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A *ras*-like Protein Is Required for a Post-Golgi Event in Yeast Secretion

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Summary

Secretion is blocked at the post-Golgi stage within 5 min of a shift of *sec4-8* cells from 25°C to 37°C. Analysis of *SEC4* predicts a protein product of 23.5 kd molecular weight that shares 32% homology with *ras* proteins and is essential for growth. The regions of best homology are those involved in the binding and hydrolysis of GTP. Duplication of *SEC4* suppresses post-Golgi-blocked mutations in three *sec* genes. These mutations are lethal when combined with *sec4-8* at 25°C. Mutations that block elsewhere on the pathway are not suppressed by the *SEC4* duplication and are not lethal when combined with *sec4-8*. We propose that the *SEC4* product is a GTP-binding protein that plays an essential role in controlling a late stage of the secretory pathway.

Introduction

Many of the features of the protein secretion pathway of animal cells are conserved in the lower eukaryote Saccharomyces cerevisiae. In both cases proteins are inserted into the lumen of the endoplasmic reticulum and pass to the Golgi, a morphologically, biochemically, and functionally distinct organelle (Palade, 1975; Novick et al., 1981). Transport to the cell surface is accomplished by fusion of Golgi-derived secretory vesicles or granules with the plasma membrane. Many cells exhibit only constitutive secretion; proteins are secreted in a continuous fashion at the same rate as they are synthesized. In these situations there is no appreciable intracellular accumulation of secretory proteins, though the transit times to the cell surface may vary from several minutes in yeast to several hours in some animal cells (Novick and Schekman, 1983; Strous and Lodish, 1980). In the specialized organs of higher eukaryotes, certain cell types may possess a constitutive secretory pathway as well as a regulated pathway (Gumbiner and Kelly, 1982). A subset of the transported proteins are stored in secretory granules and are released by exocytosis following a stimulus. There is no evidence that yeast cells possess such a stimulus-regulated secretory pathway. However, the yeast pathway is regulated in another sense. Protein secretion is restricted to the growing region of the cell known as the bud (Field and Schekman, 1980; Sloat et al., 1981; Tkacz and Lampen, 1973). The molecular mechanism by which exocytosis is limited to this specialized domain of the plasma membrane is not yet understood.

To address the molecular mechanisms of protein secretion, a large number of temperature-sensitive yeast secretion (*sec*) mutants have been isolated (Novick and Schekman, 1979; Novick et al., 1980). At their restrictive temperature these mutants block the transport of all known surface proteins, and as a result, there is an intracellular accumulation of secretory protein and an exaggeration of one of the secretory organelles. Mutations in nine *SEC* genes can result in the buildup at the endoplasmic reticulum (ER) stage. Mutations in two *SEC* genes can cause an exaggeration of the Golgi apparatus. Mutation in ten genes can result in accumulation of post-Golgi vesicles. The products of these ten genes may be required in the vectorial transport of vesicles to the bud, the recognition of the plasma membrane by the vesicles, or the fusion of the vesicles with the plasma membrane. Their biochemical functions are not yet known. In this paper we focus principally on these late-acting *SEC* genes. We present evidence that one of these genes, *SEC4*, encodes a *ras*-like protein and that there are strong genetic interactions between *SEC4* and three other late *SEC* genes.

The viral *ras* gene was originally identified by its transforming capability (Ellis et al., 1982). It is now clear that the *ras* gene family is large, diverse, and ubiquitous. Proteins that share a high degree of homology with the p21 *ras* gene product have been found in eukaryotes from yeast to man (Defeo-Jones et al., 1983; Powers et al., 1984; Shilo and Weinberg, 1981; Pawson et al., 1985). These proteins are thought to function in signal transduction (Bourne, 1986). Additional genes that share more limited homology with p21 have been found in these organisms (Gallwitz et al., 1983; Chardin and Tavitian, 1986; Madaule and Axel, 1985). In these more distantly related members of the family, homology is best conserved in four regions thought to serve collectively as a GTP-binding site (McCormick et al., 1985; Jurnak, 1985). In this paper we show that the *SEC4* gene product fits this criteria for a *ras*-like protein; it possesses the four domains necessary for GTP-binding activity.

Results

Cloning *SEC4*

The *SEC4* gene was originally identified by its ability to partially complement the *sec15-1* growth defect. NY64 (*ura3-52, sec15-1*) (Table 1) was transformed with a plasmid library of wild-type yeast inserts on a vector (YCp50) carrying the *URA3* gene and a yeast centromere. Transformants were selected at 25°C on plates lacking uracil, then replica-stamped to YPD plates, which were incubated at 37°C. Temperature-resistant colonies were picked, and the plasmids were recovered in E. coli and purified. Transformation of the *sec15-1* strain with the isolated plasmids demonstrated that six plasmids encoded complementing activity. The smallest of the complementing plasmids (pNB85) was restriction mapped (Figure 1). By subcloning portions of the insert into the YCp50 vector and testing for complementation of the *sec15-1* growth defect, the complementing region was localized to a 1.4 kb fragment

Table 1. Yeast Strains

NY3[a]	*MATa, ura3-52, sec1-1*
NY4[a]	*MATα, his4-619, sec1-1*
NY15[b]	*MATα, ura3-52, his4-619*
NY16[c]	*MATα, his4-619, sec6-4*
NY17[c]	*MATa, ura3-52, sec6-4*
NY22[c]	*MATa, ura3-52, sec5-24*
NY29[c]	*MATa, ura3-52, sec4-8*
NY44[c]	*MATa, ura3-52, sec8-9*
NY45[c]	*MATα, ura3-52, sec3-2*
NY55[c]	*MATα, his4-619, sec9-4*
NY57[c]	*MATa, ura3-52, sec9-4*
NY61[c]	*MATa, ura3-52, sec10-2*
NY63[c]	*MATα, his4-619, sec10-2*
NY64[c]	*MATa, ura3-52, sec15-1*
NY65[c]	*MATα, his4-619, sec15-1*
NY67[c]	*MATa, his4-619, sec15-1*
NY130[c]	*MATa, ura3-52, sec2-41*
NY133[c]	*MATα, his4-619, sec2-41*
NY176[c]	*MATa, ura3-52, sec7-1*
NY181[c]	*MATa, sec14-3*
NY183[c]	*MATa, sec18-1*
NY275[d]	*MATα, his4-619, act1-3*
NY376-NY379[e]	*MATa, ura3-52, sec15-1, SEC4::SEC4, URA3*
NY380[e]	*MATα, ura3-52, his4-619, SEC4::SEC4, URA3*
NY402[c]	*MATa, ura3-52, sec5-24*
NY404[c]	*MATα, his4-619, sec4-8*
NY405[c]	*MATa, ura3-52, sec4-8*
NY409[c]	*MATa, his4-619, sec4-8*
NY410[c]	*MATa, ura3-52, sec8-9*
NY412[c]	*MATa, ura3-52, sec3-2*
NY413[c]	*MATα, ura3-52, sec13-1*
NY414[c]	*MATa, ura3-52, sec13-1*
NY415[c]	*MATa, ura3-52, sec16-2*
NY416[c]	*MATα, ura3-52, sec16-2*
NY417[c]	*MATα, ura3-52, sec17-1*
NY418[c]	*MATa, ura3-52, sec17-1*
NY419[c]	*MATα, ura3-52, sec19-1*
NY420[c]	*MATα, ura3-52, sec19-1*
NY421[c]	*MATα, ura3-52, sec20-1*
NY422[c]	*MATa, ura3-52, sec20-1*
NY423[c]	*MATa, ura3-52, sec21-1*
NY424[c]	*MATα, ura3-52, sec21-1*
NY425[c]	*MATα ura3-52, sec22-3*
NY426[c]	*MATa, ura3-52, sec22-3*
NY427[f]	*MATα, ura3-52, leu2-3, 112, trp1, his4, sec12-4*
NY428[f]	*MATα, ura3-52, leu2-3, 113, his3, sec23-1*
NY430[c]	*MATa, ura3-52, sec14-3*

[a] *sec* allele initially described in Novick and Schekman (1979), then backcrossed repeatedly to NY15 until uniform temperature sensitivity was seen among the mutant meiotic products.
[b] The *ura3-52* allele was backcrossed 7 times to DBY877 (*MATα, his4-619*), a strain from the collection of Dr. D. Botstein that is isogenic with S288C.
[c] *sec* allele initially described in Novick et al. (1980), then backcrossed repeatedly to NY15 until uniform temperature sensitivity was seen among the mutant meiotic products.
[d] *act1-1* allele initially described in Shortle et al. (1984), then repeatedly backcrossed to NY15 until uniform temperature sensitivity was seen among the mutant meiotic products.
[e] Strain construction described in this paper.
[f] *sec* allele initially described in Novick et al. (1980). Strain obtained from Dr. R. Schekman.

defined by EcoRI and BamHI restriction sites (Figure 1, pNB139). Two other clones, independently isolated from the YCp50 library by complementation of *sec15-1*, also contained the same 1.4 kb fragment, but had different endpoints (data not shown). The other three complementing plasmids had restriction maps unrelated to that of pNB85, but related to each other. Inserting a synthetic linker into an internal PvuII site on pNB136 destroyed complementing activity, as did deletion of the region to the left of the HindIII site (Figure 1, pNB145). Therefore we conclude that the PvuII and HindIII sites are internal to the complementing gene.

The ability of the 1.4 kb EcoRI–BamHI fragment to complement the *sec15-1* defect on a single copy vector suggests that the cloned sequence contains the *SEC15* gene. To test this possibility, we determined whether the cloned sequence could direct a plasmid to integrate at the *SEC15* chromosomal locus. We subcloned the 1.4 kb fragment into the YIp5 vector (Figure 1, pNB141). This vector does not have a yeast origin of replication and can only give stable transformation by homologous recombination into the genome (Figure 2). Recombination into the chromosomal locus homologous with the insert sequence was stimulated by cleaving the plasmid at the unique HindIII site, which is internal to the complementing gene. Integration at this locus results in a duplication of the cloned sequence, with the *URA3* gene between the two copies. This was verified by Southern blot analysis of NY380, a Ura$^+$ transformant of the strain NY15 (*ura3-52, his4-619*). Digesting the chromosomal DNA with BamHI and probing the blot with the 1.4 kb EcoRI–BamHI fragment gave the predicted pattern (Figure 2). This result established that a single copy of the plasmid had integrated at the chromosomal locus homologous to the insert. The Ura$^+$ transformant, NY380, was crossed to NY64 (*sec15-1, ura3-52*). The diploids were induced to sporulate, and the tetrads were dissected. If the cloned sequence is *SEC15* we would expect to see complete linkage of the Ura$^+$ plasmid marker with the temperature sensitivity; that is, 2 spores would be temperature-sensitive and Ura$^-$ and 2 spores would be temperature-resistant and Ura$^+$. Instead we found that temperature sensitivity did not segregate 2:2 in the cross; many tetrads contained 3 or 4 temperature-resistant spores (Table 2). The Ura$^+$ phenotype did segregate 2:2, and all Ura$^+$ spores were temperature-resistant. These data suggest that rather than cloning *SEC15*, we have cloned an unlinked locus which, when duplicated, can suppress the *sec15-1* defect.

One possible explanation for these surprising findings is that the cloned locus normally fulfills a function similar to that of *SEC15*. Duplication would therefore compensate for the *sec15-1* defect. Candidates for such a locus are other late-acting *SEC* genes. To test whether the cloned sequence is any of the known *SEC* genes, we transformed

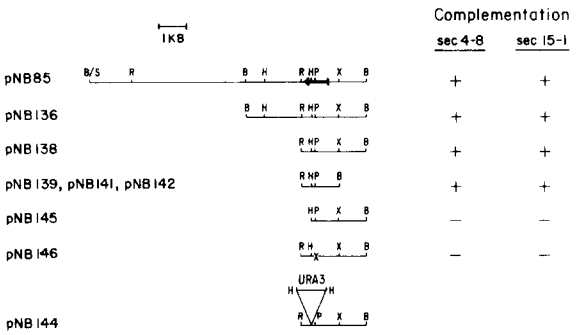

Figure 1. Complementing Activity of Plasmids

pNB85 was isolated from the CEN yeast library. The *SEC4* coding region is shown as a heavy arrow. pNB136 was constructed by insertion of the 4.4 kb BamHI fragment into the BamHI site of the CEN plasmid, YCp50. pNB138 was constructed by deletion of the EcoRI–EcoRI fragment of pNB136. pNB139 was constructed by cleavage of the pNB138 with XbaI and BamHI, filling in with Klenow, and ligation. This construction recreates a BamHI site but eliminates the XbaI site. pNB141 was constructed by insertion of the 1.4 kb EcoRI–BamHI fragment from pNB139 into the yeast integrating plasmid YIP5 cut with EcoRI and BamHI. pNB142 was constructed by insertion of the 1.4 kb EcoRI–BamHI fragment from pNB139 into the yeast episomal plasmid pRB307 (from the collection of Dr. D. Botstein). pNB145 was constructed by cleavage of pNB136 with HindIII and religation. pNB146 was constructed by the insertion of a synthetic linker into the PvuII site of pNB138, generating a +2 frameshift in the coding sequence. pNB144 was constructed by insertion of the 2.4 kb EcoRI–BamHI fragment from pNB138 into YIP5 and then inserting the yeast 1.1 kb HindIII fragment carrying the yeast *URA3* gene into the HindIII site. Complementation activity was assessed as described in Experimental Procedures. Abbreviations are as follows: B/S, junction of a BamHI site and a Sau3A site; R, EcoRI; B, BamHI; H, HindIII; P, PvuII; X, XbaI.

a variety of the late-blocked *sec* mutants with pNB85. The transformants were tested for growth at the restrictive temperature, 37°C. We found that the *sec4-8* strain acquired wild-type temperature-resistance upon transformation. To prove that the same gene was complementing both the *sec15-1* defect and the *sec4-8* defect, we transformed the *sec4-8* strain with pNB146, a plasmid in which the PvuII site had been altered by insertion of a synthetic linker. We found that this point mutation eliminated both *sec15-1* and *sec4-8* complementing activity (Figure 1).

To prove that the cloned gene was in fact *SEC4*, we crossed NY380, the strain carrying the integrated plasmid, to NY29, a *sec4-8*, *ura3-52* strain. The Ura$^+$ phenotype and the temperature-sensitive phenotype segregated 2:2, and all Ura$^+$ spores were temperature-resistant (Table 2). This result demonstrates that the site of integration is tightly linked to the *SEC4* gene and constitutes proof of the identity of the cloned sequence.

Table 2. Cloned Sequence Integrates at *SEC4* Locus and Suppresses *sec15-1*

NY64 (*MAT***a**, *ura3-52, sec15-1*)
×
NY380 (*MATα, ura3-52, his4-619, SEC4:SEC4, URA3*)
17 Tetratype: 2 Ura$^+$ TS$^+$, 1 Ura$^-$, Ts$^+$, 1 Ura$^-$, Ts$^-$
2 Parental ditype: 2 Ura$^+$ Ts$^+$, 2Ura$^-$ Ts$^-$
1 Nonparental ditype: 2 Ura$^+$ Ts$^+$, 2 Ura$^-$ Ts$^+$
NY405 (*MAT***a**, *ura3-52, sec4-8*)
×
NY380 (*MATα, ura3-52, his4-619, SEC4:SEC4, URA3*)
0 Tetratype: 2 Ura$^+$ Ts$^+$, 1 Ura$^-$ Ts$^+$, 1 Ura$^-$ Ts$^-$
16 Parental ditype: 2 Ura$^+$ Ts$^+$, 2 Ura$^-$ Ts$^-$
0 Nonparental ditype: 2 Ura$^+$ Ts$^+$, 2 Ura$^-$ Ts$^+$

SEC4 Sequence

Having localized the *SEC4* gene to a 1.4 kb region, we determined the nucleotide sequence of this fragment (Figures 3 and 4). The sequence contains a single large open reading frame. This open reading frame covers the PvuII and HindIII sites that we know to be in the gene. The predicted protein has a molecular weight of 23,479 daltons, is uncharged at neutral pH, and contains no long hydrophobic stretch.

To determine the site of the *sec4-8* mutation with respect to the restriction map, we did fine structure mapping of the gene. If a CEN plasmid is cleaved with two restriction enzymes to generate gapped molecules and the purified fragments are used to transform a mutant strain, the deleted material is repaired from the chromosomal copy and the plasmid is maintained as an episomal element. Stable integration would give a dicentric chromosome and is therefore a lethal event. If the deletion covers the mutation, then the episomal copy will be repaired with mutant information and the transformants will be temperature-sensitive. If the plasmid is cut only once, but at a site close to the mutation, then the frequency of temperature-

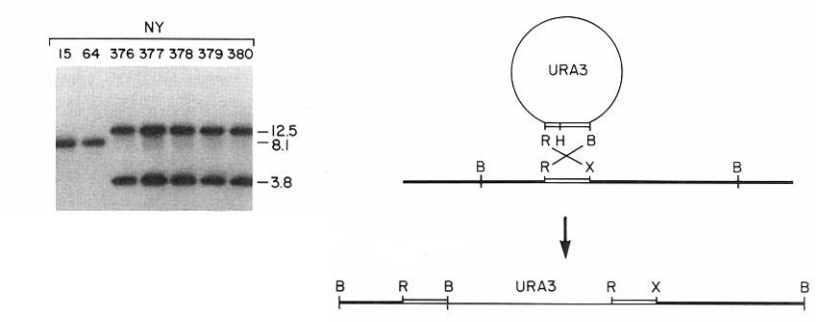

Figure 2. Integration Generates a Duplication

pNB141 was cleaved with HindIII and the linear DNA was used to transform a wild-type strain, (*MATα, ura3-52, his4-619*), and a *sec15* mutant strain, NY64 (*MAT***a**, *ura3-52, sec15-1*). Total DNA was isolated from the parental strains and from transformants derived from NY15 (NY380) and from NY64 (NY376–NY379). The DNA was digested with BamHI, electrophoresed in agarose, and transferred to a nitrocellulose filter, which was then probed. The isolated EcoRI–BamHI fragment of pNB139 was used as the probe. As predicted, the single 8.1 kb band in the parental strains is replaced with 3.8 and 12.5 kb bands. Note that the XbaI site has been converted to a BamHI site on the plasmid.

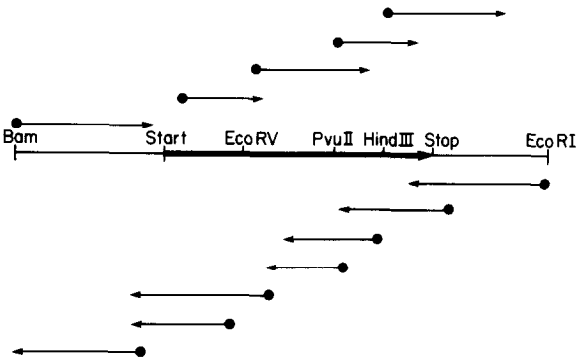

Figure 3. Restriction Map and Sequencing Strategy
The coding sequence is shown as a heavy arrow.

sensitive transformants is a function of the distance from the site of cleavage to the mutation site. Cleavage of either pNB136 or pNB139 at the PvuII site internal to *SEC4* yielded only 7% temperature-resistant transformants, while cleavage of pNB139 with HindIII gave 50% temperature-resistant transformants. Cleavage of pNB136 at sites external to the gene gave 100% temperature-resistant transformants. These data suggest that the mutation is very close to the PvuII site and somewhat more distant to the HindIII site. Deletion of the HindIII–PvuII fragment gave no temperature-resistant transformants, while deletion of the PvuII–XbaI fragment gave 7% temperature-resistant transformants. This proves that the mutation lies in the 150 bp region between the HindIII and PvuII sites, and not between the PvuII and XbaI sites.

To determine the nature of the mutation, we cloned the *sec4-8* mutant allele. This was accomplished by cleaving pNB136 with SacI and XbaI to generate a deletion covering the entire gene. Upon transformation of a *sec4-8* strain with the purified linear fragment, the deletion was repaired with the mutant information. The plasmid was recovered in E. coli, and the HindIII–BamHI fragment was subcloned into M13 for sequencing. Sequence analysis established that the only change between the PvuII site and the HindIII site is a change of a G to an A at position 843 relative to the BamHI site of pNB139, only 10 bases downstream from the PvuII site. Thus, the *sec4-8* mutation changes the glycine at amino acid 147 to an aspartate.

Sec4 Protein Is Homologous to *ras* Protein

A search for homologous sequences revealed significant homology between Sec4 and all *ras* transforming proteins (Figure 5b). With respect to human H-*ras*, 32.4% homology was found over the full length of the protein. Of the residues that can be altered to yield activated *ras* oncogenes, the glycine at position 13, the alanine at position 59, and the glutamine at position 61 are conserved in *SEC4*. The homology is strongest in the regions of the *ras* protein that have been implicated in GTP binding and hydrolysis. Over these four regions (underlined in Figure 5b), 73% identity is exhibited between *SEC4* and H-*ras*. The most prominent difference is the presence of an 18 amino acid N-terminal region that has no corresponding

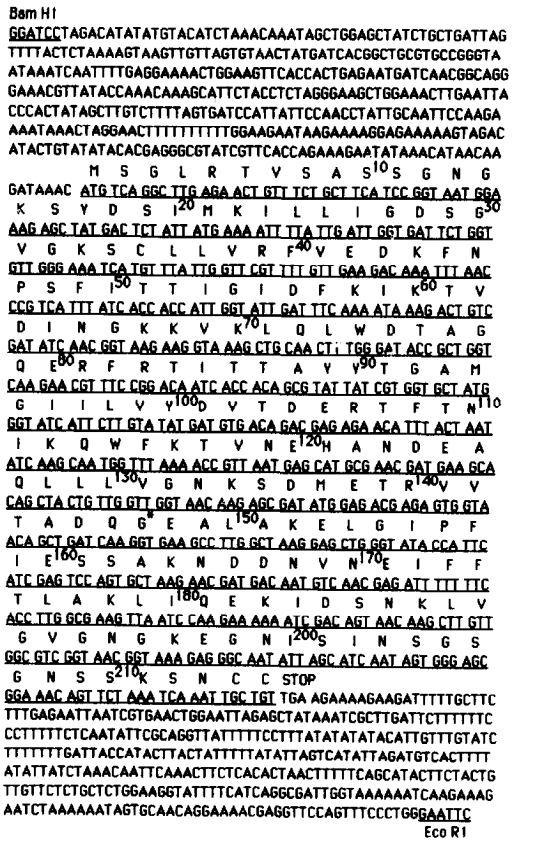

Figure 4. DNA Sequence of Complementing Region
The sequence of the 1.4 kb complementing region is shown. The single letter amino acid code is shown above the DNA sequence. The asterisk at amino acid 147 indicates the site of the *sec4-8* mutation that changes the glycine to an aspartate.

sequence in H-*ras*. Near the carboxyl terminus of N-*ras* there is a cysteine that becomes fatty acid acylated (Willumsen et al., 1984; Buss and Sefton, 1986). This modification may play a role in the localization of the protein to the inner surface of the plasma membrane. The *SEC4* sequence terminates with two cysteines. Although they do not fit the concensus palmitylation site, Cys-A-A-X, where A is an aliphatic residue and X is the carboxyl terminus, they may nevertheless be recognized by an alternate modifying enzyme. This hypothesis is supported by our recent finding that the *SEC4* gene product is membrane bound (B. Goud, A. Salminen, and P. Novick, unpublished observations).

The strongest homology was found with another yeast *ras* homolog, the *YPT1* gene product (Figure 5a). This gene, located between the actin and β-tubulin structural genes, was first identified as an open reading frame (Gallwitz et al., 1983) and is now known to be an essential locus (Schmitt et al., 1986; Segev and Botstein, 1987). *SEC4* shares 47.5% homology with this protein. The homology is maintained throughout the sequence, though the *SEC4* protein has a 12 residue amino-terminal portion with no analogue in *YPT1*. Both *SEC4* and *YPT1* encode a serine at the position corresponding to the glycine at position 12 in H-*ras*. In the case of mammalian *ras* genes, alteration

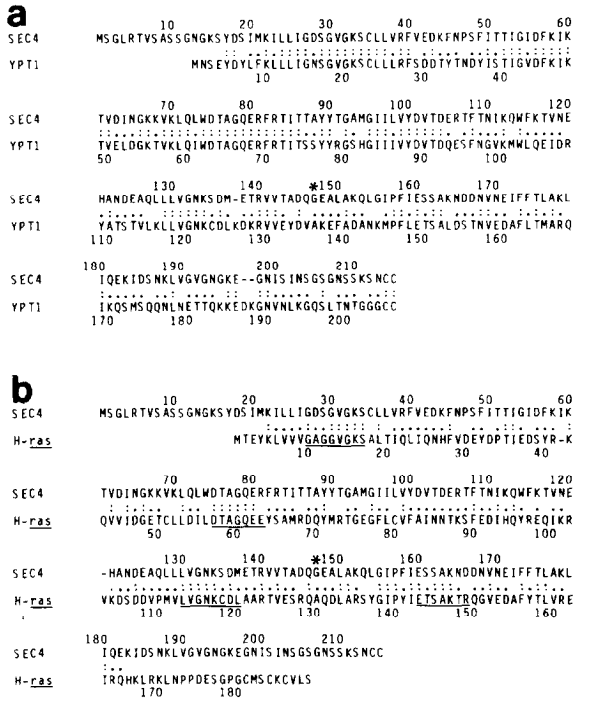

Figure 5. Comparison of SEC4 Protein with YPT1 and H-*ras* Proteins

The optimized lineup (Lipman and Pearson, 1985) of the predicted *SEC4* protein sequence with that of the *YPT1* protein is shown (a). The optimized lineup of *SEC4* protein with the H-*ras* protein (b). Double dots indicate identity and single dots indicate a conserved change. The underlined regions have been implicated in the binding of GTP. The asterisk at position 147 of *SEC4* indicates the site of the *sec4-8* mutation, a change from glycine to aspartate.

of this glycine leads to activation of the proto-oncogene. The unusual double cysteines at the carboxyl terminus of *SEC4* are conserved in *YPT1*. The function of these cysteine residues is not known, yet in the case of *YPT1* they are essential for gene function (Schmitt et al., 1986).

Two other yeast *ras* homologs have also been well characterized. These are the *RAS1* and *RAS2* genes. These two genes play interchangeable roles; neither one is essential but one of the pair must be functional for cell viability (Kataoka et al., 1984; Tatchell et al., 1984). The proteins encoded by these two genes have 107 residue carboxy-terminal extensions with no analogue in the mammalian proteins, but are otherwise significantly more homologous to N-*ras* than are the *SEC4* or *YPT1* gene products.

SEC4 Is an Essential Locus

Because of the existence of three other yeast genes that share homology with *ras*, we determined whether the *SEC4* gene was essential in its own right. Although the isolation of a recessive conditional lethal allele of *sec4* strongly suggests that the gene product is essential, we verified this by the construction of an insertion mutation. A 1.1 kb HindIII fragment carrying the yeast *URA3* gene was inserted into the HindIII site of pNB139, which is internal to the *SEC4* gene (Figure 1, pNB144). A linear fragment defined by the EcoRI and BamHI sites flanking the disrupted gene was used to transform a diploid strain homozygous for the *ura3-52* mutation, thereby replacing one chromosomal copy of *SEC4* with the disrupted allele. The Ura$^+$ diploid transformants were shifted to sporulation media, and tetrads were dissected. Two spores from each tetrad were inviable, and all viable spores were Ura$^-$. The non-growing colonies were arrested at the one or two cell stage. Although the insertion was at the carboxyl terminus of the coding sequence and therefore allowed for the synthesis of a truncated gene product, the expected result was obtained. This result establishes the *SEC4* gene as an essential locus and this putative null allele as a recessive lethal mutation. Furthermore, the result implies that the *SEC4* gene does not play an interchangeable role with *YPT1* or *RAS1* and *RAS2*.

Secretion Is Rapidly Blocked in *sec4-8* Cells

The finding that the *SEC4* product is homologous to *ras* caused us to reevaluate the role of *SEC4* in protein secretion. If the *SEC4* protein plays a direct role in secretion, we would expect to see a rapid block following a shift of mutant cells to 37°C. Previous studies (Novick et al., 1980) measured secretion and intracellular accumulation in *sec4-8* cells only after a 1 hr period at the restrictive temperature. Therefore, invertase secretion and accumulation were continuously followed in *sec4-8* cells during a shift from the permissive temperature to the restrictive temperature. By performing the experiment during the period of peak invertase synthesis we can accurately determine the rapidity of the secretory block. Cells were grown at 25°C in media containing 2% glucose to repress the synthesis of invertase. The cells were shifted to media containing 0.1% glucose to derepress invertase synthesis, and after 35 min of derepression, the culture was shifted to 37°C. Within 5 min of the shift in temperature, the secretion block is greater than 80% (Figure 6); that is, of the differential increase of total invertase activity during the 5 min period, more than 80% of the increase is intracellular rather than cell surface. By 15 min after the temperature shift the secretion block is essentially 100%. Therefore, the rapidity of the secretion block is consistent with a direct role in protein secretion. An indirect block in secretion resulting from some imbalance in cell growth would presumably take a longer time to act.

Suppression by *SEC4* Duplication

The suppression of *sec15-1* by multiple copies of *SEC4* was examined more thoroughly. A wild-type (NY15) and a *sec15-1* strain (NY64) were transformed with plasmids carrying the 1.4 kb EcoRI–BamHI *SEC4*-containing fragment. The vectors used were the CEN plasmid, YCp50, which is maintained in low copy number (Figure 1, pNB139); pRB307, a plasmid carrying a portion of the yeast 2 μm circle, which is maintained at about 20 copies per cell (pNB142); or YIp5, a vector that must integrate in order to replicate and is therefore stably maintained at low copy number (pNB141). Plasmid stability was tested in the case of the pNB142 transformants to prove that the plasmid was episomal and had not integrated. The pNB141 transfor-

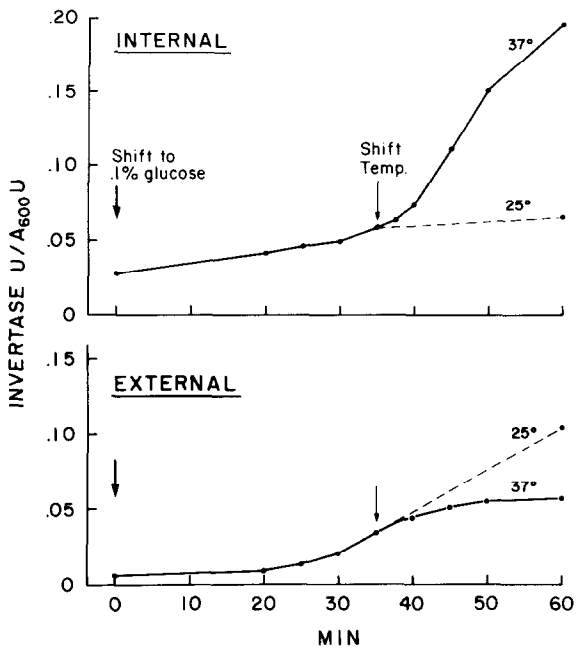

Figure 6. Secretion and Accumulation of Invertase in *sec4-8*

NY404 (*MATα, his4-619, sec4-8*) was grown overnight at 25°C in YP media containing 2% glucose. At 0 time the cells were shifted to YP media containing 0.1% glucose. After 35 min at 25°C the culture was shifted to 37°C. At time points, samples were removed and assayed for internal and external invertase as described in Experimental Procedures.

mants were tested by Southern blot analysis to establish that only one plasmid had integrated, duplicating *SEC4* (Figure 2). The growth rates of the transformants and the parental strain were measured following a shift from 25°C to 37°C. All of the *SEC15* transformants grew with a doubling time of 102 min at 37°C. The parental *sec15-1* strain stopped growing soon after the shift. The *sec15-1* strains carrying a second copy of *SEC4* integrated into the genome or on the CEN vector grew at 37°C with a doubling time of 130 min. Further increase in *SEC4* copy number by transformation with a 2 μm-derived plasmid did not result in better growth of *sec15-1* strains.

Suppression of the secretory defect of *sec15-1* was assessed quantitatively. While NY64 cells accumulate 70% of the newly synthesized invertase intracellularly during a 1 hr derepression, NY376 accumulated only 17% intracellularly. Wild-type cells show no increase in the internal invertase level under these conditions.

Suppression of *sec15-1* by duplication of *SEC4* was also evaluated by thin section electron microscopy. Although we did not attempt to quantitate vesicle accumulation, the results are qualitatively consistent with our measurements of growth rate and invertase secretion. The parental *sec15-1* strain, NY64, shows accumulation of many 100 nm vesicles after 2 hr at 37°C (Figure 7). When a second copy of *SEC4* is introduced on an integrating vector (NY376) the accumulation of vesicles is much reduced, though still greater than in wild-type cells. Therefore, duplication of *SEC4* suppresses the full range of *sec15-1* phenotypes. In all parameters the suppression, while dramatic, is not complete.

To determine whether the pattern of gene interaction extends beyond *SEC4* and *SEC15*, we transformed representative mutants of all of the *SEC* complementation groups with a CEN plasmid carrying *SEC4* (pNB139). The parental mutants and the transformants were tested for growth at 37°C, 33.5°C, 30°C, and 25°C. The results are shown in Table 3. At intermediate temperatures duplication of *SEC4* can partially suppress the growth defects of *sec2-41* and

Table 3. Growth Properties of *sec* Strains with and without an Extra Copy of *SEC4*

		Parental Strain				pNB139 Transformed Strain			
Strain	Genotype	25°C	30°C	33.5°C	37°C	25°C	30°C	33.5°C	37°C
NY3	*sec1-1*[a]	+	+	−	−	+	+	+/−	−
NY130	*sec2-41*[a]	+	+/−	−	−	+	+	+	+/−
NY45	*sec3-2*	+	+	−	−	+	+	−	−
NY29	*sec4-8*[a]	+	+/−	−	−	+	+	+	+
NY22	*sec5-24*[a]	+	+	−	−	+	+	+/−	−
NY17	*sec6-4*	+	+	+/−	−	+	+	+/−	−
NY176	*sec7-1*	+	+	+	−	+	+	+	−
NY44	*sec8-9*[a]	+	−	−	−	+	+	+	−
NY57	*sec9-4*	+	+	+/−	−	+	+	+/−	−
NY61	*sec10-2*[a]	+	+	+/−	−	+	+	+	−
NY427	*sec12-4*	+	+	−	−	+	+	−	−
NY413	*sec13-1*	+	+	−	−	+	+	−	−
NY430	*sec14-3*	+	+	+	−	+	+	+	−
NY64	*sec15-1*[a]	+	+	−	−	+	+	+	+
NY416	*sec16-2*	+	+/−	−	−	+	−	−	−
NY417	*sec17-1*	+	+	−	−	+	+	−	−
NY431	*sec18-1*	+	+	−	−	+	+	−	−
NY419	*sec19-1*[a]	+	+	−	−	+	+	+	−
NY421	*sec20-1*	+	+	−	−	+	+	−	−
NY424	*sec21-1*	+	+	+	−	+	+	+	−
NY425	*sec22-3*	+	+	−	−	+	+	−	−
NY428	*sec23-1*	+	+	−	−	+	+	−	−

[a] Strain exhibited detectable suppression by increase in *SEC4* copy number.

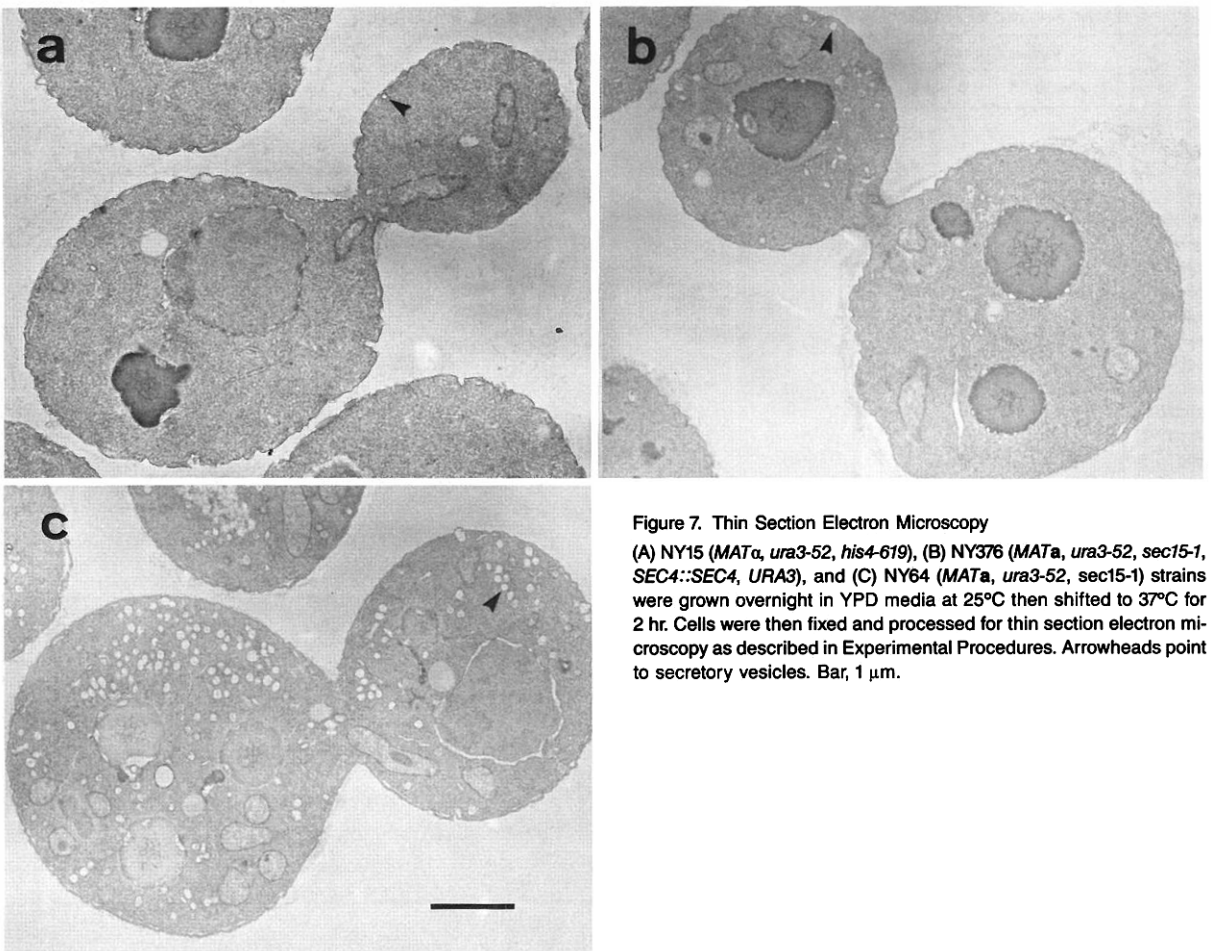

Figure 7. Thin Section Electron Microscopy

(A) NY15 (*MATα*, *ura3-52*, *his4-619*), (B) NY376 (*MAT***a**, *ura3-52*, *sec15-1*, *SEC4::SEC4*, *URA3*), and (C) NY64 (*MAT***a**, *ura3-52*, sec15-1) strains were grown overnight in YPD media at 25°C then shifted to 37°C for 2 hr. Cells were then fixed and processed for thin section electron microscopy as described in Experimental Procedures. Arrowheads point to secretory vesicles. Bar, 1 μm.

sec8-9 in addition to *sec15-1* (Table 3). Suppression can also be seen over a quite limited temperature range in the cases of *sec1-1*, *sec 5-24*, *sec10-2*, and *sec19-1*. With the exception of *sec19-1*, all of these suppressible mutations appear to block late steps on the secretory pathway; at the restrictive temperature they accumulate post-Golgi vesicles. It is not clear where on the pathway *sec19-1* is blocked; at the restrictive temperature ER, Golgi-like structures, and vesicles accumulate in this mutant. None of the ER- or Golgi-blocked mutants are suppressed by the *SEC4* duplication, and not all vesicle-accumulating mutants are suppressed by the duplication. *sec3-2*, *sec6-4*, and *sec9-4* show no detectable change in their growth properties when transformed with the *SEC4* gene on CEN plasmids. When a high copy number plasmid carrying *SEC4* was used the results were essentially the same (data not shown). Examination of allele specificity can help to determine the mechanism of suppression; unfortunately only one allele of each *SEC* complementation group has been saved (R. Schekman, personal communication).

Double Mutants Are Lethal at 25°C

Another test for genetic interaction is to evaluate the phenotype of double mutants. Crosses were performed between *sec4-8* and a representative allele of all other *sec* complementation groups. In seven crosses inviability was seen among approximately one-fourth of the meiotic products. The pattern of inviability suggests that it is the combination of *sec* defects that is lethal. In tetrads with 4 viable spores, all are temperature-sensitive. When 3 spores are viable, 2 of them are temperature-sensitive, and when 2 spores are viable, both are temperature-resistant (see Table 4). The lethal combinations are *sec4-8* with *sec2-41*, *sec3-2*, *sec5-24*, *sec8-9*, *sec10-2*, *sec15-1*, and *sec19-1*. Complementation analysis was performed on all meiotic products of the cross between *sec4-8* and *sec15-1* to confirm the genotype of the viable spores (see Table 5). This analysis verified that the inviable spores were double mutants. All of the other crosses to *sec4-8* gave excellent spore viability. Therefore, with the exception of *sec1-1*, all of the mutations that can be partially suppressed by the *SEC4* duplication are lethal when combined with *sec4-8*. We also crossed *sec15-1* to all of the vesicle-accumulating mutants to determine whether other combinations were lethal as well. We found that *sec15-1* is lethal when combined with the same set of mutants. The only exception to this rule is *sec19-1*; this mutant is suppressed by the *SEC4* duplication and is lethal when combined with *sec4-8*, yet

Table 4. Tetrad Analysis of *sec* Crosses

sec4-8 ×	4 Viable Spores				3 Viable Spores			2 Viable Spores		
	4−:0+[a]	3−:1+	2−:2+	1−:3+	3−:0+	2−:1+	1−:2+	0−:2+	1−:1+	2−:0+
sec1-1	1	7	2		1					
sec2-41[b]	3	1				8	1	4	1	
sec3-2[b,c]	5					1		6		
sec5-24[b]	2		1	1		5	1	2		
sec6-4	1	9	2							
sec7-1	1	9	1					1		
sec8-9[b]	1				1	6		1	1	
sec9-4[c]	6	1	4							
sec10-2[b]	2		1		1	4		2		
sec12-4	2	4	4			1				1
sec13-1	4	6				2				
sec14-3	2	6	2						1	
sec16-2	2	8	2							
sec17-1	1	9			2					
sec18-1	1	9			2					
sec19-1[b]	2					3		6	1	
sec20-1		7	2			2			1	
sec21-1	3	8				1				
sec22-3	3	6	1			1				
sec23-1	2	9							1	
sec15-1 ×										
sec1-1	1	7	2							
sec2-41[b]	5	1				14	1	1		1
sec3-2[b]	2					10				
sec5-24[b]	1					9		1	1	
sec6-4	2	8			1					
sec8-9[b]	3				1	14		2	4	
sec9-4	1	7	3							
sec10-2[b]	3		1			8	1	3	1	
sec19-1	1	7	3						1	
sec23-1	2	9	1							
act1-3 ×										
sec1-1	1	7	3			1				
sec2-41	1	8	3							
sec4-8	2	8								
sec6-4		8	3							
sec9-4	1	8	1			1			1	
sec10-2	1	7	3							
sec15-1	2	5	2			1				

Mating, sporulation, and germination was done at 25°C.

[a] In a ratio such as 4−:0+ the (−) implies temperature-sensitive and the (+) implies temperature-resistant.

[b] Cross in which lethality of double mutants was demonstrated.

[c] Both temperature-sensitive mutations are centromerically linked and therefore parental ditypes and nonparental ditypes are prevalent.

Table 5. *sec4-8* Is Lethal When Combined with *sec15-1*

NY65 (*MATα, his4-619, sec15-1*)
×
NY29 (*MATa, ura3-52, sec4-8*)
10 Tetratype: 1 *SEC4, SEC15*; 1 *SEC4, sec15-1*; 1 *sec4-8, SEC15*; 1 inviable
1 Parental ditype: 2 *SEC4, sec15-1*; 2 *sec4-8, SEC15*
3 Nonparental ditype: 2 *SEC4, SEC15*; 2 inviable

is not lethal when combined with *sec15-1*. This is also the only mutant of the set that does not exclusively accumulate vesicles. One mutant, *sec3-2*, is lethal when combined with *sec4-8* or *sec15-1*, yet showed no detectable suppression when transformed with a plasmid carrying *SEC4*.

Another gene that may play a role late on the secretory pathway is the actin gene, *ACT1* (Novick and Botstein, 1985). Crosses were performed between a temperature-sensitive actin mutant, *act1-3*, and many of the late-blocked *sec* mutants. Excellent spore viability was seen in all crosses to *act1-3* (Table 4).

Discussion

We have shown that Sec4, a protein that plays an essential role in the transport of secretory proteins from the Golgi to the cells surface, shares significant homology with the p21 *ras* protein family. More extensive homology is seen with another yeast *ras* homolog, *YPT1*. We have also

Table 6. Summary of Interactions between *sec4* and Other *sec* Genes

Mutations Either Suppressed by *SEC4* Duplication or Lethal When Combined with *sec4-8*	Viability of Double Mutant				
	sec4-8 ×	*sec15-1* ×	*act1-1* ×	Suppression by 2 × *SEC4*	Phenotype
sec1-1	+	+	+	+/−	Ves
sec2-41	−	−	+	+	Ves
sec3-2	−	−		−	Ves
sec4-8		−	+	+	Ves
sec5-24	−	−		+/−	Ves
sec8-9	−	−		+	Ves
sec10-2	−	−	+	+/−	Ves
sec15-1	−		+	+	Ves
sec19-1	−	+		+/−	ER-Golgi-ves
Mutations Not Suppressed by Duplication of *SEC4* and Not Lethal When Combined with *sec4-8*					
sec6-4	+	+	+	−	Ves
sec7-1	+			−	Golgi
sec9-4	+	+	+	−	Ves
sec12-4	+			−	ER
sec13-1	+			−	ER
sec14-3	+			−	Golgi-ves
sec16-2	+			−	ER
sec17-1	+			−	ER
sec18-1	+			−	ER
sec20-1	+			−	ER
sec21-1	+			−	ER
sec22-3	+			−	ER
sec23-1	+	+		−	ER

demonstrated an extensive pattern of genetic interactions between *SEC4* and a subset of the other *SEC* genes. Here, we will discuss these results and place them in the larger context of eukaryotic secretion and *ras* oncogene function.

Ras proteins are ubiquitous among eukaryotes. The general theme that has emerged implicates these proteins in signal transduction (Bourne, 1986). They are thought to act by a mechanism analogous to that of the G-proteins, which regulate adenylate cyclase: upon stimulation by a cell surface receptor, the protein binds GTP and then activates an effector enzyme. The activation is curtailed by slow hydrolysis of the bound GTP. The sequence of the *SEC4* gene product exhibits homology with H-*ras* in the four regions thought to constitute a GTP-binding site. Therefore we can predict that the *SEC4* protein probably binds GTP. While a variety of GTP-binding proteins are found in both prokaryotes and eukaryotes, Chardin and Tavitian (1986) have shown that a strictly conserved domain corresponding to amino acids 57–63 of K-*ras* defines a distinct set of *ras*-like proteins. Although the *SEC4* gene product has only moderate homology with K-*ras* (32%), it does fit this criteria for a *ras*-like (*ral*) protein.

Does the homology signify anything else? While the regulatory nature of *ras* proteins may be conserved, the activating stimuli and regulated effector pathways are certainly diverse. Three *ras* homologs have been previously identified in yeast: *RAS1*, *RAS2*, and *YPT1* (Defeo-Jones et al., 1983; Powers et al., 1984; Gallwitz et al., 1983). *RAS1* and *RAS2* share excellent homology with H-*ras* in the amino-terminal portion of the proteins, yet they have long carboxy-terminal extensions not found in the mammalian gene. A combination of genetic and biochemical evidence has established *RAS1* and *RAS2* as regulatory elements in the synthesis of cAMP (Toda et al., 1985). Recent evidence indicates that H-*ras* does not regulate cAMP levels in mammalian cells (Beckner et al., 1985); rather it is thought to couple the activation of cell surface receptors to the production of inositol triphosphate and diacylglycerol and ultimately to the activation of protein kinase C (Wakelam et al., 1986; Fleischman et al., 1986). *YPT1* shares less homology with H-*ras*, yet is closer in size to the mammalian gene. The target of *YPT1* is not known, though it has been suggested that *YPT1* might regulate microtubule organization (Schmitt et al., 1986), and effects on actin assembly, sporulation, and nitrogen starvation response have also been demonstrated (Segev and Botstein, 1987). Therefore, we cannot assume that the moderate degree of structural homology between the *SEC4* product and p21 *ras* implies a similar target. It also appears unlikely that *SEC4* fulfills a function identical to that of *RAS1*, *RAS2*, or *YPT1*. *SEC4* is an essential gene in its own right, while *RAS1* and *RAS2* can each replace the other's function (Tatchell et al., 1984; Kataoka et al., 1984). *SEC4* shows the best homology (47.5%) with *YPT1*, yet since *YPT1* and *SEC4* are each essential in their own right, it is unlikely that *SEC4* and *YPT1* fulfill similar roles. Supporting this conclusion, we find that *YPT1* on a high copy number vector does not even partially complement the *sec4-8* mutation, and *SEC4* on a high copy number vector does not complement a cold-sensitive allele of *YPT1* (Novick, Salminen, Segev, and Botstein, unpublished observations).

Why is a putative signal transduction protein needed for yeast secretion? Such a role might be anticipated in the case of a stimulated secretory pathway. In fact, Gomperts (1986) has demonstrated GTP-stimulated release of granules in permeabilized neutrophils and has postulated a GTP-binding protein on the granule surface. However, the yeast pathway is constitutive. No known stimulus needs to be transduced for exocytosis to occur. Three possible explanations are offered. The first possibility is that the *SEC4* product is not a regulatory protein. Although the sequence predicts a capacity for binding GTP, other properties of the protein may have diverged significantly from the H-*ras* protein. It should be noted that signal transduction roles have not been established for other proteins having moderate homology with H-*ras*, for example, *ral*, *rho*, and *YPT1*. The second possibility is that the yeast secretory pathway is regulated, not temporally, but spatially. The *SEC4* protein may be necessary to limit the site of vesicle fusion to the bud. This would be a novel role for a *ras*-like protein, yet it is consistent with the localization of *ras* proteins to the inner surface of the plasma membrane. The third explanation is that the same type of cellular machinery is necessary for both constitutive and regulated secretion. In the case of the constitutive pathway, the effector enzymes may be regulated at a constant level by a *ras*-like protein.

Recent experiments on the role of p21 *ras* protein in endocytosis indirectly support the notion of the *SEC4* protein as a steady-state regulator of membrane traffic. Bar-Sagi and Feramisco (1986) have shown that microinjection of the p21 *ras* protein into fibroblasts leads to a transient increase in pinocytosis and that injection of oncogenic *ras* protein causes a prolonged response. Conversely, Reizman et al. (1986) has shown that all of the vesicle-accumulating *sec* mutants, including *sec4-8*, block fluid phase uptake at the restrictive temperature. While the activated *ras* protein cannot hydrolyze GTP and is therefore stuck in its stimulatory mode, the *sec4-8* protein may fail to activate its effector protein at the restrictive temperature. Thus the two mutations would have opposite effects on endocytosis. This hypothesis is consistent with the dominant nature of the oncogenic *ras* mutations and the recessive nature of the *sec4-8* mutation. It is not clear whether this extended analogy will stand up to closer scrutiny.

How can we explain the extensive genetic interaction seen between *SEC4* and the other three late *SEC* genes? We will consider three types of suppression: informational suppression, suppression by interaction, and bypass suppression. Informational suppression can be ruled out because a temperature-sensitive allele of *SEC4* shows a rapid secretory block. Suppression by interaction can be observed if alterations in one protein can compensate for defects in the other member of a protein–protein complex (Jarvik and Botstein, 1975). This mechanism of suppression seems unlikely because of the large number of gene products involved and because suppression by *SEC4* is achieved not by alteration, but by overexpression. Suppression by overproduction usually suggests that the two gene products fulfill similar functions. Overproducing one gene product bypasses the need for the other gene product. A recent example of this mechanism was demonstrated by Shatz et al. (1986). In this case, overexpression of one α-tubulin gene was found to bypass the requirement for a second α-tubulin gene. It is unlikely that the *SEC4* product has a structural similarity to the other three *SEC* proteins. The *SEC15* gene is much larger than *SEC4*, and the two genes do not cross-hybridize on Southern blots (Salminen and Novick, unpublished results). Nevertheless, *SEC4* may function on a pathway that is parallel to the one on which the other five gene products function. By this model, overexpression of *SEC4* would compensate for a partial defect on the alternate pathway, while a partial defect on both pathways would be synergistically deleterious and therefore lethal at all temperatures. Alternatively, there may only be one linear pathway and *SEC4* functions as a master regulator. Overexpressing *SEC4* could therefore compensate for a partial defect elsewhere on the pathway, while a partial loss of *SEC4* function would make other partial defects more severe. What might be the ultimate target of the pathway(s)? It is too early to anticipate an answer to this question, but we are obtaining antibodies to the products of *SEC4* and other late *SEC* genes to start to address their biochemical functions directly.

Experimental Procedures

Yeast Genetic Techniques

Yeast strains used in this study are listed in Table 1. Cells were grown on rich medium (YPD), containing 1% Bacto yeast extract, 2% Bacto-Peptone (Difco), and 2% glucose, or on minimal medium (SD), 0.7% yeast nitrogen base without amino acids (Difco), and 2% glucose, supplemented for auxotrophic requirements as described by Sherman et al. (1974) when necessary.

The yeast genomic library was constructed by inserting fragments produced by partial Sau3A digestion of chromosomal DNA into the BamHI site of plasmid YCp50 (Rose, Novick, Thomas, Botstein, and Fink, unpublished observation). The YCp50 vector was originally constructed by C. Mann, and a map can be found in Kuo and Cambell (1983).

Yeast transformation was done by the method of alkali cation treatment (Ito et al., 1983). Transformants were selected on SD medium at 25°C.

For the complementation and suppression assays, transformants were suspended in the wells of a 96 well microtiter plate. From the wells, the cells were transferred with a multi-prong transfer device onto YPD plates, which were incubated at 37°C. For more accurate determination of suppression, the cell suspensions were first transferred to a second microtiter plate. These twice-diluted suspensions were then transferred onto YPD plates, which were incubated at 37°C, 33.5°C, 30°C, and 25°C. This dilution protocol allowed the detection of single colony growth. For the determination of doubling times, the cells were grown in YPD broth at 25°C with agitation to an OD_{600} of 0.5, then shifted to 37°C. The growth was monitored at 1 hr intervals by following the OD_{600} of the cultures over an 8 hr period.

Plasmid recovery from transformed yeast cells was done as follows. Total yeast DNA was extracted by the method of Holm et al. (1986). E. coli cells were transformed with approximately 50 ng of this preparation. E. coli transformants were selected on LB plates containing ampicillin at 100 μg/ml. The plasmid DNA was then recovered from E. coli and purified.

The crosses, sporulation of diploids, and dissection of tetrads were done as described by Sherman et al. (1974).

Nucleic Acid Techniques

E. coli strain DH1 (F^-, recA1, endA1, gyrA96, thi-1, hsdR17, supE 44, red A1, lambda$^-$) was used for all cloning experiments. Plasmid preparations were done essentially as described elsewhere (Silhavy et al., 1984).

Plasmid pNB85 was isolated from a plasmid library of wild-type

yeast inserts in YCp50, a single copy shuttle vector containing yeast CEN4, URA3$^+$; amp^r, tet^r. pNB136 was constructed by inserting the 4.4 kb BamHI fragment from pNB85 into the BamHI site of YCp50. Insert orientation was determined by EcoRI digestion. The construction of plasmid pNB138 involved the deletion of the 2.4 kb EcoRI fragment of pNB136. Plasmid pNB139, containing the 1.4 kb EcoRI–BamHI complementing fragment, was constructed from pNB138 by deleting the 1.0 kb XbaI–BamHI fragment. The recessed 3′ ends were filled using E. coli pol I and Klenow fragment (Boehringer Mannheim) and blunt-end-ligated. This ligation recreates the BamHI site but removes the XbaI site. To mutate the unique PvuII site in the insert, pNB136 was digested with PvuII and religated in the presence of a synthetic linker (dpGGAATTCC, New England Biolabs #1020), creating plasmid pNB146. Plasmid pNB145 was constructed from pNB136 by digestion with HindIII and religation. To construct the integrating plasmid, pNB141, and the multicopy plasmid, pNB142, the 1.4 kb EcoRI–BamHI fragment from pNB139 was inserted to the EcoRI and BamHI sites of the integrating vector, YIp5 (Struhl et al., 1979), and the 2 μm vector, pRB307 (from collection of D. Botstein).

DNA Sequence and Protein Homology Analyses

Nucleotide sequencing was carried out 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). The sequencing reactions were electrophoresed on 8% acrylamide gels (40:1.3, acrylamide:bis) containing 8 M urea.

The template DNA was obtained either by cloning the 1.4 kb EcoRI–BamHI fragment from pNB139 in M13 phage derivatives mp18 and mp19 and producing sequential series of overlapping clones (Dale et al., 1985), or by subcloning restriction fragments into either one of the phage derivatives.

The predicted protein sequence was compared with the National Biomedical Research Foundation library by the FASTP program in the ktup-1 mode (Lipman and Pearson, 1985).

Southern Blot Hybridization

Total yeast DNA was extracted from cells by the method of Holm et al. (1986). DNA (1 μg) was digested with BamHI, fractionated by agarose gel electrophoresis, and transferred to nitrocellulose filters (Southern et al., 1975). The prehybridization, hybridization, and washing of the blots and the preparation of the probe were essentially as described elsewhere (Emanuel et al, 1986). In each hybridization experiment the isolated EcoRI–BamHI fragment from pNB139 was used as a probe.

Thin Section Electron Microscopy

Liquid cultures were grown to early log phase at 25°C in YPD media, then shifted to 37°C for 2 hr. Approximately 5 × 10^8 cells were then harvested by filtration onto nitrocellulose, washed on the filters with 10 ml of 0.1 M cacodylate (pH 6.8), and immediately resuspended in 8 ml of a solution containing 0.1 M cacodylate and 3% glutaraldehyde. After a 2 hr fixation at 25°C, the cells were collected by centrifugation in a clinical centrifuge and washed 2 times with 50 mM potassium phosphate buffer (pH 7.5) and resuspended in 2 ml of phosphate buffer containing 0.125 mg of Zymolyase 100T. After digestion of the wall for 40 min at 30°C, the cells were washed 2 times in cacodylate buffer and resuspended in cacodylate buffer containing 2% OsO_4 at 0°C. After a 1 hr incubation, the cells were washed three times in H_2O, then incubated for 2 hr in 2% aqueous uranyl acetate. The cells were washed 2 times with H_2O, then formed into agar blocks. The blocks were dehydrated in graded ethanol and embedded in Spurr media. Thin sections were stained with uranyl acetate and lead citrate and observed in a Phillips 301 microscope at 80 kV.

Invertase Secretion

Cells were grown to early log phase in liquid YPD media at 25°C. Approximately 1.5 × 10^8 cells were sedimented in a clinical centrifuge, then resuspended in YP media containing 0.1% glucose at 25°C. After 35 min in low glucose media, the culture was transferred to 37°C. At time points, a 1 ml aliquot was removed, sedimented for 1 min, and resuspended in 1 ml of 10 mM NaN_3 at 0°C. To prepare the spheroplast lysates, 0.5 ml of the cell suspension was mixed with 0.5 ml of a solution containing 2.8 M sorbitol, 100 mM potassium phosphate (pH 7.5), 10 mM NaN_3, 50 mM 2-mercaptoethanol, and 0.1 mg/ml of Zymolyase 100T. After incubation at 37°C for 45 min, the resulting spheroplasts were sedimented, then lysed in 0.5 ml of 0.5% Triton X-100. Invertase activity was assayed as described by Goldstein and Lampen (1975).

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