT1* and a *Bam*HI restriction site downstream of the termination codon. The construct was then transformed into *E. coli* BL21(DE3). Large-scale induction of Ypt1 protein in *E. coli* and preparation of a sonicated supernatant was carried out as described for bacterial expression of Sec4 (34). The sonicated supernatant was then purified as described by Wagner et al. (48) except that all buffers contained 5 mM instead of 10 mM $MgCl_2$ and gel filtration chromatography was performed before ion-exchange chromatography on DEAE-Sephacel (Pharmacia). The Ypt1 protein was then exchanged into 40 mM HEPES (pH 8.0)–5 mM $MgCl_2$–1 mM DTT as described above for Sec4.

The Ypt1/GTPγS complex was prepared by preincubation of 28 μM Ypt1 with 28 μM GTPγS as described for the preparation of Sec4/GTPγS except that the preincubation buffer contained 2 mM EDTA instead of 5 mM $MgCl_2$. After incubation at 37°C for 30 min, the complex was placed on ice, $MgCl_2$ was added to a final concentration of 5 mM, and then aliquots were added to GAP assay mixes. The effect of Ypt1/GTPγS was determined by comparison with the rate of stimulated GTP hydrolysis on Sec4/GTP in the absence of Ypt1/GTPγS.

Miscellaneous methods. Protein was determined by the method of Bradford (9). SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis were done as described by Walworth and Novick (50) and Goud et al. (18). Thin-section electron microscopy was performed as described by Salminen and Novick (36).

RESULTS

Sec4-Leu79 is defective for GTP hydrolysis. To assess the role of GTP hydrolysis on Sec4 function, a substitution of leucine for glutamine was made at position 79 of Sec4, in the putative phosphoryl binding site (49), based on the prediction that the mutant protein would exhibit a lower rate of GTP hydrolysis. To determine the effects of this alteration on the biochemical properties of the protein, Sec4-Leu79 was purified from a high-speed supernatant fraction derived from galactose-grown yeast cells containing multiple copies of the gene under the control of the inducible *GAL1* promoter (22). The Sec4-Leu79 accumulated in these cells to approximately the same extent as we had previously observed with the wild-type Sec4 protein by using the same expression vector under identical conditions (22). This represents a 200-fold amplification of the soluble Sec4 pool. Thus, the mutant protein is present in vast excess of the wild-type protein produced from the chromosomal copy of the gene.

Analysis of purified wild-type Sec4 protein suggests that it is bound to GDP (22). If the Sec4-Leu79 protein has a lowered rate of nucleotide hydrolysis, purified mutant protein may be partially or completely bound to GTP. While GDP dissociates from wild-type Sec4 at a rate of 0.23 min^{-1}, GTP dissociates from wild-type Sec4 with a rate of 0.002 min^{-1} under our standard assay conditions (22). Thus, the GTP-bound pool of protein does not undergo significant nucleotide exchange during a 60-min assay. The purified Sec4-Leu79 protein, although essentially a single molecular species as revealed by Coomassie blue staining of an SDS-polyacrylamide gel (Fig. 1A), possessed a low specific activity with respect to guanine nucleotide binding (Table 2). While a representative preparation of wild-type Sec4 bound [^{35}S]GTPγS with a stoichiometry of 0.49 mol/mol of protein, Sec4-Leu79 bound at 0.09 mol/mol of protein. This could reflect the presence of a sizable pool of Sec4-Leu79 prebound to GTP or a defect in the ability of Sec4-leu79 to bind nucleotide. Since Sec4 fails to bind [^{35}S]GTPγS at magnesium concentrations below 10^{-7} M (22), a preincubation at low magnesium can be used to remove prebound nucleotide from Sec4. We therefore determined whether we could increase [^{35}S]GTPγS binding activity by briefly exposing Sec4-Leu79 to conditions of lowered magnesium. Sec4 or Sec4-Leu79 was incubated for 4 minutes at 30°C in the presence of 1 mM EDTA to give a final concentration of free magnesium ions of 50 nM. Magnesium chloride was added to elevate the free-magnesium concentration to 5 mM concurrent with the addition of [^{35}S]GTPγS, and the reaction mixtures were incubated for 60 min. While the brief reduction of magnesium levels had no effect on the maximum level of [^{35}S]GTPγS binding achieved by the wild-type protein, binding of the nucleotide to the mutant protein was stimulated 2.6-fold (Table 2). This result is consistent with the presence of GTP in the binding site of approximately 60% of the Sec4-Leu79 protein pool.

To determine whether the Sec4-Leu79 protein is defective in the rate of guanine nucleotide binding, the kinetics of the association of GTPγS to both wild-type and mutant proteins were examined (Fig. 1B). At 30°C, the rate of binding of GTPγS to Sec4-Leu79 was only moderately reduced to 60% of the binding rate of the wild-type protein.

The inherent GTPase activity of Sec4-Leu79 was measured by using the charcoal binding assay to quantitate the release of $^{32}P_i$ over time following incubation of the protein with [γ-^{32}P]GTP as described previously (22). While in this experiment, the wild-type protein hydrolyzed GTP with a rate of 0.0021 min^{-1}, the intrinsic GTPase activity of the mutant protein was not measurable (Fig. 1C). Identical results were obtained following brief preincubation of the mutant protein in the presence of nanomolar concentrations of free magnesium ions (data not shown). Under the conditions of our assay, we could detect a rate one-fifth that of the wild-type protein over the background due to spontaneous hydrolysis of GTP.

***sec4-leu79* is a cold-sensitive loss-of-function mutation.** A strain was constructed in which the mutant allele serves as the sole copy of Sec4 in the cell (see Materials and Methods). The *sec4-leu79* allele was integrated at the *LEU2* locus in a diploid yeast strain containing one copy of *SEC4* disrupted by *URA3*. As a control, an equivalent construction was made by using wild-type *SEC4*. Sporulation of these strains resulted in one-quarter of the haploid progeny containing

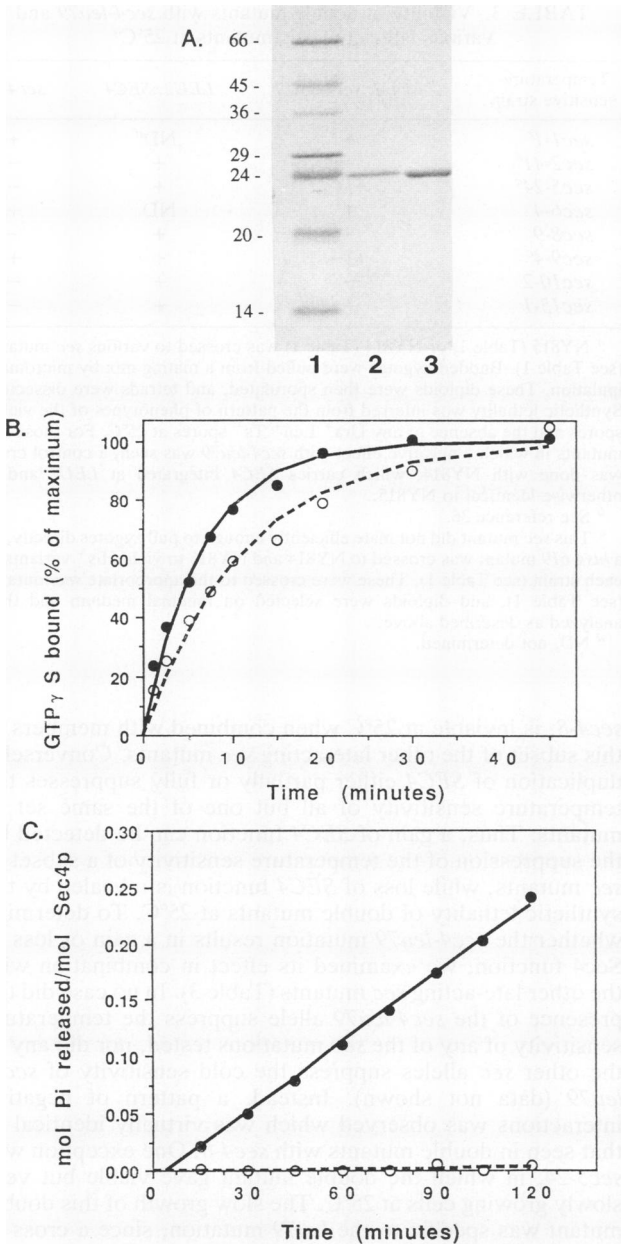

FIG. 1. (A) SDS-PAGE analysis of purified wild-type Sec4 and Sec4-Leu79. Lanes: 1, molecular weight standards (indicated in thousands); 2, 1.25 μg of wild-type Sec4 protein; 3, 1.25 μg of Sec4-Leu79 protein. The proteins were resolved on an SDS–15% polyacrylamide minigel and visualized by staining with Coomassie blue. (B) Kinetics of GTPγS binding. Purified Sec4 proteins (200 nM GTPγS binding sites) in 50 mM HEPES (pH 8.0)–200 mM NaCl–1 mM EDTA–1 mM DTT–5 mM $MgCl_2$–1 mg of BSA per ml–0.1% Lubrol 12A9 were incubated with 2 μM [^{35}S]GTPγS (specific activity, 7,000 cpm/pmol) at 30°C. At the specified times, 25-μl aliquots were withdrawn, and bound GTPγS was quantitated as previously described (22). Data shown are averages of duplicate determinations from a single experiment which is representative of two similar experiments. Maximum binding was approximately 5 pmol. Closed circles, wild-type Sec4, association rate = 0.15 min^{-1}; open circles, Sec4-Leu79, association rate = 0.09 min^{-1}. (C) Intrinsic GTPase activity. Purified Sec4 proteins (Sec4p) (200 nM GTPγS binding sites) were incubated at 30°C with 500 nM [γ-^{32}P]GTP (specific activity, 10,000 cpm/pmol) in 50 mM HEPES (pH 8.0)–200 mM NaCl–1 mM EDTA–10 mM $MgCl_2$–1 mM DTT–0.1% Lubrol. At the times indicated, 50-μl aliquots were removed, and the $^{32}P_i$ liberated was determined by the charcoal binding method previously described (22). Data shown are averages of duplicate determinations from one experiment which is representative of two similar experiments. Closed circles, wild-type Sec4, rate = 0.0021 mol of P_i released per mol of Sec4 per min; open circles, Sec4-Leu79.

TABLE 2. Binding of GTPγS to Sec4 and Sec4-Leu79[a]

Protein	EDTA pretreatment	nmol of GTPγS bound/mg of protein
Sec4	−	20.9
	+	19.7
Sec4-Leu79	−	3.7
	+	9.8

[a] Purified Sec4 (45 ng) or purified Sec4-Leu79 (180 ng) was incubated in 50 mM HEPES (pH 7.6)–200 mM NaCl–1 mM EDTA–5 mM $MgCl_2$–1 mM DTT–1 mg of BSA per ml–0.1% Lubrol for 4 min at 30°C (−EDTA pretreatment). Alternatively, the proteins were incubated in the buffer without $MgCl_2$ and with 5 mM EDTA, resulting in a calculated free-magnesium concentration of 50 nM (22) (+EDTA pretreatment). The concentration of magnesium was then adjusted to 5 mM with $MgCl_2$ prior to the addition of an equal volume of buffer containing 4 μM [^{35}S]GTPγS (specific activity, 7,200 cpm/pmol). Guanine nucleotide binding was quantitated following a 60-min incubation at 30°C.

both the integrated copy of *sec4-leu79* or *SEC4* (marked by leucine prototrophy) and the disrupted copy of *SEC4* (marked by uracil prototrophy). Replica plating of these tetrads showed that all the Leu^+ Ura^+ haploids exhibited near-normal growth at 25 and 37°C. This result is consistent with our earlier observation that the *sec4-leu79* allele is at least partially functional, as determined by its ability to suppress the temperature-sensitive growth defect of a *sec4-8* strain (49). However, the Leu^+ Ura^+ haploids containing the *sec4-leu79* allele, but not those containing the *SEC4* allele, showed impaired growth at 14°C. Measurement of growth rates indicated that at 37°C, both strains grew with a doubling time of 80 min and that at 25°C, *SEC4* cells doubled every 130 min, while *sec4-leu79* cells exhibited a slight defect, doubling every 155 min. At 14°C, the defect was more prominent (Fig. 2A). The doubling time of *SEC4* cells was 8 h; during the first 12 h after the shift to 14°C, *sec4-leu79* cells had a doubling time of 14 h, and during the next 24 h, the doubling time increased to 25 h. A heterozygous *sec4-leu79/SEC4* diploid grew at the same rate as did a *SEC4/SEC4* diploid, both doubling every 8 h at 14°C. Thus, *sec4-leu79* is a recessive, cold-sensitive allele.

To measure the effect of the *sec4-leu79* mutation on protein secretion, we performed pulse-chase studies at 13°C, using regenerated spheroplasts overproducing the secretory protein invertase (see Materials and Methods). After a 15-min pulse-labeling period with ^{35}S-Trans label, a chase period was initiated by the addition of 50 mM cysteine and methionine. At time points, aliquots were removed, the cells were pelleted, and invertase was immunoprecipitated from the medium. The *SEC4* spheroplasts secreted radiolabeled invertase after a 10-min lag, reflecting the transit time of the newly synthesized protein (Fig. 2B), while the *sec4-leu79* spheroplasts secreted invertase only after a 28-min lag. Thus, secretion is not blocked but is substantially slowed. A slowing of the secretory pathway should result in the accumulation of membrane-bound organelles indicative of the stage of the pathway at which the partial block is imposed. Thin-section electron microscopy revealed that the mutant cells accumulated secretory vesicles 100 nm in diameter

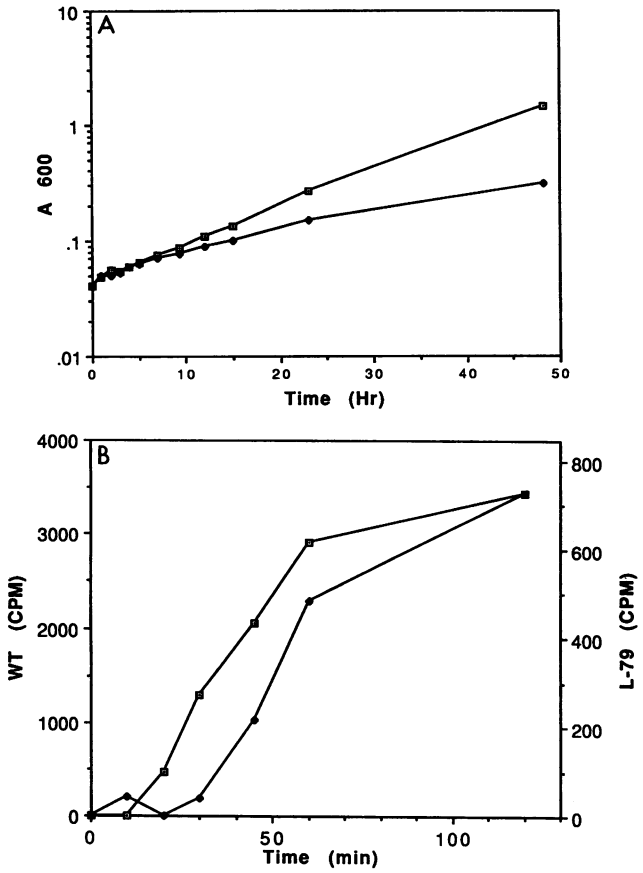

FIG. 2. (A) Evidence that *sec4-leu79* cells are cold sensitive for growth. NY815 (Table 1) (closed symbols) and NY814 (Table 1) (open symbols) were grown to early exponential phase at 25°C in YPD medium and then shifted at time 0 to 14°C. The A_{600} was measured at various time points. (B) Evidence that *sec4-leu79* cells are slowed in the secretion of invertase. NY943 (Table 1) (closed symbols) and NY945 (Table 1) (open symbols) were transformed to Ura^+ with pRB58, a high-copy-number plasmid carrying the *SUC2* gene. The invertase-overexpressing cells were grown at 25°C and shifted to 0.2% glucose for 30 min to start the derepression of invertase synthesis. Spheroplasts were formed and allowed to regenerate at 14°C for 4 h. The spheroplasts were then pulse-labeled with 300 μCi of ^{35}S-Trans label for 15 min. The chase period was initiated by the addition of 50 mM cysteine and methionine, and at various time points aliquots were removed and the cells were pelleted. Invertase was recovered from the supernatants by immunoprecipitation and quantitated by liquid scintillation. The background counts at time 0 were subtracted from all samples, and the scales were adjusted to account for differences in incorporation. WT, wild type.

during an incubation at the lowered temperature (Fig. 3A), while the control strain carrying wild-type *SEC4* did not (Fig. 3B). Western immunoblot analysis of fractionated lysates revealed no significant differences in the level of expression or solubility of Sec4 between the mutant (NY815) and wild-type (NY814) strains at either 25 or 14°C (data not shown). Therefore, the effect of the *leu79* mutation in those cells is unlikely to be a result of defects in expression or membrane association of Sec4-Leu79.

SEC4 is known to have strong genetic interactions with a subset of *sec* mutants which are blocked at the final stage of the secretory pathway (36). The temperature-sensitive allele,

TABLE 3. Viability of double mutants with *sec4-leu79* and various late-acting *sec* mutants at 25°C[a]

Temperature-sensitive strain	*LEU2::sec4-leu79*	*LEU2::SEC4*	*sec4-8*[b]
sec1-1[c]	+	ND[d]	+
sec2-41[c]	−	+	−
sec5-24[c]	+/−	+	−
sec6-4	+	ND	+
sec8-9	−	+	−
sec9-4[c]	+/−	+	+
sec10-2	−	+	−
sec15-1	−	+	−

[a] NY815 (Table 1) or NY814 (Table 1) was crossed to various *sec* mutants (see Table 1). Budded zygotes were pulled from a mating mix by micromanipulation. These diploids were then sporulated, and tetrads were dissected. Synthetic lethality was inferred from the pattern of phenotypes of the viable spores and the absence of any Ura^+ Leu^+ Ts^- spores at 25°C. For those *sec* mutants in which a negative effect with *sec4-leu79* was seen, a control cross was done with NY814, which carries *SEC4* integrated at *LEU2* and is otherwise identical to NY815.

[b] See reference 36.

[c] This *sec* mutant did not mate efficiently enough to pull zygotes directly, so a *his4-619* mutant was crossed to NY814 and NY815 to yield His^- variants of each strain (see Table 1). These were crossed to the appropriate *sec* mutants (see Table 1), and diploids were selected on minimal medium and then analyzed as described above.

[d] ND, not determined.

sec4-8, is inviable at 25°C when combined with members of this subset of the other late-acting *sec* mutants. Conversely, duplication of *SEC4* either partially or fully suppresses the temperature sensitivity of all but one of the same set of mutants. Thus, a gain of *SEC4* function can be detected by the suppression of the temperature sensitivity of a subset of *sec* mutants, while loss of *SEC4* function is revealed by the synthetic lethality of double mutants at 25°C. To determine whether the *sec4-leu79* mutation results in a gain or loss of Sec4 function, we examined its effect in combination with the other late-acting *sec* mutants (Table 3). In no case did the presence of the *sec4-leu79* allele suppress the temperature sensitivity of any of the *sec* mutations tested, nor did any of the other *sec* alleles suppress the cold sensitivity of *sec4-leu79* (data not shown). Instead, a pattern of negative interactions was observed which was virtually identical to that seen in double mutants with *sec4-8*. One exception was *sec5-24*, in which the double mutant gave viable but very slowly growing cells at 25°C. The slow growth of this double mutant was specific to the *leu79* mutation, since a cross to NY814, which is isogenic to NY815 except for this mutation, showed no growth-impaired progeny. All of the *sec* mutants which showed synthetic lethality in crosses to NY815 were also crossed to NY814 to ensure that the lethality was due to the *sec4-leu79* mutation rather than an effect of integration of the *sec4* allele at the *LEU2* locus. Together, these data indicate that the *sec4-leu79* mutation, unlike the analogous mutation in *ras*, has a negative rather than positive effect on overall function and establish this allele of *sec4* as a loss of function mutation.

A Sec4 GTPase-stimulating activity is associated with a membrane fraction. Studies of oncogenic *ras* mutations indicate that the intrinsic hydrolysis rate is not as relevant in vivo as the stimulated rate achieved by interaction with a GAP (28). For this reason, we sought to identify an activity in a yeast lysate which would stimulate the hydrolysis of GTP bound to Sec4 in a manner analogous to that of the GAP specific for $p21^{ras}$. To assay for stimulation of GTP hydrolysis activity by Sec4, [γ-^{32}P]GTP was prebound to Sec4 and

the complex was incubated at 37°C in the presence or absence of a yeast lysate (see Materials and Methods). Protein-bound radioactivity was determined following rapid filtration. This assay cannot distinguish between stimulation of GTP hydrolysis and stimulation of nucleotide dissociation. However, data from the charcoal binding assay (see below) establish that loss of protein-associated radioactivity reflects the hydrolysis of [γ-^{32}P]GTP and subsequent release of free $^{32}P_i$ from Sec4. Therefore, we will refer to the activity as Sec4-GAP activity.

When Sec4/[γ-^{32}P]GTP is incubated at 37°C for 30 min, a rate of hydrolysis of 0.001 mol of GTP hydrolyzed per mol of Sec4 per min is observed. This rate is comparable to that obtained at 30°C in the charcoal binding assay, which measures the production of free P_i (22). As shown in Fig. 4A, the rate of release of ^{32}P is stimulated (in this case, 5.6-fold) in the presence of a yeast lysate (127 μg of a total lysate of osmotically lysed yeast spheroplasts; see Materials and Methods). Both the intrinsic GTPase activity of Sec4 and the stimulated activity are inhibited by incubation on ice. Pretreatment of the extract at 95°C for 15 min abolishes the stimulating activity (data not shown).

Sec4 is stable to proteolysis during a 60-min incubation at 37°C, as determined by Western blot (immunoblot) analysis (data not shown). Thus, the loss of ^{32}P bound to the filters is not due to degradation of Sec4 and release of [γ-^{32}P]GTP. Like p21^{ras} GAP, this activity is sensitive to salt concentration. At 60 mM NaCl, 70% of the activity is detected, whereas at 120 mM NaCl, only 46% is observed (data not shown). The stimulating activity is inhibited to a small extent (20% inhibition) by the addition of 20 mM free GTP in the assay. Changing the pH at which the assay is done to 7.4 or 6.8 did not significantly alter the level of activity (data not shown).

A 450 × *g* supernatant of a total yeast lysate (S1) was subfractionated by differential centrifugation, and the various fractions were assayed for stimulation of Sec4 GTPase (Fig. 4B). While the 100,000 × *g* supernatant cytosolic fraction (S3) contains 23% of the total GAP activity assayed in S1, the sum of the 10,000 × *g* and 100,000 × *g* membrane fractions (P2 plus P3) accounts for 50% of the total GAP activity. Expressed as specific GAP activities, the P2 and P3 fractions are enriched 1.8- and 1.3-fold, respectively, relative to the S1 starting lysate, while the specific activity of S3 is decreased. For most of the experiments discussed below, a membrane fraction prepared by centrifugation of S1 at 30,000 × *g* for 30 min was used. This P30 was found to contain 48% of the total S1 activity. Only an additional 7% pellets at 100,000 × *g*, and this material is of lower specific activity than that found in P30 (data not shown). Stimulated release of ^{32}P increases with protein concentration in a linear fashion (Fig. 4C).

To determine whether the pelletable Sec4-GAP activity was membrane bound or in a proteinaceous particle, the activity was partially purified on a sucrose density gradient. When the P30 fraction was loaded at the bottom of the gradient (see Materials and Methods), the majority of the Sec4-GAP activity, as measured by the filter binding assay, floated up and was found in two peaks (Fig. 5). That the pelletable Sec4-GAP activity floats in this gradient confirms that it is actually membrane bound.

To formally establish that the observed activity was stimulating GTP hydrolysis rather than nucleotide release, we tested the sucrose gradient fractions for their ability to stimulate Sec4-dependent hydrolysis by using the charcoal binding method, which measures phosphate production. The

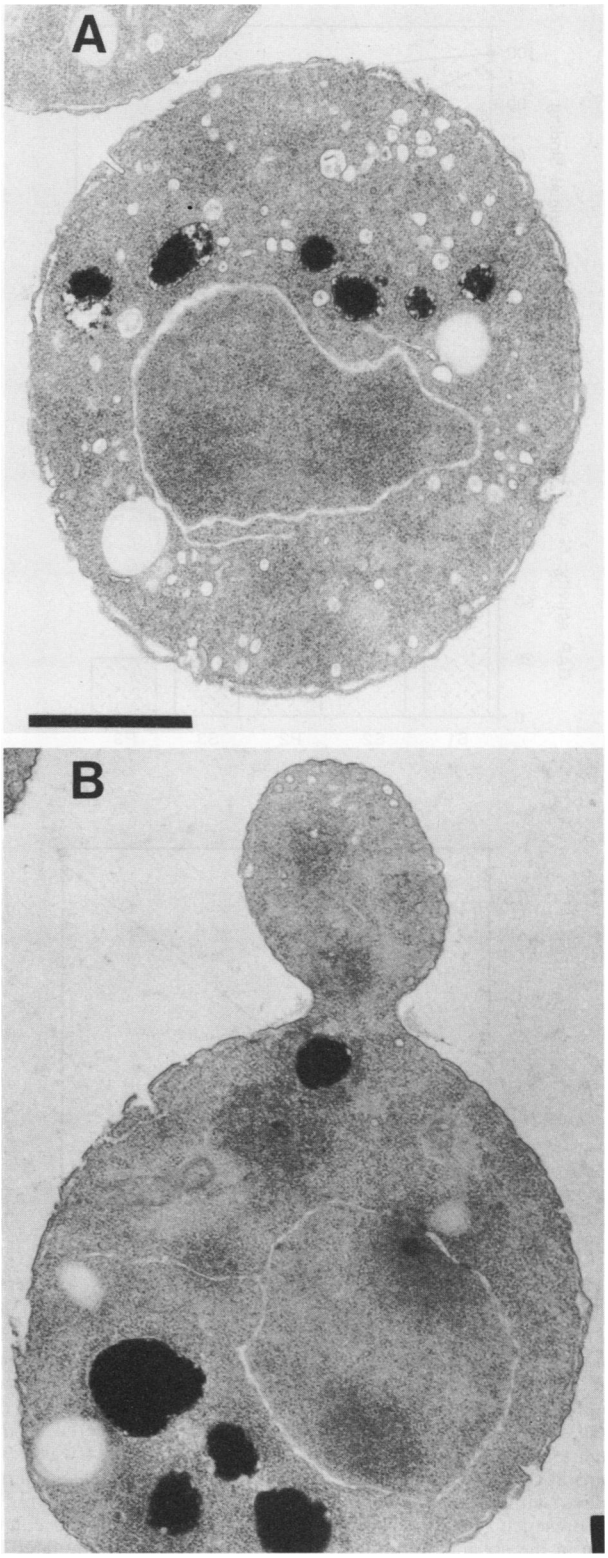

FIG. 3. Accumulation of secretory vesicles by *sec4-leu79* cells. NY815 (Table 1) (A) and NY814 (Table 1) (B) cells were grown to early exponential phase in YPD medium at 25°C, then the cultures were shifted to 14°C, and after 24 h, the cells were fixed and processed for thin-section electron microscopy. The bar equals 1 μm.

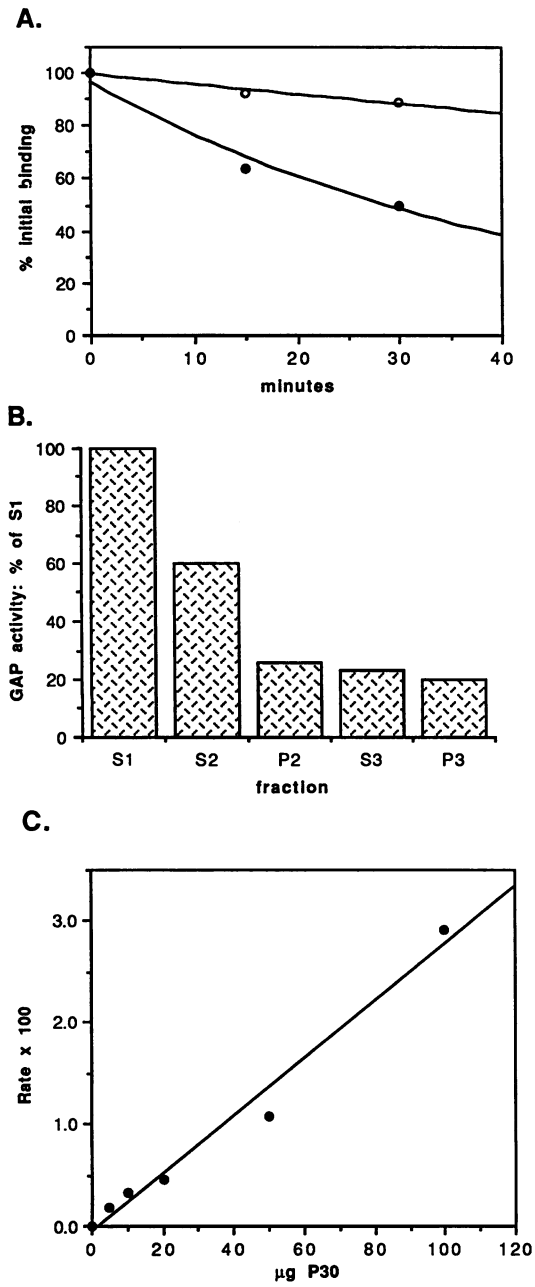

FIG. 4. (A) Stimulation of GTP hydrolysis by lysate. [γ-^{32}P]GTP (300 nM) was prebound to Sec4 (300 nM GTPγS binding sites) and then diluted 10-fold into assay mixes at 0°C. As a control, an equal amount of free [γ-^{32}P]GTP was added to a parallel set of assay mixes. The assay mixes were then incubated at 37°C. Duplicate aliquots, removed after mixing at 0°C and over time during the incubation at 37°C, were diluted into cold buffer and filtered. In this experiment, the initial binding in the presence of Sec4 was 4,917 cpm; in the absence of Sec4, the value was 216 cpm. The control values (without Sec4) were subtracted from the values measured in the presence of Sec4. The rate of decrease in filter-bound counts seen in the absence of added lysate (open circles) is stimulated by the addition of 127 µg (2.5 mg/ml) of a 450 × *g* supernatant of a total cell lysate (S1) (closed circles). (B) Detection of GAP activity in both membrane and cytosol fractions. S1 was fractionated by centrifugation at 10,000 × *g* for 10 min to yield S2 and P2. S2 was further fractionated at 100,000 × *g* for 1 h to yield a high-speed membrane fraction (P3) and cytosol (S3). The exponential rate of decay of filter-bound counts as a function of time observed in the presence of Sec4 and 1 mg of BSA per ml (no added lysate) was measured to determine the intrinsic hydrolysis rate. This rate was subtracted from that measured in the presence of the different fractions to give the stimulated rate. The data shown represent the total activity of each fraction and are averages of three assays done on independent fractionations of wild-type cells. Approximately 50% of the total activity measured in S1 is recoverable in membrane fractions if P2 and P3 are summed. (C) Linearity of GAP activity over a range of protein concentrations. A 30,000 × *g* membrane fraction (P30) was used as the source of GAP activity in 50-µl assay mixes. The exponential rate of decay of filter-bound counts as a function of time was used as a measure of the activity. GAP activity reflects the rate of loss of filter-bound counts in the presence of membranes minus the intrinsic GTPase rate measured for Sec4 alone. At 100 µg of added membranes, the stimulated rate of decay was 0.0291 min^{-1} and represents a stimulation of 25-fold over the intrinsic rate of 0.00115 min^{-1}, as measured in this experiment.

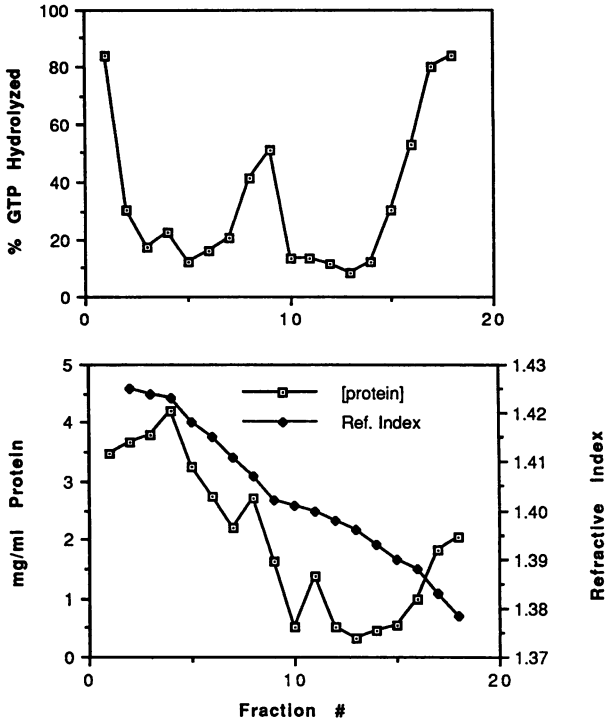

FIG. 5. Sucrose density gradient analysis of Sec4-GAP. A 30,000 × *g* pellet fraction derived from 700 A_{600} units of cells was resuspended in 55% sucrose–10 mM TEA (pH 7.2)–1 mM EDTA and loaded at the bottom of the tube. Sucrose solutions were overlaid in a stepwise manner (25% at the top) as described in Materials and Methods. After centrifugation, fractions were collected from the bottom of the tube. Fraction 1 is the pellet fraction, and fraction 18 is the top of the gradient. Sec4-GAP activity was determined as described in Materials and Methods for each fraction by the rapid filtration method, taking 0- and 30-min time points for each fraction.

Sec4/[γ-^{32}P]GTP complex was first resolved from free GTP by chromatography on a Sephadex G-25 column. Then Sec4/[γ-^{32}P]GTP or an equivalent amount of [γ-^{32}P]GTP was added to assay tubes containing BSA or a sucrose gradient fraction in the presence of 20 mM cold GTP (Fig. 6A). In the absence of any subcellular fraction, Sec4 has low intrinsic hydrolytic activity (+Sec4p/BSA columns). However, in the presence of the sucrose gradient fractions containing the

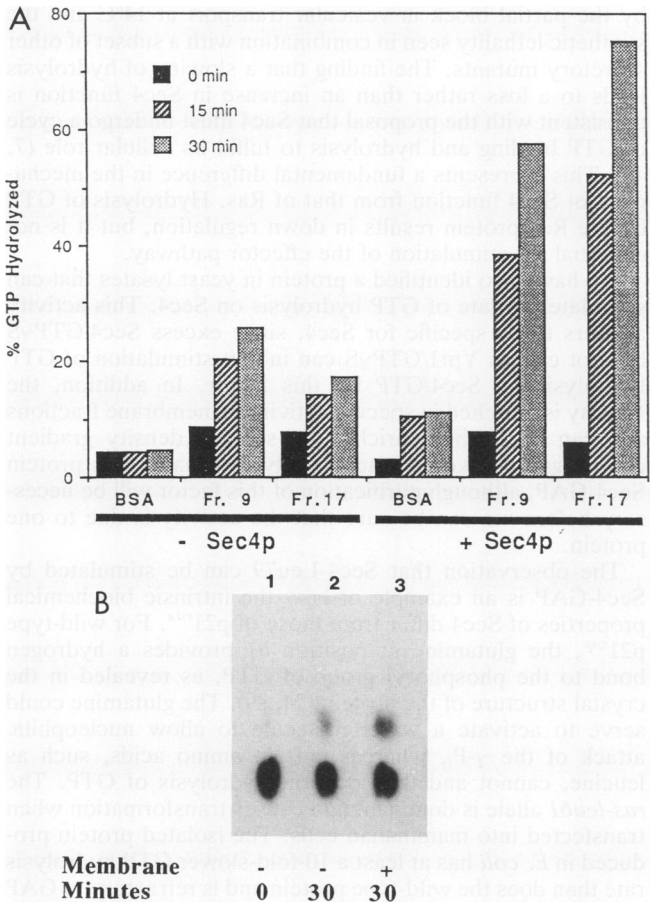

FIG. 6. (A) Dependence of high-level GTPase activity on the presence of both Sec4 protein (Sec4p) and yeast membrane fractions. In +Sec4p reactions, the Sec4/[γ-^{32}P]GTP complex was purified on a Sephadex G-25 column and then incubated with sucrose gradient-purified membrane fractions (fractions 9 and 17 in Fig. 4). In −Sec4p reactions, an equivalent amount of unbound [γ-^{32}P]GTP was added to a parallel set of reactions. Aliquots were removed after 0-, 15-, and 30-min incubations at 37°C, and the amount of $^{32}P_i$ released was determined by the charcoal binding method (see Materials and Methods). (B) Conversion of GTP to GDP in the presence of Sec4 or of Sec4 and a sucrose gradient fraction. Sec4/[α-^{32}P]GTP complex was purified on a Sephadex G-25 column and then incubated in GAP assay mixes. Aliquots were removed over time and incubated at 65°C in the presence of 20 mM EDTA–1% SDS for 4 min. Nucleotides were separated on polyethyleneimine-cellulose plates and visualized by autoradiography as described in Materials and Methods. At 0 min, only GTP is found associated with Sec4. After 30 min at 37°C, a small fraction of the GTP is converted to GDP. Conversion of GTP to GDP is stimulated (~3-fold in the experiment shown) by including 6.1 μg of the lighter membrane fraction from a sucrose density gradient (0.08 mg/ml in the assay mix).

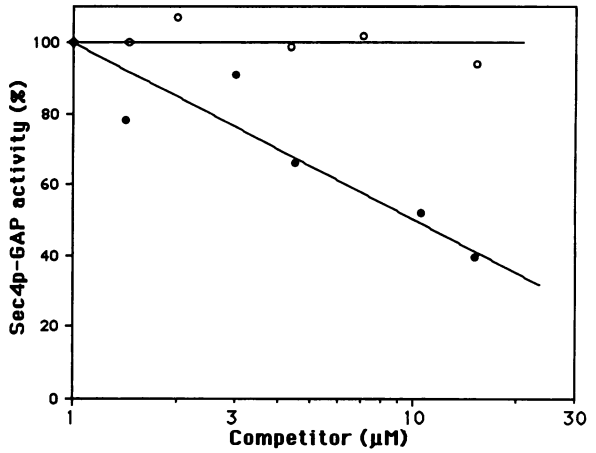

FIG. 7. Inhibition of Sec4-GAP activity by Sec4/GTPγS but not by Ypt1/GTPγS. Competition experiments were performed by adding increasing amounts of either Sec4 protein (Sec4p) or Ypt1, prebound to GTPγS, into the GAP assay mix as described in Materials and Methods. Inhibition of GAP activity is expressed as a percentage comparison with the Sec4-GAP activity detected with 20 nM Sec4/[γ-^{32}P]GTP in the absence of competitor. The source of Sec4-GAP was a 30,000 × *g* pellet fraction (75 μg per assay). Closed circles, Sec4 prebound to GTPγS; open circles, Ypt1 prebound to GTPγS.

peak activity (9 or 17), the hydrolytic activity is dramatically stimulated. In the absence of Sec4, hydrolysis of free GTP by the membrane fractions is low. Thus, we can exclude the possibility that the stimulation seen by addition of membranes to Sec4-GTP is due to release of GTP and subsequent hydrolysis by a contaminating hydrolytic activity, since even complete release of GTP at the start of the incubation could not result in the level of hydrolysis observed.

To confirm that the putative Sec4-GAP activity is stimulating conversion of GTP to GDP, product analysis of the nucleotide was determined by thin-layer chromatography. The assay was set up as described above except that [α-^{32}P]GTP rather than γ-labeled GTP was used. For this reaction, the denser peak of Sec4-GAP activity from a gradient prepared equivalently to that seen in Fig. 5 was used as the source of Sec4-GAP. Separation of GTP from GDP by chromatography on polyethyleneimine-cellulose plates was carried out as described in Materials and Methods. A representative autoradiogram (Fig. 6B) compares the hydrolysis of GTP to GDP by Sec4 in the presence or absence of Sec4-GAP. The GTP bound initially to Sec4 (lane 1) is slowly converted to GDP after 30 min (lane 2) by the intrinsic GTPase activity of Sec4. Conversion of GTP to GDP is stimulated by the sucrose gradient fraction. Free GTP in the absence of Sec4 is stable when added to a sucrose fraction (not shown). Quantitation of the thin-layer chromatography plates indicates that the stimulation with this amount of membrane was about 3.5-fold over the rate of loss of GTP in the absence of added membranes. Therefore, the activity defined as Sec4-GAP does indeed stimulate the conversion of GTP to GDP in the presence of Sec4.

To study the interaction of Sec4 with Sec4-GAP, GAP assays were performed in the presence of excess Sec4/GTPγS. As can be seen in Fig. 7, the presence of increasing amounts of Sec4/GTPγS was found to inhibit the GAP-stimulated hydrolysis of Sec4/GTP, with 50% inhibition at 10.5 μM Sec4/GTPγS. Addition of 20 μM free GTPγS does not inhibit the activity (not shown).

To study the specificity of the GAP activity for Sec4, we tested the effect of Ypt1/GTPγS on the interaction between Sec4/GTP and Sec4-GAP. The *YPT1* gene was expressed in *E. coli*, and the Ypt1 protein was purified (see Materials and Methods). This was found to be 80 to 90% pure as analyzed by SDS-PAGE and Coomassie brilliant blue staining of the gel and demonstrated intrinsic nucleotide binding and hydrolysis properties similar to those described by Wagner et

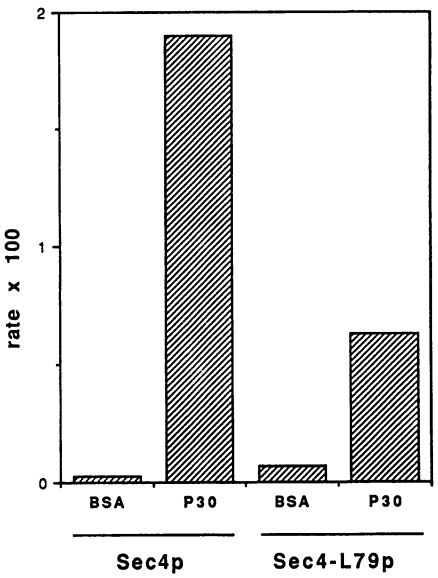

FIG. 8. Stimulation of GTP hydrolysis by Sec4-Leu79 by Sec4-GAP but not to the same degree as by wild-type Sec4. Sec4 or Sec4-Leu79 was incubated with [γ-^{32}P]GTP and then diluted into assay mixes with 50 μg (1 mg/ml in assay mix) of a 30,000 $\times$ *g* pellet fraction (P30 columns) or with BSA (BSA columns). Equivalent amounts of [γ-^{32}P]GTP-bound Sec4 or Sec4-Leu79 were used, and aliquots were removed at 0, 15, and 30 min. The rates of hydrolysis were calculated as described in Materials and Methods.

al. (48). It was shown that this bacterially expressed Ypt1 protein (up to the maximum possible concentration of 15 μM) did not compete with Sec4/GTP for Sec4-GAP (Fig. 7). In addition, it was found that the rate of GTP hydrolysis by Ypt1 was not stimulated by incubation with a P30 fraction containing Sec4-GAP activity (data not shown). Thus, it appears that Sec4 interacts with a distinct GAP (Sec4-GAP) which is specific for Sec4.

Having established the presence of a Sec4-GAP activity, we tested the sensitivity of the Leu79 mutant protein to stimulation. The wild-type and mutant Sec4 proteins were incubated with [γ-^{32}P]GTP prior to the addition of membranes, and the loss of protein bound radioactivity was quantitated over time. Under these conditions, a stimulated GTPase activity was observed for Sec4-Leu79 which reached 30% of that of the stimulated wild-type protein (Fig. 8). This activity was not due to small amounts of wild-type Sec4 present in our mutant protein preparation, since similar results were obtained when Sec4-Leu79 was isolated from cells containing the *sec4-8* allele as the only other copy of *SEC4* (data not shown).

DISCUSSION

A key finding presented here is that lowering the level of GTP hydrolysis by introducing *sec4-leu79* as the sole source of Sec4 results in a net loss of Sec4 function rather than a gain of function. In the case of Ras, an impairment of hydrolysis leads to an increase in the fraction of p21^{ras} in the GTP-bound form, and this in turn leads to an increase in Ras function, resulting in a signal for cell proliferation. Impairing the hydrolysis of GTP by Sec4 leads to an increase in the GTP-bound pool in a Sec4-Leu79-overproducing strain and presumably does so at normal levels of expression as well. However, this leads to a loss of Sec4 function, as indicated by the partial block in vesicular transport at 14°C and the synthetic lethality seen in combination with a subset of other secretory mutants. The finding that a slowing of hydrolysis leads to a loss rather than an increase in Sec4 function is consistent with the proposal that Sec4 must undergo a cycle of GTP binding and hydrolysis to fulfill its cellular role (7, 49). This represents a fundamental difference in the mechanism of Sec4 function from that of Ras. Hydrolysis of GTP by the Ras protein results in down regulation, but it is not essential for stimulation of the effector pathway.

We have also identified a protein in yeast lysates that can stimulate the rate of GTP hydrolysis on Sec4. This activity appears to be specific for Sec4, since excess Sec4/GTPγS but not excess Ypt1/GTPγS can inhibit stimulation of GTP hydrolysis on Sec4/GTP by this factor. In addition, the activity is enriched in specific activity in membrane fractions and can be further enriched by sucrose density gradient centrifugation. We have tentatively designated this protein Sec4-GAP, although purification of this factor will be necessary before we can be sure that the activity is due to one protein.

The observation that Sec4-Leu79 can be stimulated by Sec4-GAP is an example of how the intrinsic biochemical properties of Sec4 differ from those of p21^{ras}. For wild-type p21^{ras}, the glutamine at position 61 provides a hydrogen bond to the phosphoryl group of GTP, as revealed in the crystal structure of the protein (24, 29). The glutamine could serve to activate a water molecule to allow nucleophilic attack of the γ-P_i, whereas certain amino acids, such as leucine, cannot and thus prevent hydrolysis of GTP. The *ras-leu61* allele is dominant and causes transformation when transfected into mammalian cells. The isolated protein produced in *E. coli* has at least a 10-fold-slower GTP hydrolysis rate than does the wild-type protein and is refractory to GAP stimulation (1, 16). Nonetheless, p21-Leu61 can compete with wild-type p21^{ras} for interaction with GAP, which suggests that although it interacts with GAP, it cannot hydrolyze GTP (25). Sec4-Leu79 has no detectable intrinsic GTP hydrolysis activity. However, unlike p21-Leu61, Sec4-Leu79 can be stimulated to hydrolyze GTP. Since Sec4-Leu79 can serve as the sole copy of Sec4 in the cell, it is reasonable to assume that the mutant protein can interact with components of the secretory machinery necessary for Sec4 function, even though the GTP hydrolysis activity is diminished. The fact that the cells exhibit a cold-sensitive phenotype suggests that the mutant protein does not function as efficiently as the wild-type protein, and it is likely that the defective GTPase activity is responsible for this phenotype.

Because hydrolysis of GTP by Sec4 is necessary for its function, we might expect that a defect in Sec4-GAP would lead to a block in vesicular transport. A number of temperature-sensitive mutants which are defective at various stages of the secretory pathway exist (31, 33). All of the *sec* and *bet* mutant strains which are defective in endoplasmic reticulum-to-Golgi transport, intra-Golgi transport, or post-Golgi-to-plasma membrane transport were tested for GAP activity. The cells were grown at the permissive temperature, lysates were prepared, and then the lysates were shifted in vitro to 37°C before assays for GAP activity. None of the mutant lysates were significantly defective for Sec4-GAP activity (not shown).

The only other yeast GAP activity which has been biochemically demonstrated to date is attributed to the *IRA1* and *IRA2* gene products, which function as negative regulators of the *RAS1* and *RAS2* gene products. The phenotype of

cells carrying disruptions of the *IRA1* and *IRA2* genes is similar to that of cells expressing $RAS2^{Val-19}$, they have an elevated level of cyclic AMP, and they lose viability as they approach stationary phase (42, 44). Preparation of lysates from strains harboring single or double disruptions of Ira1 or Ira2 have somewhat lower Sec4-GAP activity than do wild-type lysates (data not shown), yet activity is still present in the double-disruption strain. Furthermore, lysates prepared from a strain transformed with a plasmid (pKT16) which overexpresses the GAP domain of Ira2 (41) failed to show any increase in Sec4-GAP activity relative to lysates from the untransformed strain (data not shown). We presume, therefore, that the reduced activity seen in the deletion strains reflects the altered growth and physiological properties of these mutants rather than a specific effect of Ira1 or Ira2 on Sec4-GAP activity.

Purification of the proteins which influence the biochemical properties of Sec4 should help to elucidate the mechanism by which Sec4 mediates vesicular traffic in budding yeast. Our original model (49) for Sec4 function suggests that GTP hydrolysis could be coupled to release of Sec4 from the plasma membrane. Once GAP and other proteins regulating Sec4 function are localized at the subcellular level, a more detailed understanding of the coupling between the biochemical functions and localization of Sec4 will be achieved. This may serve as a useful model for the roles of other low-molecular-weight GTP-binding proteins thought to regulate vesicular transport events in all eukaryotic cells.

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