The EMBO Journal vol.8 no.6 pp.1685 - 1693, 1989

Mutational analysis of *SEC4* suggests a cyclical mechanism for the regulation of vesicular traffic

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Communicated by P.de Camilli

Mutant alleles of *SEC4*, an essential gene required for the final stage of secretion in yeast, have been generated by *in vitro* mutagenesis. Deletion of the two cysteine residues at the C terminus of the protein results in a soluble non-functional protein, indicating that those two residues are required for normal localization of Sec4p to secretory vesicles and the plasma membrane. A mutant allele of *SEC4* generated to mimic an activated, transforming allele of H-*ras*, as predicted, does not bind GTP. The presence of this allele in cells containing wild-type *SEC4* causes a secretory defect and the accumulation of secretory vesicles. The results of genetic studies indicate that this allele behaves as a dominant loss of function mutant and as such prevents wild-type protein from functioning properly. We propose a model in which Sec4p cycles between an active and an inactive state in order to mediate the fusion of vesicles to the plasma membrane.
Key words: GTP binding protein/secretion/Sec4p/ras homolog/vesicular traffic

Introduction

The *SEC4* gene of *Saccharomyces cerevisiae* encodes a 24 kd GTP-binding protein which plays an essential role in the final stage of secretion (Salminen and Novick, 1987). Post-Golgi secretory vesicles are transported to a specific region of the plasma membrane called the bud and subsequently fuse with this membrane (Novick *et al.*, 1980; Salminen and Novick, 1987). A recessive temperature-sensitive mutation in *sec4* causes cells to accumulate secretory vesicles at the restrictive temperature (Novick *et al.*, 1980). The localization of the wild-type protein, Sec4p, on the cytoplasmic surface of secretory vesicles is consistent with it participating in the regulation of transport or recognition between secretory vesicles and the plasma membrane (Goud *et al.*, 1988).

GTP-binding proteins have been shown to mediate diverse cellular processes. Members of this family of proteins include factors involved in protein synthesis such as elongation factor Tu (EF-Tu), the G proteins involved in signal transduction, and the product of the *ras* oncogene (see Kaziro, 1978; Barbacid, 1987; Gilman, 1987, for reviews). These proteins share the ability to assume different conformations depending on whether GTP or GDP is bound and therefore each can act as a regulatory switch. Generally, in the GDP bound form, the protein is in the resting or inactive state. Exchange of GTP for GDP results in activation of the protein until the GTP is hydrolyzed. The intrinsic hydrolysis activity as well as the further exchange of the bound GDP for a new molecule of GTP are processes which can be affected by interaction with other proteins (Trahey and McCormick, 1987; Neer and Clapham, 1988).

Two basic mechanisms by which GTP-binding proteins perform their functions have been described. The mammalian G proteins, heterotrimeric protein complexes responsible for the transduction of extracellular signals, couple cell surface receptors to intracellular enzymes which produce regulatory molecules within the cell. In the GTP bound state, the activated G protein stimulates an effector molecule to increase the intracellular level of such second messengers as cAMP, Ca^{2+} or inositol lipid products. Thus, the G protein initiates the amplification of the signal, beginning a cascade of events which leads to a cellular response (Gilman, 1987).

An alternative mechanism of action for a GTP-binding protein is to ensure that a reaction occurs in a unidirectional fashion. The bacterial protein synthesis factor EF-Tu mediates the attachment to and subsequent release of a macromolecular complex from the ribosome. The process of protein synthesis is stopped *in vitro* in the presence of the non-hydrolyzable GTP analog, GTP-γ-S. EF-Tu, trapped in the GTP bound state, cannot change conformation and come off of the ribosome to allow another round of delivery to occur. Thus, for a single round of nucleotide binding and hydrolysis, a single event (delivery) takes place (Kaziro, 1978).

We have taken a genetic approach to determine if either of these two general mechanisms could account for the regulation of secretion by Sec4p. Since a large amount of information is available about the structure of GTP-binding proteins and the function of specific amino acids within that structure, we have undertaken a mutational analysis of Sec4p based on well-characterized mutations in other proteins (Jurnak, 1985; LaCour *et al.*, 1985; de Vos *et al.*, 1988). Point mutations that alter the ability of p21 H-*ras* to bind or hydrolyze GTP are known and several of these confer upon the protein the capacity to induce transformation of the mammalian cells in which they are expressed (Barbacid, 1987).

From mutational studies of the yeast RAS proteins that regulate the entry of cells into the mitotic cell cycle at G_1, it is clear that an intact carboxy terminus is required for proper localization and normal function of these proteins (Fujiyama and Tamanoi, 1986; Deschenes and Broach, 1987). The same is true for oncogenic mammalian *ras* proteins, which have transforming ability only when attached to the inner surface of the plasma membrane, the usual location of the normal cellular version of the protein (Willumsen *et al.*, 1984a,b). Ypt1p, a yeast *ras*-like protein

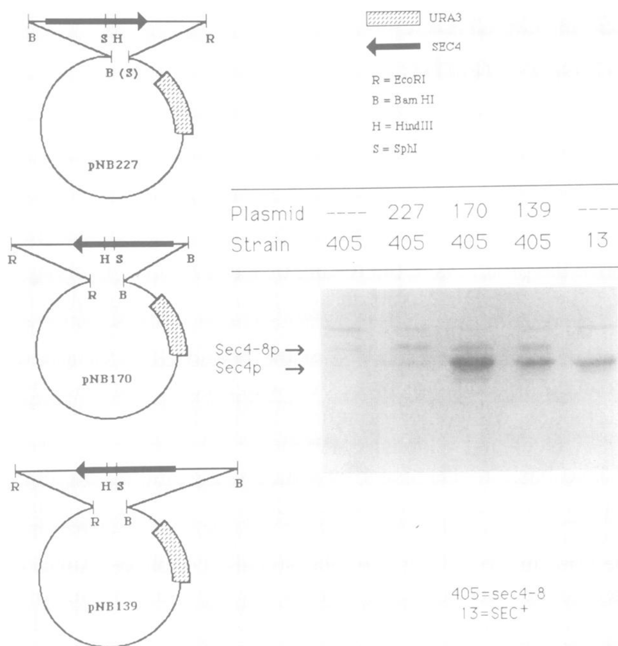


Fig. 1. Documentation of Sec4p expression levels from various plasmid constructions. The amount of Sec4p made from the plasmids shown varies depending on (i) the amount of genomic DNA 5′ to the initiator ATG included in the insert and (ii) the orientation of the insert in the plasmid with respect to the *URA3* gene. On the right, total yeast lysates prepared by the glass bead lysis method and transferred from an SDS−polyacrylamide gel to a nitrocellulose filter were incubated with anti-Sec4p antibody and alkaline phosphatase conjugated secondary antibody. NY405, a *sec4-8* mutant strain, was transformed with each plasmid expressing the wild-type *SEC4* gene. The protein synthesized from the plasmid (Sec4p) can be distinguished from the endogenous protein (Sec4-8p) because the two have different mobilities. NY13 is a wild-type strain. In plasmid pNB227 the *SEC4* gene is oriented with the 3′ end near *URA3* and ~150 bp of 5′ non-coding sequence present in the insert. Sec4p is expressed at a low level from pNB227. Plasmid pNB170 has the same size insert as 227, but the orientation of the insert is reversed, and the protein is expressed at high levels. Plasmid pNB139 expresses Sec4p at a level which is approximately equivalent to the amount of Sec4p found in the untransformed wild-type strain, NY13. pNB139 is in the same orientation as pNB170 but has ~400 bp of the non-coding region of *SEC4* at the 5′ end. Plasmids 227, 170 and 139 are centromere-containing plasmids. Various plasmids used in this report include those which are the integrating forms of these plasmids as well as centromere and integrating plasmids expressing mutant forms of *SEC4*.

that has been implicated to function at an earlier stage of the secretory pathway (Segev *et al.*, 1988), terminates in two cysteine residues which are essential for proper functioning of this protein (Molenaar *et al.*, 1988). Sec4p also terminates with two cysteines.

We have made specific amino acid changes in the GTP-binding domains of Sec4p, as well as at the C terminus, in an attempt to alter its biochemical activities and study the effects of such changes on the function of the protein *in vivo*. Using these mutants, we hoped to distinguish between the two mechanisms described. Like the signal transducing G proteins, Sec4p could serve to amplify a signal required for secretion by regulating the level of a second messenger within the cell. Alternatively, like EF-Tu, Sec4p could function to direct a macromolecular structure to a specific location in the cell. The phenotypes of mutants described in this paper suggest that the mechanism of action of Sec4p may more closely resemble that of EF-Tu. We propose a model in which membrane-associated Sec4p functions in a cyclical fashion to direct the fusion of post-Golgi secretory vesicles with the plasma membrane.

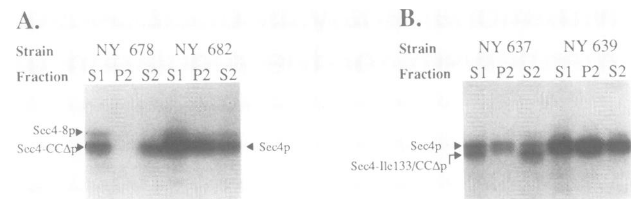


Fig. 2. Deletion of the C-terminal cysteines results in soluble Sec4p. Lysates of exponentially growing cells were prepared by osmotic lysis of spheroplasts in 0.8 M sorbitol followed by a 450 *g* centrifugation to remove unlysed cells (S1). S1 was then spun at 100 000 *g* to yield membrane (P2) and cytosolic fractions (S2). (**A**) NY678 is a *sec4-8* strain (NY405) transformed with pNB254 which expresses the *sec4-CCΔ* gene. NY682 is a *sec4-8* strain (NY405) transformed with pNB170 which expresses the wild-type *SEC4* gene. In both cases the plasmid encoded protein can be distinguished from the endogenous protein, Sec4-8p. (**B**) NY637 is a SEC$^+$ strain (NY15) transformed with pNB286 which expresses the *Sec4-Ile133,CCΔ* gene. NY639 is a SEC$^+$ strain (NY15) transformed with pNB141, the integrating version of pNB139, expressing wild-type Sec4p.

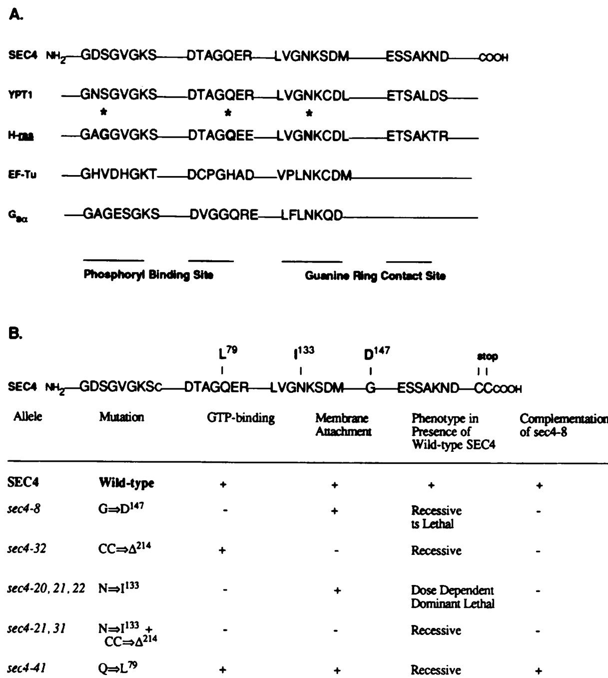
Results

Deletion of the C-terminal cysteines prevents membrane attachment

To assess a possible role in membrane attachment for the two C-terminal cysteine residues of Sec4p, we have deleted them by site-specific mutagenesis (*sec4-CCΔ*, see Materials and methods). Plasmid pNB254 (Figure 1), which allows for the expression of Sec4-CCΔp at higher than wild-type levels, was introduced into a temperature-sensitive *sec4-8* strain. This strain cannot grow at 37°C unless it is rescued by a functional copy of *SEC4*. The *sec4-CCΔ* gene on pNB254 cannot rescue this strain, suggesting that it encodes a non-functional protein. The same plasmid in wild-type cells has no effect on cell growth, indicating that this allele is recessive to the wild-type allele. Overproduction of the Sec4-CCΔp behind the *GAL1* promoter does not complement the temperature-sensitive growth defect of a *sec4-8*, GAL$^+$ strain at 37°C (data not shown).

To confirm the hypothesis that Sec4-CCΔp is not a functional protein, we performed a gene replacement experiment that makes *sec4-CCΔ* the sole copy of *SEC4* in a cell. An integrating plasmid containing *sec4-CCΔ* was introduced into a diploid strain such that one chromosome has a complete wild-type copy of the gene and the other chromosome has as its only complete copy the *sec4-CCΔ* gene marked by the *URA3* selectable marker. Upon sporulation and dissection of the resulting tetrads no viable spores were found which were Ura$^+$ (data not shown). This result demonstrates that the *sec4-CCΔ* gene is not sufficient to support growth as the only copy of *sec4* in the cell.

To determine the intracellular location of Sec4-CCΔp, isogenic strains were constructed in which plasmid pNB254 (*sec4-CCΔ*, NY678) or pNB170 (*SEC4*, NY682) was introduced into NY405. Lysates were prepared by osmotic lysis of spheroplasts, and membrane and soluble fractions were generated by centrifugation at 100 000 *g* (see Materials and methods). On a Western blot, the proteins expressed from the plasmids can be distinguished from the endogenous



Allele	Mutation	GTP-binding	Membrane Attachment	Phenotype in Presence of Wild-type SEC4	Complementation of sec4-8
SEC4	**Wild-type**	+	+	+	+
sec4-8	G⇒D^{147}	-	+	Recessive ts Lethal	-
sec4-32	CC⇒Δ^{214}	+	-	Recessive	-
sec4-20,21,22	N⇒I^{133}	-	+	Dose Dependent Dominant Lethal	-
sec4-21,31	N⇒I^{133} + CC⇒Δ^{214}	-	-	Recessive	-
sec4-41	Q⇒L^{79}	+	+	Recessive	+

Fig. 3. (**A**) Comparison of regions involved in GTP binding. The amino acid sequence of the portions of Sec4p which make up the putative GTP binding site is compared to the GTP binding regions of other known guanine nucleotide binding proteins. Based on the crystal structure of the mammalian H-*ras* protein (de Vos *et al.*, 1988) the two conserved domains at the amino terminal end interact with the phosphoryl group. The other conserved domains at the carboxy end of the protein form a pocket for interaction with the guanine ring. Positions where single amino acid substitutions alter the GTP binding or hydrolysis activity of H-*ras* p21 and result in oncogenic transformation of mammalian cells are indicated by * (G 12, Q 61, N 116). Sequences obtained from: SEC4 (Salminen and Novick, 1987); YPT1 (Gallwitz *et al.*, 1983); H-*ras* (Capon *et al.*, 1983); EF-Tu (Arai *et al.*, 1980); $G_{s\alpha}$ (Robishaw *et al.*, 1986). (**B**) Summary of the behavior of *SEC4* alleles. The regions of Sec4p that make up the GTP binding site are shown, as well as sites of the *sec4-8* and *sec4-CCΔ* mutations. GTP binding was assayed by the nitrocellulose filter binding assay and/or in solution. Membrane attachment was determined by centrifugation at 100 000 *g*. The phenotypes listed reflect the behavior of the various alleles in a SEC$^+$ background. 'Dose-dependent dominant lethal' reflects the fact that low levels of this protein can be tolerated, normal levels cause a severe growth and secretion defect in wild-type cells and high levels are lethal in a SEC$^+$ background. Complementation of *sec4-8* tests whether or not the encoded protein is functional.

Sec4-8 because the mobility of the latter is slower than that of Sec4p or Sec4-CCΔp. As shown in Figure 2A, in contrast to the wild-type protein which is primarily in the high-speed pellet (P2), the Sec4-CCΔ protein is found only in the soluble fraction (S2), which suggests that the two carboxy-terminal cysteines are necessary for membrane attachment. Sec4-CCΔp does retain its ability to bind GTP (data not shown).

SEC4-Ile133 is a dominant lethal mutation

The crystal structures of EF-Tu and of the mammalian H-*ras* protein have allowed the conclusive identification of the regions of these proteins that interact with the bound guanine nucleotide (Jurnak, 1985; LaCour *et al.*, 1985; de Vos *et al.*, 1988). Four distinct domains which are higly conserved among a large variety of GTP-binding proteins (Figure 3A, Dever *et al.*, 1987) form a pocket in which the nucleotide rests. Particular mutations in these domains in H-*ras* result in alleles which lead to oncogenic transformation when expressed in mammalian cells (Barbacid, 1987).

We have changed the asparagine at position 133 in Sec4p to an isoleucine. The analogous mutation at position 116 in H-*ras* results in oncogenic transformation despite the inability of the protein to detectably bind GTP (Walter *et al.*, 1986). To vary the level of Sec4-Ile133p expressed, the mutant gene was cloned into various plasmids (see Figure 1 and Materials and methods). The efficiency with which plasmid pNB256 (which should synthesize the mutant protein at a low level) could be transformed into wild-type cells is equivalent to that of plasmid pNB227 (containing the normal gene). However, if plasmid pNB260, which should synthesize wild-type levels of Sec4-Ile133p is used, then the transformation efficiency is reduced ~ 10-fold compared to transformation with the control plasmid, pNB139. No transformants are obtained with pNB225, which should synthesize high levels of the mutant protein, even though the analogous wild-type plasmid, pNB170, does yield colonies. However, if pNB225 is first digested with restriction enzymes to create a 70 bp gap over the Ile133 mutation, transformation does occur because the gap is repaired using the endogenous wild-type gene as template (Orr-Weaver *et al.*, 1981). Thus, the inability to obtain transformants with the pNB225 construction is due to sequence located in the deleted 70 bp rather than elsewhere on the plasmid. The only change from the wild-type sequence in this region encodes the Ile133 mutation.

In order to study the phenotype induced by the mutant protein we constructed a strain which, in addition to the wild-type *SEC4* gene present at the chromosomal locus, has *SEC4-Ile133* (pNB276) integrated at the *URA3* locus (NY640). This plasmid should synthesize the mutant protein at levels that are approximately equivalent to endogenous Sec4p expression levels. As mentioned above, the transformation frequency of wild-type cells with the mutant plasmid is ~ 10-fold lower than with the equivalent wild-type plasmid (pNB141). In addition, the growth rate of cells expressing Sec4-Ile133p is reduced. The doubling time of wild-type is ~2 h whereas the doubling time for NY640, cells expressing Sec4-Ile133p in addition to wild-type Sec4p, is at least 4 h (data not shown).

We examined the efficiency of secretion in these cells by assaying the release of the secreted enzyme invertase at the cell surface. Approximately 40% less invertase was secreted from NY640, cells expressing the mutant protein, as compared to wild-type cells (data not shown). NY640 and an analogous strain bearing an extra copy of *SEC4* were also examined by electron microscopy. A dramatic increase in the number of secretory vesicles is seen in NY640 (Figure 4), despite the presence of wild-type Sec4p in these cells. Thus, *SEC4-Ile133* induces a dominant secretory defect. The vesicles in this strain are similar in size and appearance to the vesicles present in the other late secretory mutants that accumulate post-Golgi vesicles when incubated at the restrictive temperature (Novick *et al.*, 1980).

To confirm the phenotype conferred by the presence of Sec4-Ile133p, the gene was cloned behind the inducible *GAL1* promoter and integrated at the *URA3* locus in SEC$^+$GAL$^+$ cells (NY519). The control strain, NY496, has the wild-type *SEC4* gene cloned behind *GAL1* and integrated at *URA3*. After 0, 6 and 12 h of induction on galactose, cells were harvested for the preparation of lysates to measure Sec4p expression and for examination by electron

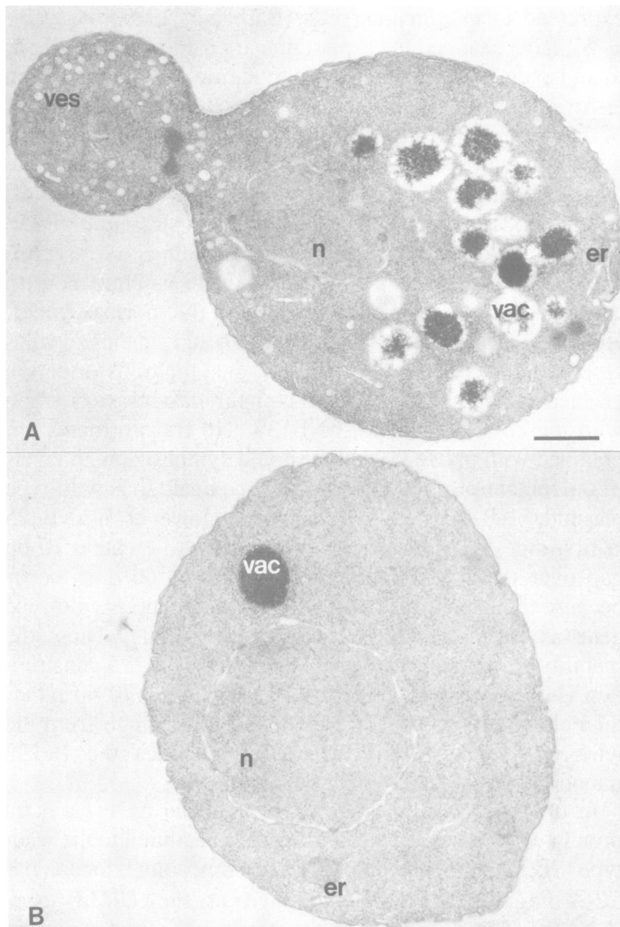


Fig. 4. Cells expressing Sec4-Ile133p contain a large number of secretory vesicles despite the presence of wild-type Sec4p. Cells were harvested during growth in exponential phase and fixed for electron microscopy. (**A**) NY640 has the wild-type *SEC4* gene at the chromosomal locus and the *SEC4-Ile133* gene integrated at the *URA3* locus. The integrated plasmid, pNB276, should synthesize normal levels of mutant protein. Bar = 0.5 μm. (**B**) Wild-type strain containing an extra copy of the wild-type *SEC4* gene at the *URA3* locus shown at the same magnification as the cell in (A). Typical of wild-type cells, no secretory vesicles are seen in this section.

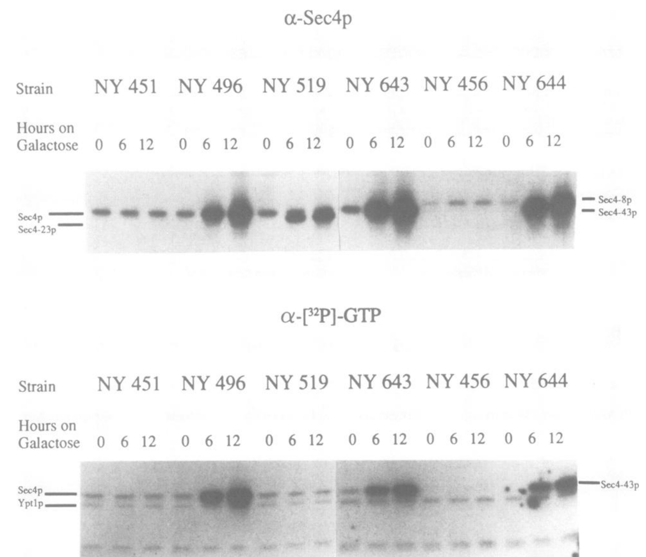


Fig. 5. GTP binding ability of mutant Sec4 proteins. Gal$^+$ strains with *Sec4* genes under control of the inducible GAL1 promoter were induced with galactose for 12 h. Aliquots of cells were harvested at 0, 6 and 12 h of induction, lysed with glass beads and boiled in sample buffer. Samples were separated on an SDS–polyacrylamide (15%) gel and transferred to nitrocellulose. The filters shown in the top panel were incubated with anti-Sec4p antibody followed by [^{125}I]protein A and exposed onto X-ray film. In the bottom panel, the filters were incubated with [α-^{32}P]GTP and exposed onto X-ray film. Strains are as follows (transformed strains have the plasmid integrated at the *URA3* locus): NY451 is an untransformed Gal$^+$ strain. NY496 is NY451 with *SEC4* under control of the *GAL1* promoter (Sec4p). NY519 is NY451 with *SEC4-Ile133* under control of *GAL1* (Sec4-23p). NY643 is NY451 with *sec4-Leu79* under control of *GAL1* (Sec4-43p). NY456 is a *sec4-8*, Gal$^+$ strain. NY644 is NY456 with *sec4-Leu79* under control of *GAL1*.

microscopy. The Western blot shown in Figure 5 indicates that the level of Sec4p in NY496 increases upon induction with galactose, as compared to NY451, the parental strain which does not contain a plasmid. Similarly, in NY519 total Sec4p is overexpressed although not to the same extent as in NY496. This could be due to greater instability of the mutant protein. Sec4-Ile133p has a slightly faster mobility than the wild-type protein and the accompanying GTP binding blot in Figure 5 indicates that the mutant protein does not bind significant amounts of radiolabeled nucleotide by this assay. This observation has been confirmed by measuring GTP-γ-S binding activity in solution of lysates of cells in which the protein has been overexpressed (Table I). Sec4-Ile133p is found in both the 10 000 *g* and the 100 000 *g* pellet fractions following differential centrifugation of lysed spheroplasts indicating that the protein does attach to membranes (data not shown).

By thin-section electron microscopy, a dramatic accumulation of secretory vesicles can be seen in NY519 as early as 6 h after induction when there is only twice as much mutant protein as wild-type protein present in the cell (Figure 6A). Cells containing five times more wild-type protein at this time do not accumulate secretory vesicles (Figure 6B). After 12 h of growth on galactose, overexpression of the mutant protein causes the accumulation of a large number of secretory vesicles, while overexpression of wild-type Sec4p results in a slight accumulation of membranes including a few secretory vesicles (not shown). The growth rate of NY519 begins to drop within 4 h of inducing expression of the mutant protein (Figure 6C), whereas overexpression of the wild-type protein in NY496 for up to 12 h has no effect on doubling time. Thus, production of Sec4-Ile133p results in a secretory defect that correlates with a decreased growth rate, despite the presence of the wild-type protein in these cells.

Sec4-Ile133p is a dominant loss of function mutant

When the *SEC4-Ile133* gene is introduced into a *sec4-8* strain on pNB256, the plasmid which produces low levels of the protein, the transformed cells grow at 25°C but are not able to grow at 37°C. As a control we have shown that *sec4-8* cells transformed with pNB227 expressing the wild-type allele are able to grow at 37°C. Thus, Sec4-Ile133p does not appear to have wild-type function. Genetic analysis of the interaction between *SEC4* and a subset of other late acting secretory mutants allows the distinction to be made between the gain or loss of Sec4p function. A gain of Sec4p function is achieved by duplicating the wild-type *SEC4* gene, which results in suppression of the temperature-sensitive growth defect in *sec2-41*, *sec8-9* and *sec15-1* strains. A loss of Sec4p

Table I. GTP-γ-S binding of Sec4 proteins overexpressed in *sec4-8* background

Induced protein (strain)	Specific GTP-γ-S binding[a] (pmol/mg × 10)	Relative amount of Sec4 protein[b]	Binding normalized to Sec4p level (pmol/mg × 10)	% wild-type Sec4p binding
Membrane fraction[c]				
Sec4p (NY500)	14	1	14	100
Sec4-Ile133p (NY520)	0.5	0.72	0.7	5
Sec4-Leu79p (NY644)	8.0	1.1	7.3	52
Soluble fraction[d]				
Sec4p (NY500)	14	1	14	100
Sec4-Ile133p (NY520)[e]	–	0.25	–	–
Sec4-Leu79p (NY644)	11	1.2	9.2	66

[a]Cells were grown in the presence of galactose for 24 h to induce expression of the indicated proteins. Spheroplasts were lysed osmotically and centrifuged at 100 000 *g* for 1 h. The assay measures the binding of [^{35}S]GTP-γ-S in solution using a rapid filtration technique. Specific binding was calculated as the difference between binding in the presence of 1 mM AppNHp and binding in the presence of 1 mM GppNHp. Binding specific to the induced protein was calculated by subtracting the amount detected in the equivalent fraction from strain NY456, a GAL$^+$, *sec4-8* strain. Values represent the average of two binding experiments in which duplicate samples were assayed.
[b]The levels of Sec4 protein in each fraction were determined by quantitative Western blot analysis. Counts were normalized to the number of counts detected in the equivalent fraction of NY500, the strain overproducing Sec4p.
[c]Pellet fraction from 100 000 *g* centrifugation. Specific binding values represent total binding minus the amount detected in the equivalent fraction of NY456: 5.5 pmol/mg × 10.
[d]Supernatant fraction from 100 000 *g* centrifugation. Specific binding values represent total binding minus the amount detected in the equivalent fraction of NY456: 2.2 pmol/mg × 10.
[e]The amount of Sec4-Ile133p present in this fraction was too low to assess binding.

function cannot be tolerated by these particular mutants as strains bearing both the *sec4-8* mutation and either *sec2-41*, *sec8-9* or *sec15-1* cannot be constructed (Salminen and Novick, 1987).

Analysis of the behavior of the *SEC4-Ile133* allele in crosses with other *sec* strains indicates that it behaves as a loss of function allele. This behavior is dominant, as in each case a wild-type *SEC4* gene is always present. Strains containing *SEC4-Ile133* (marked by Ura$^+$) and strains bearing other *sec* mutations (marked by temperature sensitivity) were crossed and the resulting diploids sporulated. The *SEC4-Ile133* mutant gene synthesized at wild-type levels is lethal when combined with the same subset of *sec* mutants with which the recessive, temperature-sensitive *sec4-8* mutant is lethal (Salminen and Novick, 1987) even when a functional copy of *SEC4* is present (Tables II and III). In crosses between *SEC4-Ile133* and, for example, *sec15-1*, many tetrads had one or two inviable spores. Phenotypic analysis of the remaining viable spores indicated that the inviable spore would have contained both the *sec15-1* and the Ura$^+$ marked *SEC4-Ile133* allele. Thus, the combination of *sec15-1* and *SEC4-Ile133* is a lethal event. The same results were obtained for *sec2-41* and *sec8-9*. This phenotype is specific, as crosses with vesicle accumulating mutants *sec1-11* and *sec6-4*, as well as crosses with the Golgi accumulating mutant *sec7-1* and the endoplasmic reticulum accumulating mutant *sec18-1*, all yielded viable temperature-sensitive, ura$^+$ spores. If *SEC4-Ile133* is present on the low expression plasmid, then viable ura$^+$, temperature-sensitive spores can be obtained indicating that low levels of the mutant protein can be tolerated by the other *sec* mutants when a functional copy of *SEC4* is also present (tetrad data not shown, but summarized in Table III).

Analysis of Sec4-Ile133, CCΔ

Since deletion of the cysteine residues causes Sec4p to become soluble and non-functional, we have tested whether their removal from the dominant Sec4-Ile133p would prevent this protein from being detrimental. A gene was constructed in which the C-terminal portion of the *SEC4-Ile133* gene was replaced with the same portion of the *sec4-CCΔ* gene bearing the cysteine deletion. When this gene was introduced into a wild-type SEC$^+$ background no obvious effect on growth was observed whether the gene is expressed at a normal level (pNB286) or at a high level (pNB285). Western blot analysis of cell fractionation experiments indicates that the protein has the same mol. wt as Sec4-Ile133p and that it is soluble (Figure 2B). Thus, removal of the two cysteines, which makes Sec4-Ile133p soluble in wild-type cells, relieves the dominant lethality of the mutant protein.

Sec4-Leu79p binds GTP less efficiently, but is still functional

A mutation in the domain of the GTP binding site responsible for interacting with the phosphoryl group of GTP causes *ras* to have transforming activity (see Figure 3A). This mutation, which changes the glutamine at position 61 to leucine, causes the protein to hydrolyze GTP at a 10-fold slower rate. If the glutamine is changed to other amino acids besides leucine, the hydrolysis rate is also decreased but the protein has various degrees of transforming ability depending on the specific amino acid substituted (Der *et al.*, 1986). We have changed the analogous glutamine at position 79 in Sec4p to leucine.

Sec4-Leu79p is able to bind GTP by the filter assay (Figure 5) although in the quantitative solution assay the mutant protein binds nucleotide at 50–60% of the wild-type levels (Table I). Neither of these assays rule out the possibility that the on rate or off rate of GTP binding to the mutant protein is different from the rates for the wild-type protein. Introduction of this protein into wild-type cells has no obvious effect on cell growth. When introduced into temperature-sensitive *sec4-8* cells and expressed at normal levels (pNB287), the protein does rescue the temperature-sensitive growth defect of these cells. Thus, although Sec4-Leu79p binds GTP with altered characteristics as compared to the wild-type protein, it is functional. However, if the protein is expressed at higher than normal levels in

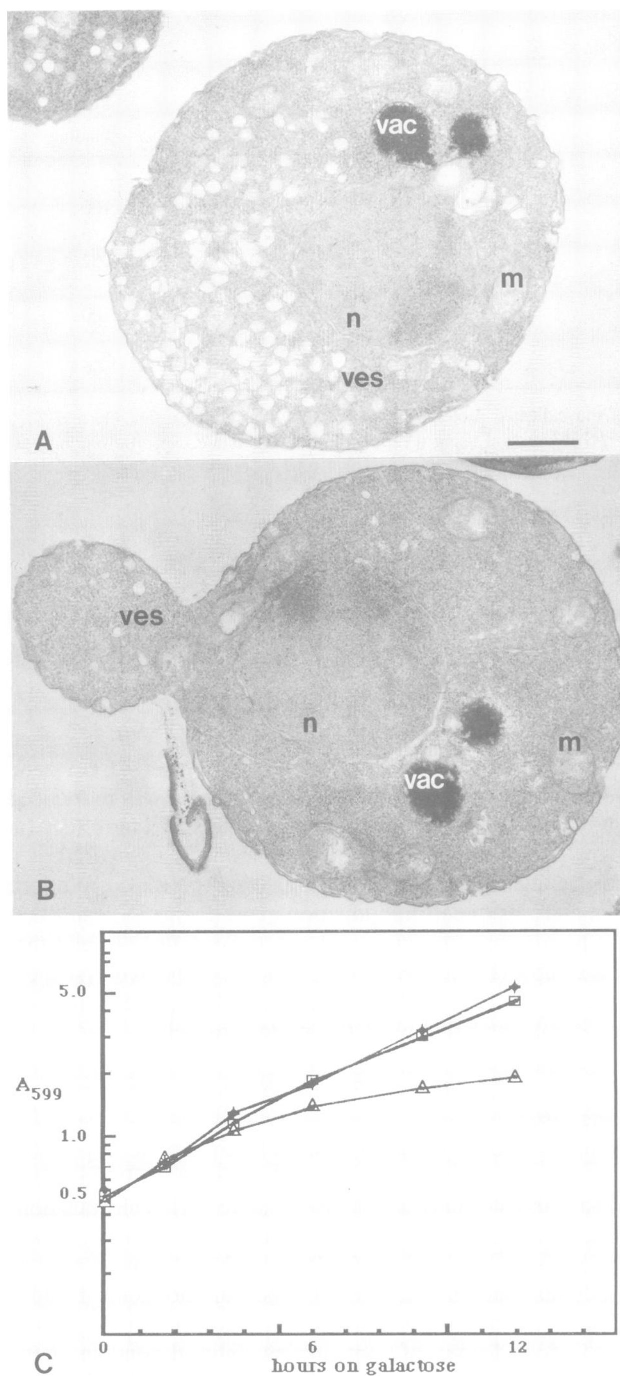


Fig. 6. Induced expression of Sec4-Ile133p causes accumulation of secretory vesicles and a decrease in growth rate. The growth rate of cells expressing wild-type Sec4p or Sec4-Ile133p under control of *GAL1* was followed after induction with galactose and cells were harvested for electron microscopy. (**A**) After 6 h of induction with galactose, cells expressing Sec4-Ile133p (NY519) accumulate a large number of secretory vesicles. Bar = 0.5 μm. (**B**) At the same time point, cells expressing Sec4p (NY496) display a normal morphology. (**C**) Up to 12 h of induction with galactose the growth rate of cells expressing wild-type Sec4p is normal whereas at 6 h the growth rate of cells expressing the mutant Sec4-Ile133p is slowed. Open squares = NY451. Closed diamonds = NY496. Open triangles = NY519.

sec4-8 (on pNB293) then these cells grow at a slower rate at 37°C than cells expressing the same level of wild-type Sec4p (data not shown). This phenotype may reflect the altered binding activity of this protein.

Discussion

The behaviors of various Sec4 mutant proteins are summarized in Figure 3B. The proteins have been examined for their capacity to bind GTP and to attach to membranes. Complementation of the temperature-sensitive growth defect of *sec4-8* has been used to determine whether or not they are functional. The phenotypes induced by the mutant proteins in wild-type cells and in cells containing other secretory defects have been documented. The behavior of these mutants is consistent with Sec4p playing a cyclical role in mediating transport of secretory vesicles to the plasma membrane in yeast.

Relationship of Sec4p localization to its function

We have shown previously that Sec4p is found in the cell stably associated with the plasma membrane and secretory vesicles despite the hydrophilic nature of the protein sequence. A soluble pool of 10–15% of total Sec4p is maintained and the evidence indicates that the protein can recycle from the plasma membrane to secretory vesicles most probably through a soluble intermediate (Goud *et al.*, 1988). The structurally related *ras* family of GTP-binding proteins, including the yeast Ypt1p, attach to the cytoplasmic surfaces of cellular membranes via a lipid moiety linked to a C-terminal cysteine (Chen *et al.*, 1985; Buss and Sefton, 1986; Fujiyama and Tamanoi, 1986; Molenaar *et al.*, 1988). Ypt1p has been shown to be palmitoylated and deletion of the cysteines results in a soluble, unmodified protein (Molenaar *et al.*, 1988). Like Ypt1p, Sec4p terminates with two cysteines (Salminen and Novick, 1987). Deletion of these two amino acid residues results in the majority of the protein remaining soluble. The protein is not functional even though it retains the ability to bind GTP. This suggests that the cysteines are necessary for mediating or stabilizing the attachment of Sec4p to membranes and that this association is required for normal function. As yet a modification such as palmitoylation has not been detected.

SEC4-Ile133 is a dominant secretory mutant

SEC4-Ile133 was constructed based on the behavior of a transforming mutant of H-*ras* and a dominant lethal mutant of *YPT1* (Schmitt *et al.*, 1986; Walter *et al.*, 1986). Mutations in p21 H-*ras* at position 116 or 119 in the third domain of the GTP-binding site decrease the protein's affinity for GTP and GDP. Despite this lowered affinity for nucleotide, particular substitutions at 116 and 119 result in transformation competent *ras* proteins, which are assumed to be constitutively active (Sigal *et al.*, 1986; Walter *et al.*, 1986). A mutation in the same domain of the yeast *YPT1* gene at position 121 changes asparagine to isoleucine and prevents the protein from binding GTP. Schmitt *et al.* (1986) have proposed that the dominant lethal phenotype induced by this mutant protein is due to it being locked in an active conformation. The *SEC4-Ile133* mutation was constructed to mimic this situation. As expected, the Sec4-Ile133p is defective in GTP binding. When this protein is expressed at normal levels in cells already expressing the wild-type Sec4p, the cells grow very slowly. In addition, these cells secrete lower levels of invertase and accumulate an excess of secretory vesicles in the cytoplasm. We postulate that there is a reversible interaction between Sec4p and an effector molecule. This interaction may be irreversible in the case of the mutant protein which, therefore, prevents wild-type

Table II. Tetrad analysis of *URA3* :: *SEC4-Ile133* crossed to ts$^-$ *sec* mutants

		Categories of tetrads: distribution in crosses				
		2 ts$^+$ura$^+$ 2 ts$^-$ura$^-$	1 ts$^+$ura$^+$ 1 ts$^-$ura$^-$ 1 ts$^+$ura$^-$ 1 ts$^-$ura$^+$	2 ts$^+$ura$^-$ 2 ts$^-$ura$^+$	1 ts$^+$ura$^+$ 1 ts$^-$ura$^-$ 1 ts$^+$ura$^-$ 1 inviable[a]	2 ts$^+$ura$^-$ 2 inviable[a]
URA3 :: *SEC4-Ile133* ×	*sec4-8*	3			1	8
NY640	NY405					
URA3 :: *SEC4-Ile133* ×	*sec15-1*				9	2
NY640	NY64[b]					
URA3 :: *SEC4-Ile133* ×	*sec2-41*	3			8	1
NY640	NY130					
URA3 :: *SEC4-Ile133* ×	*sec8-9*	1			8	2
NY640	NY410[c]					
URA3 :: *SEC4-Ile133* ×	*sec6-4*	2	8	1		
NY640	NY17[d]					
URA3 :: *SEC4-Ile133* ×	*sec1-11*	1	6	3		
NY640	NY24[e]					
URA3 :: *SEC4-Ile133* ×	*sec7-1*		8			
NY640	NY176[f]					
URA3 :: *SEC4-Ile133* ×	*sec18-1*	1	9	1		
NY640	NY431[g]					

[a]The absence of ts$^-$ura$^+$ spores indicates that the combination of the ura$^+$ marked *SEC4-Ile133* or *SEC4-Ile133,CCΔ* allele with the indicated ts$^-$ *sec* allele is a lethal event.
Additional tetrads
[b]1 tetrad; 1 viable spore: ts$^-$ura$^-$.
[c]1 tetrad; 2 viable spores: ts$^-$ura$^-$; ts$^+$ura$^-$.
[d]1 tetrad; 4 viable spores: ts$^+$ura$^+$; 2 ts$^-$ura$^-$; ts$^+$ura$^-$.
[e]2 tetrads; 4 viable spores: ts$^+$ura$^+$; 2 ts$^-$ura$^-$; ts$^+$ura$^-$.
[f]1 tetrad; 4 viable spores: ts$^+$ura$^+$; ts$^+$ura$^-$; 2 ts$^-$ura$^+$; 1 tetrad; 4 viable spores: 2 ts$^+$ura$^+$; ts$^-$ura$^-$; ts$^-$ura$^+$; 1 tetrad; 4 viable spores: ts$^+$ura$^+$; ts$^-$ura$^-$; ts$^-$ura$^+$; ts$^+$ura$^?$; 1 tetrad; 3 viable spores: ts$^+$ura$^+$; ts$^+$ura$^-$; ts$^-$ura$^?$.
[g]1 tetrad; 3 viable spores: ts$^+$ura$^+$; ts$^-$ura$^-$; ts$^-$ura$^+$.

protein function and is responsible for the dominant nature of the mutation.

Evidence that *SEC4-Ile133* is a dominant loss of function mutant comes from genetic experiments. Previous studies (Salminen and Novick, 1987) have shown that a gain of Sec4p function as in the case of *SEC4* duplication can suppress the temperature-sensitive growth defects of a set of vesicle-accumulating *sec* mutants including *sec15-1* and *sec2-41*. A loss of Sec4p function, as exemplifed by the presence of *sec4-8* as the sole copy of *SEC4*, is lethal in strains bearing these other mutations. The *SEC4-Ile133* gene, when introduced into these strains at a level that permits viability in a wild-type background, behaves as a loss of function allele, i.e. the combination of *SEC4-Ile133* with *sec15-1* or *sec2-41* is lethal. Importantly, the inviability is seen specifically in those strains which are known to interact at the genetic level with *SEC4*. Introduction of *SEC4-Ile133* into *sec6-4*, *sec1-11*, *sec7-1* or *sec18-1* is not lethal and these are mutations that are neither suppressed by duplication of *SEC4* nor inviable when combined with *sec4-8* (Salminen and Novick, 1987).

Sec4p functions as part of a cycle

Two models for the mechanism of action of GTP-binding proteins have been discussed (see Introduction). The model shown in Figure 7 is based loosely on the mechanism by which EF-Tu functions to deliver an aminoacyl-tRNA to the ribosome (Kaziro, 1978; Bourne, 1988). At steady state, Sec4p is found in the cell in a soluble form, as well as bound to secretory vesicles and bound to the plasma membrane. Sec4p binds GTP and we postulate that in the GTP-bound

Table III. Summary of genetic interactions: double mutant viability at 25°C

ts$^-$ strain	Site of block at 37°C	NY405 *sec4-8*	NY640 *sec4-21*[a] *SEC4*	NY617 *sec4-20*[b] *SEC4*
sec4-8	vesicle		−	+
sec2-41	vesicle	−	−	+
sec8-9	vesicle	−	−	+
sec15-1	vesicle	−	−	+
sec1-11	vesicle	+	+	
sec6-4	vesicle	+	+	
sec7-1	Golgi	+	+	
sec18-1	ER	+	+	

[a]*sec4-21*: *SEC4-Ile133* at normal level.
[b]*sec4-20*: *SEC4-Ile133* at low level.

conformation Sec4p recognizes an attachment site on a newly formed secretory vesicle. This complex is recognized by a hypothetical effector on the cytoplasmic surface of the plasma membrane triggering membrane fusion and exocytosis. By analogy, release of Sec4p from the effector is coupled to and dependent on hydrolysis of the bound GTP leading to a conformational change.

A failure to bind nucleotide efficiently could leave Sec4p in either the inactive or the active conformation. The phenotype of cells expressing Sec4-Ile133p is consistent with the hypothesis that it is trapped in the active conformation. Thus, it may be competent to attach to vesicles and enter the cycle, but because it cannot relax to the inactive conformation it blocks the cycle. Vesicles are formed but

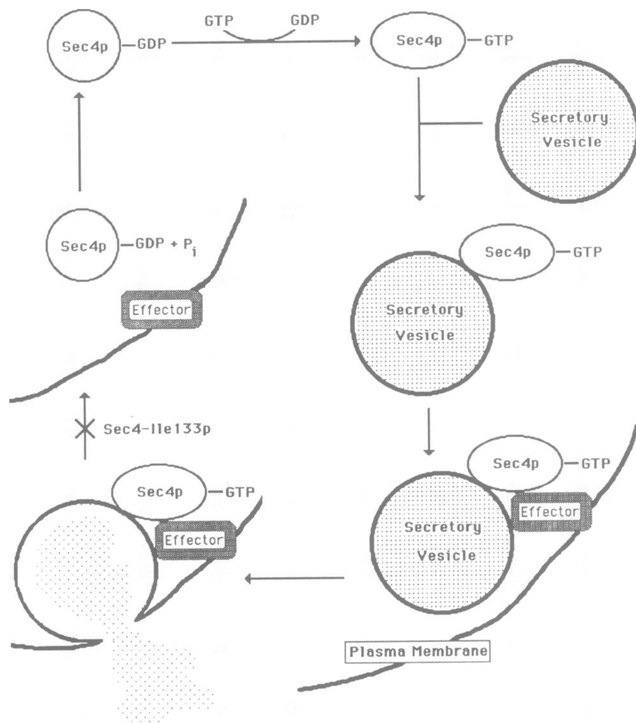


Fig. 7. Model for Sec4p function. The GDP bound by Sec4p is exchanged for GTP. In the GTP-bound conformation Sec4p recognizes an attachment site on a newly formed secretory vesicle. This complex is recognized by an effector on the cytoplasmic surface of the plasma membrane-triggering membrane fusion and exocytosis. Release of Sec4p from the effector is coupled to hydrolysis of the bound GTP.

an effector that is necessary for vesicle delivery to take place is unavailable as it is bound to activated Sec4p from the previous round. As a dominant mutant, Sec4-Ile133p prevents wild-type protein from functioning in a manner that suggests that Sec4p interacts directly with some specific component(s) of the cycle. Lowering the level of mutant protein allows wild-type protein to compete and function effectively. A higher level of the mutant protein does not permit wild-type protein to function at all. Significantly, it is at normal levels of synthesis that the secretory defect induced by the presence of Sec4-Ile133p is seen.

In the yeast Ras/adenylate cyclase system a number of components of the regulatory pathway were identified through suppressor analysis (Toda *et al.*, 1985; Cannon *et al.*, 1986). We attempted to identify suppressors of a loss of Sec4p function by isolating revertants of a *sec4-8* strain. However, only intragenic suppressors were obtained (data not shown). Our failure to obtain extragenic suppressors may reflect a fundamental difference between the mechanism by which Ras2p regulates the cell cycle and the mechanism by which Sec4p regulates vesicular traffic. That is, in the adenylate cyclase system that involves the amplification of a second messenger molecule, constitutive activation of downstream components of the pathway suppresses the loss of function of the Ras protein, the upstream regulatory molecule. Loss of Sec4p function may not be suppressible in a similar manner if it must operate in a cyclical fashion.

Future studies on the role of Sec4p are directed toward the identification of other components that interact with Sec4p and function as part of a cycle. Candidates for proteins that are involved are the products of those genes that have been shown to interact with *SEC4* at the genetic level. The dominant *SEC4-Ile133* allele provides another tool with which to probe for interacting proteins at both the genetic and the biochemical level. Furthermore, studies of the biochemical properties of Sec4p and the identification of factors that influence those properties promise to be revealing.

Table IV. Strains used

Strain	Genotype
NY13	*MATa, ura3-52*
NY15	*MATα, ura3-52, his4-619*
NY17	*MATa, ura3-52, sec6-4*
NY24	*MATa, ura3-52, sec1-11*
NY64	*MATa, ura3-52, sec15-1*
NY130	*MATa, ura3-52, sec2-41*
NY176	*MATa, ura3-52, sec7-1*
NY405	*MATa, ura3-52, sec4-8*
NY410	*MATa, ura3-52, sec8-9*
NY451	*MATa, ura3-52, Gal*$^+$
NY456	*MATa, ura3-52, Gal*$^+$*, sec4-8*
NY496	*MATa, ura3-52 :: (URA3, GAL1-SEC4), Gal*$^+$
NY500	*MATa, ura3-52 :: (URA3, GAL1-SEC4), sec4-8, Gal*$^+$
NY519	*MATa, ura3-52 :: (URA3, GAL1-SEC4-23), Gal*$^+$
NY520	*MATa, ura3-52 :: (URA3, GAL1-SEC4-23), sec4-8, Gal*$^+$
NY617	*MATa, ura3-52 :: (URA3, sec4-20), his4-619*
NY637	*MATα, ura3-52 :: (URA3, sec4-21,31), his4-619*
NY639	*MATα, ura3-52 :: (URA3, SEC4), his4-619*
NY640	*MATα, ura 3-52::(URA3, SEC4-21), his4-619*
NY643	*MATa, ura3-52 :: (URA3, GAL1-sec4-43), Gal*$^+$
NY644	*MATa, ura3-52 :: (URA3, GAL1-sec4-43), Gal*$^+$
NY678	*MATa, ura3-52, pNB254 (URA3, sec4-32), sec4-8*
NY682	*MATa, ura3-52, pNB170 (URA3, SEC4), sec4-8*
sec4 alleles	
sec4-8	*Sec4-Asp147*
sec4-20	*Sec4-Ile133* (low)
sec4-21	*Sec4-Ile133p* (normal)
sec4-21,31	*Sec4-Ile133p,CCΔ* (normal)
sec4-23	*Gal/Sec4-Ile133*
sec4-32	*Sec4-CCΔ* (high)
sec4-43	*Gal/Sec4-Leu79*

Materials and methods

Yeast genetic techniques

Yeast strains used in this study are listed in Table IV. Cells were grown in rich medium (YPD) containing 1% Bacto-yeast extract, 2% Bacto-Peptone (Difco Laboratories, Inc., Detroit, MI) with 2% glucose as the carbon source except where indicated. When selective conditions were required cells were grown on minimal medium containing 0.7% yeast nitrogen base without amino acids (Difco) and 2% glucose, supplemented with auxotrophic requirements, as described by Sherman *et al.* (1974), when necessary.

Yeast transformation was done by the method of alkali cation treatment (Ito *et al.*, 1983). Transformants were selected on minimal medium at 25°C. Crosses, sporulation of diploids and dissection of tetrads were done as described by Sherman *et al.* (1974).

Nucleic acid techniques

Escherichia coli strain DH1 was used for all cloning experiments. Plasmid preparations were done essentially as described elsewhere (Silhavy *et al.*, 1984). Centromere-containing plasmids pNB139 and pNB170 encoding the SEC4 genes are described in Salminen and Novick (1987) and plasmid pNB227 was constructed by sub-cloning the *Bam*HI–*Eco*RI fragment of pNB170 into the *Bam*HI–*Sph*I site of YCp50, which reverses the orientation of the SEC4 gene with respect to the URA3 gene on the plasmid. Mutant

SEC4 genes were cloned into these plasmids or integrating versions of these plasmids (YIp5 derivatives) from the M13 vectors on which the mutants were constructed (see below). The parent integrating plasmid containing the GAL1−SEC4 construct (pNB219) is described in Goud *et al.* (1988) and mutant derivatives were constructed by direct mutagenesis of a portion of the plasmid and sub-cloning from the mutagenized M13 into pNB219.

Mutagenesis was carried out using the Amersham oligonucleotide directed *in vitro* mutagenesis kit according to the manufacturer's instructions (Amersham, Arlington Heights, IL). Oligonucleotides were synthesized by the Yale University DNA Synthesizing Service. All sequences of the mutant genes were confirmed by the dideoxy chain termination method (Sanger *et al.*, 1977) in the presence of [α-^{35}S]dATP (650 Ci/mmol; Amersham) (Williams *et al.*, 1986). To construct the Ile33 mutation the *Eco*RI−*Hin*dIII fragment from pNB219 was subcloned into mp18, an M13 derivative and mutagenized using a 25 base oligonucleotide encoding Ile instead of Asn: 5'CATATCGCTCTTAATACCAACCAAC3'. To construct the CCΔ mutant the *Hin*dIII−*Eco*RI fragment from pNB170 was cloned into the M13 derivative mp19 and mutagenized using a 30 base oligonucleotide with the two codons for the cysteines at the C terminus deleted: 5'AGTTCTAA-ATCAAATΔTGAAGAAAAGAAGAT3'. To construct the Leu 79 mutant the *Bam*HI−*Hin*dIII fragment from pNB139 and the *Eco*RI−*Hin*dIII fragment from pNB190 (the YCp50 version of pNB219) were cloned independently into mp18 and mutagenized using a 24-base oligonucleotide encoding Leu instead of Gln: 5'GGAAACGTTCCAAACCAGCGGTAT3'. M13 plasmids were propagated in *E.coli* strain TG1 (for mutagenesis) or JM101 (for sequencing).

Expressing SEC4 from the GAL1 promoter

Cells were grown in YP medium containing 2% lactate. Expression from the GAL1 promoter was induced using 0.5% galactose, and 0.1% glucose, included to maintain a moderate level of expression (Adams, 1972).

Other methods

Electron microscopy was carried out as described in Walworth and Novick (1987). Electrophoresis and immunoblotting are described in Goud *et al.* (1988). Glass bead lysates were prepared by harvesting 5×10^7 cells, washing in 1 ml of cold 10 mM Tris, pH 7.5, 10 mM NaN_3, resuspending in 75 μl of the same buffer, and adding glass beads to reach the meniscus. Lysis was achieved by mixing for 3 min with a Vortex mixer (VWR Scientific) in a 4°C cold room followed immediately by addition of SDS sample buffer and boiling. Osmotic lysis of spheroplasts was done essentially as in Goud *et al.* (1988). Following homogenization, cell lysates were spun at 450 *g*, the pellets washed once and the pooled supernatants spun at 100 000 *g* in a SW50.1 rotor (Beckman Instruments) at 33 000 r.p.m. for 1 h to generate membrane and soluble fractions. GTP binding on nitrocellulose filters was done according to Lapetina and Reep (1987). GTP-γ-S binding in solution was measured with a rapid filtration technique utilizing HA MF-type membrane filters (Millipore, Bedford, MA) as described in Northup *et al.* (1982). From soluble and membrane fractions, 7.5−10 μg of protein was assayed in 25 μl of 50 mM Hepes, pH 8.0, 50 mM $MgCl_2$, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Lubrol and 2 μM [^{35}S]GTP-γ-S (Amersham, 5×10^5 c.p.m.) at 30°C for 60 min. Where appropriate, guanosine-5'-(β,γ-imino)triphosphate [Gpp(NH)p] or adenine-5'-(β,γ-imino)triphosphate [App(NH)p] was added to 1 mM (Boehringer Mannheim).

Acknowledgements

We thank Drs P.DeCamilli, I.Mellman, H.Bourne, S.Powers, A.Tamir and J.Northup for helpful discussions. We thank M.Talmor for synthesis of oligonucleotides, H.Stukenbroek and R.Dowin for assistance in thin section electron microscopy and A.Curley-Whitehouse for assistance in photography. This work was supported by grants GM35370 and CA46218 from the National Institutes of Health. N.C.W. was supported by a training grant from the National Institutes of Health, B.G. was supported by a grant from the Swebilius Foundation and A.K.K. was supported by a grant from the Jane Coffin Childs Foundation.

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Received 27th January, 1989; revised 20th March, 1989