The GTP-binding Protein Ypt1 Is Required for Transport In Vitro: The Golgi Apparatus Is Defective in ypt1 Mutants

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Abstract. The YPT1 gene encodes a raslike, GTP-binding protein that is essential for growth of yeast cells. We show here that mutations in the ypt1 gene disrupt transport of carboxypeptidase Y to the vacuole in vivo and transport of pro-α-factor to a site of extensive glycosylation in the Golgi apparatus in vitro. Two different ypt1 mutations result in loss of function of the Golgi complex without affecting the activity of the endoplasmic reticulum or soluble components required for in vitro transport. The function of the mutant Golgi apparatus can be restored by preincubation with wild-type cytosol. The transport defect observed in vitro cannot be overcome by addition of Ca++ to the reaction mixture. We have also established genetic interactions between ypt1 and a subset of the other genes required for transport to and through the Golgi apparatus.

GTP-binding proteins play diverse roles in the cell (Bourne, 1986). Despite this diversity in function, they may share a common mechanism. Many are thought to operate as molecular switches, changing conformation depending on the nucleotide bound. In their GTP-bound state, they each interact with a specific cellular component to stimulate or inhibit its function. Hydrolysis of bound GTP to GDP (guanosine 5'-diphosphate) by the binding protein catalyzes interaction. Exchange of GTP for GDP is often under control of another protein. This common scheme can be used for signal transduction (Neer and Clapham, 1988), as in the case of G, G, and transducin (Stryer and Bourne, 1986), or to coordinate the events of a multistage reaction as in the cases of the elongation factors (Kaziro, 1978). One subset of GTP-binding proteins has been defined as raslike because all of the members share significant sequence identity with the mammalian ras proto-oncogene products. While one of these proteins, the RAS2 protein of yeast, the product of the YPT2 gene, has been clearly implicated in regulating the functions of the other raslike proteins are less clear.

We have focused on the function of one specific raslike protein of yeast, the product of the YPT1 gene. First identified as an open reading frame adjacent to the actin gene (Gallwitz et al., 1983), the YPT1gene has been shown by in vitro mutagenesis and gene replacement to be essential for vegetative growth of yeast (Schmitt et al., 1986; Segev and Botstein, 1987). Study of conditional lethal mutants has led to two very different hypotheses regarding the function of the YPT1 product. Schmitt et al. (1988) have argued that its primary function is the regulation of intracellular calcium levels. This proposal is based on the finding that a ypt1 mutant strain exhibits increased "Ca++" uptake and has a growth defect that can be partially suppressed by high extracellular levels of Ca++. Both Schmitt et al. (1988) and Segev et al. (1988) have noted a block in the secretion of invertase at an early stage of transport that is analogous to the role of the YPT1 product in vesicular transport through the Golgi complex. Their argument is based on the apparent association of the Ypt1 protein with the Golgi apparatus in yeast. They also note the presence of a cross-reacting Yptl homologue in the Golgi complex of a mammalian cell line. By this proposal, the Ypt1 protein would play a role at an early stage of transport that is analogous to the role shown for the structurally related Sec4 protein at a later stage, vesicular transport from the Golgi complex to the plasma membrane (Salminen and Novick, 1987; Goud et al., 1988).

In this report, we investigate the role of the YPT1 gene product in the transport of pro-α-factor from the ER to a site of outer chain mannose addition in the Golgi apparatus. We have used a recently developed in vitro transport assay (Ruohola et al., 1988) to probe the site of action of the YPT1 gene product and to clarify the ambiguity regarding the primary defect in the ypt1 mutant. We have also screened for genetic interactions between the YPT1 gene and other genes that are required for early stages of transport.

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1. Abbreviations used in this paper: CPY, carboxypeptidase Y; GDP, guanosine 5'-diphosphate; HSP, high speed (100,000 g) pellet; HSS, high speed (100,000 g) supernatant; PYC, permeabilized yeast cells.
Materials and Methods

Genetic Techniques

Yeast strains used in this study are listed in Table I. Genetic crosses, sporulation of diploids, and dissection of tetrads were done as described by Sherman et al. (1974). Transformation of yeast was done by the method of alkali cation treatment (Iio et al., 1983). Growth properties were assessed by replica stamping strains onto YPD (1% yeast extract, 2% Bactopeptone, and 2% glucose) plates that were then incubated at 25°C, 30°C, 34°C, and 37°C and scored after 24 h.

In Vitro Transport Assay

Prepro-α-factor was transcribed and translated (Ruohola et al., 1988) by a modification of the procedure used by Hansen et al. (1986). Yeast lysates containing 35S]methionine labeled prepro-α-factor were used immediately after translation or frozen at −80°C. The transport assay was performed in two stages as described before (Ruohola et al., 1988). In the first stage of the reaction, prepro-α-factor translated in a yeast translation lysate was translated into the ER lumen retained within the PIC. Cells, containing the ER form of α-factor, were pelleted, washed once with transport buffer, resuspended in the same buffer, and used to perform the second stage of the reaction. At the end of this reaction, the Golgi form of α-factor resides outside the PIC. To separate the reaction product from the PIC, the cells were pelleted during a 23 s centrifugation in a microfuge (Fisher Scientific Co., Pittsburgh, PA) and the supernatant was treated with trypsin (470 µg/ml) for 20 min at 0°C and then with trypsin inhibitor (940 µg/ml) for 5 min at 0°C. Samples were heated to 100°C in the presence of 1% SDS and α-factor was immunoprecipitated with anti-α-factor antibody as described before (Ruohola et al., 1988) or by binding to Con A Sepharose (Sigma Chemical Co., St. Louis, MO) in the presence of high salt wash (500 mM NaCl, 1% Triton X-100, 20 mM Tris, pH 7.5). When Con A Sepharose was used, samples were incubated for 2 h at room temperature in the presence of 90 µl of a 20% (vol/vol) solution. The beads were washed twice with 1 ml of low salt wash buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 15 mM Tris, pH 7.5), twice with 1 ml of urea wash buffer (2 M urea, 0.2 M NaCl, 1% Triton X-100, 0.1 M Tris, pH 7.5), once with 1 ml of high salt wash buffer and once with 1 ml of Trit-salt buffer (50 mM NaCl, 10 mM Tris (pH 7.5). PIC, S3, HSS, and HSP fractions were prepared as described before (Ruohola et al., 1988), or, were prepared from regenerated spheroplasts that were kept as a packed pellet overnight at 0°C or frozen at −80°C and lysed after thawing. To compare the yptl and sec4 mutants with wild type cells, S3 fractions and PIC were assayed at the same protein concentration. The protein concentration of each fraction was measured using the Bradford assay (1976) with ovalbumin as a protein standard. Samples were electrophoresed in a 12% SDS polyacrylamide slab gel.

In Vivo Labeling and Immunoprecipitation

Cells grown overnight at 25°C in minimal medium were supplemented with 100 µm ammonium sulfate, 2% glucose, histidine, and uracil. Cells (1 OD600 U) were pelleted, resuspended in 0.5 ml of minimal medium supplemented with 25 µm ammonium sulfate, histidine, uracil, and 200 µCi of 35S sulfate. At the end of a 30-min incubation at 37°C, the cells were washed with 1 ml of cold 10 mM sodium azide, converted to spheroplasts and lysed as described before (Newman and Ferro-Novick, 1987). The lysate was centrifuged at 100,000 g for 1 h in a rotor (Beckman Instruments Inc., Palo Alto, CA) and carboxypeptidase Y (CPY) was immunoprecipitated from the clarified lysate with anti-CPI antibody as described before (Newman and Ferro-Novick, 1987).

Results

Isolation of a New Allele of yptl

A new allele of yptl was found during a screen of existing secretory (sec) mutants for defects in GTP-binding proteins. Lysates were prepared from mutants that were grown at 25°C in YPD medium, and shifted to 37°C for 1 h. Cellular proteins were separated by electrophoresis on SDS polyacrylamide gels. On transfer to nitrocellulose, GTP-binding proteins were revealed by the method of Lapetina and Reep (1987). Cultures grown overnight at 25°C in minimal medium were supplemented with 100 µm ammonium sulfate, 2% glucose, histidine, and uracil. Cells (1 OD600 U) were pelleted, resuspended in 0.5 ml of minimal medium supplemented with 25 µM ammonium sulfate, histidine, uracil, and 200 µCi of [35S] sulfate. At the end of a 30-min incubation at 37°C, the cells were washed with 1 ml of cold 10 mM sodium azide, converted to spheroplasts and lysed as described before (Newman and Ferro-Novick, 1987). The lysate was centrifuged at 100,000 g for 1 h in a rotor (Beckman Instruments Inc., Palo Alto, CA) and carboxypeptidase Y (CPY) was immunoprecipitated from the clarified lysate with anti-CPY antibody as described before (Newman and Ferro-Novick, 1987).

Table I. Strain List

| Strain | Characteristics | Source |
|--------|-----------------|--------|
| NY 15  | MATα, ura3-52, his4-619 |        |
| NY 45  | MATα, ura3-52, sec-2, yptl-2 |        |
| NY 371 | MATα, his4-619, pNB142 (SEC4, URA3, 2 µ) |        |
| NY 404 | MATα, his4-619, sec4-8 |        |
| NY 431 | MATα, ura3-52, sec18-1 |        |
| NY 435*| MATα, ura3-52, yptl-1 |        |
| NY 703 | MATα, ura3-52, yptl-2 |        |
| NY 710 | MATα, ura3-52, yptl-2, pNB166 (YPT1, URA3, CEN4) |        |
| NY 711 | MATα, ura3-52, yptl-2, pNB167 (YPT1, URA3, CEN4) |        |
| SFNY26-6A | MATα, his4-619 |        |

* DBY 1803 (MATα, ura3-52, his4-539, lys2-801, yptl-1) was backcrossed four times to wild type.
ing in the position of the Ypt1 protein (Fig. 1, compare lanes 1 and 2). The same strain transformed with a single copy number plasmid (pNB166) showed restored binding (Fig. 1, lane 3) and when transformed with a multi-copy plasmid (pNB167) showed increased binding (Fig. 1, lane 4). Therefore, the mutation must be in the yptl gene itself and not in a second gene whose product functions to activate the binding activity of the Ypt1 protein. The band above Ypt1 on the blot is the Sec4 protein as shown by its absence in a sec4-8 strain (Fig. 1, lane 5) and its overproduction in a strain carrying SEC4 on a 2-μm plasmid (Fig. 1, lane 6).

**The yptl Mutant Blocks Transport to the Vacuole In Vivo**

Conditional lethal mutations in the yptl gene disrupt the transit of invertase (Segev et al., 1988; Schmitt et al., 1988). This block in export leads to the accumulation of a network of ER and an aberrant form of the Golgi apparatus at the restrictive temperature (Schmitt et al., 1988; Segev et al., 1988). It was previously shown that the bet and sec ER-accumulating secretory mutants block the transport of the vacuolar protease CPY (Stevens et al., 1982; Newman and Ferro-Novick, 1987). CPY follows the early portion of the secretory pathway. In the ER, it is glycosylated at four sites, yielding the 67-kD, pl form (Haslik and Tanner, 1978). Subsequent transport to the Golgi apparatus leads to extension of the carbohydrate chains resulting in the 69-kD, p2 form. Just before or upon arrival at the vacuole, an 8-kD NH2 terminal propeptide is removed, producing the mature form. Early blocked secretory mutants arrest transit from the ER to the Golgi complex at 37°C, accumulating the pl form of CPY (67 kD).

CPY transport was examined in the conditionally lethal yptl-1 allele. Mutant cells were grown at 25°C and then immediately labeled with [35S]methionine at 37°C. Cells were converted to spheroplasts by enzymatic removal of the yeast cell wall, lysed with detergent, and CPY was immunoprecipitated with anti-CPP antibody. As shown in Fig. 2, lane 1, wild-type cells synthesized the glycosylated, processed, mature form of CPY (61 kD); trace amounts of pl and p2 CPY were also detected. In contrast, the yptl-1 mutant synthesized a form of CPY similar in mobility to the pl form (lane 2). This form of CPY was also synthesized in sec18-1 (lane 3), a previously identified ER-accumulating mutant (Novick et al., 1980).

In some experiments the form of CPY in yptl-1 lysates was slightly higher in apparent molecular mass than was the pl form in sec18-1 lysates. The significance of this slight shift is addressed in the Discussion. Although the yptl-2 strain (NY703) is not a conditional lethal mutant, pulse chase experiments revealed that transit of CPY to the vacuole, as measured by the rate of appearance of the mature form, was slower than in wild-type cells (not shown).

**A Defect in the Ypt1 Protein Disrupts Transport In Vitro**

Mutations in the yptl gene disrupt transit through the early stage of the secretory pathway in vivo (Segev et al., 1988; Schmitt et al., 1988). To determine whether this defect could be reproduced in vitro, we isolated fractions from NY703 and examined their ability to support transport.

Transport from the ER to the Golgi complex in vitro can be monitored using a gel assay (Ruohola et al., 1988). The marker protein used in this assay is a precursor to the secreted pheromone α-factor (prepro-α-factor). In vivo prepro-α-factor is synthesized as a 19-kD proprotein that is converted to a 26-kD polypeptide by the addition of three NH2-linked core oligosaccharide units in the lumen of the ER. Outer chain carbohydrate, a highly branched structure consisting of 50–150 mannose residues (Ballou, 1982), is added to pro-α-factor in the Golgi complex (Julius et al., 1984). This modification results in a protein that migrates as a higher molecular mass heterogeneous smear on SDS polyacrylamide gels. In the assay we developed, transport is monitored by following the processing of pro-α-factor. In the first stage of this two-stage assay, in vitro translated prepro-α-factor enters PYC and is translocated across the lumen of the ER where it is glycosylated (26-kD polypeptide). In the second stage, the ER form of α-factor is converted to the high molecular mass Golgi form. Conversion to the Golgi form is dependent upon the presence of ATP and the supernatant of a yeast lysate spun at 3,000 g (S3 fraction). An advantage of our transport assay (Ruohola et al., 1988) is the resolution of the membrane components: the ER, or donor compartment, resides in the permeabilized cells while the acceptor Golgi compartment is added exogenously to the cells. At the end of the reaction, the high molecular mass Golgi form is found outside the PYC with the exogenously added acceptor.

Fig. 3 A illustrates that an S3 fraction, obtained from the yptl-2 mutant, failed to support transport to a site of extensive outer chain addition in vitro when assayed with wild-type PYC (Fig. 3, compare lanes 1 and 2 with 3). Although the high molecular mass Golgi species was not formed, α-factor...
Figure 3. The S3 fraction from the yptl-2 mutant is defective for transport. A, permeabilized yeast cells (60 µg of protein), containing the ER form of α-factor, were incubated in the absence (lane 1) or presence (lane 2) of a wild-type (SFNY26-6A) S3 fraction (1.0 mg of protein). The yptl-2 mutant (NY703) S3 fraction (1.0 mg of protein) failed to support transport (lane 3). This defect was no longer observed when an S3 fraction (1.0 mg of protein) was obtained from a yptl-2 mutant strain (NY710) harboring a wild-type copy of YPT1 on a CEN plasmid (lane 4).

B, yptl-2 PYC (lane 2) supported transport as efficiently as wild-type (SFNY26-6A) cells (lane 1) when assayed in the presence of a wild-type S3 fraction. However, mutant HSP failed to support transport when assayed with a wild-type HSS and wild-type PYC (Fig. 4, compare lanes 1 and 5). The yptl-1 allele was also examined in the in vitro assay. Fractions from the yptl-1 mutant were prepared from cells grown at 25°C and then assayed at 20°C. As was the case with yptl-2, the HSP was the only fraction that was found to be defective in the yptl-1 lyse (not shown). Thus, mutations in the Yptl protein specifically affect the Golgi compartment but not the permeabilized cells or the soluble factors required for transport.

Figure 4. The Golgi complex is defective in the yptl mutant. The S3 fraction from a yptl-2 mutant was defective for transport when assayed with a wild-type HSS and wild-type PYC (Fig. 4, compare lanes 1 and 5). The yptl-2 S3 fraction was subfractionated at 100,000 g for 1 h to generate HSS and HSP fractions (lane 3). The yptl-2 HSS efficiently supported transport when assayed with a wild-type HSP (compare lanes 1 and 4); however, the yptl-2 HSP failed to support transport when assayed in the presence of a wild type HSS (lane 5). Mutant HSS failed to transport the ER form of α-factor to the Golgi complex in the absence of a wild-type HSP and wild-type HSS failed to support transport in the absence of a wild-type HSP (not shown). The two major bands appearing below the 26-kD species represents partially core glycosylated pro-α-factor.
Cell pellet
Cell sup (ypt1 S3)
Cell sup (ypt1 HSP, w.t. HSS)

Figure 5. The 28-kD species is found outside the permeabilized cells. Wild-type donor cells were incubated with either a yptl-2 (NY 703) S3 fraction (lanes 1 and 2) or with wild-type (SFNY26-6A) cytosol in the presence of a mutant HSP fraction (lane 3). At the end of the assay, the samples were centrifuged for 23 s at room temperature in a microfuge (Fisher Scientific Co.) and processed as described in Materials and Methods. The forms of α-factor residing in the cell pellet and released into the cell supernatant were examined by SDS-PAGE. Most of the 26-kD species and all of the 28-kD form of α-factor was released into the supernatant during the reaction (compare lanes 1 and 2). The appearance of the 28-kD species was dependent upon addition of a HSP fraction that contained the mutant Golgi complex (lane 3). The 28-kD species was not observed when the PYC were incubated with mutant HSS and ATP (not shown).

Figure 6. The defect in the yptl mutant can be complemented in vitro. Transport was observed when a wild-type (SFNY26-6A) S3 fraction was incubated with wild-type PYC (lane 1). This S3 fraction was subfractionated into HSS and HSP fractions that were active (lane 2). No activity was seen (lane 3) when the wild-type HSS was preincubated with the yptl mutant Golgi (NY703). However, transport was restored if the wild-type HSS was preincubated with the yptl mutant HSP (not shown). The use of calcium/EGTA buffers allows a more accurate definition of the free calcium concentration. Unfortunately EGTA causes an inhibition of the assay that cannot be overcome by the addition of calcium (not shown). This may reflect the chelation of a trace divalent cation required for the assay. A likely candidate is manganese, since this ion is required for the activity of the mannosyl transferases that add outer chain carbohydrate (Nakajima and Ballou, 1975). The ER and mitochondria can take up Ca++, and thus, when Ca++ is added to the transport assay, they can act as Ca++ sinks.

YPT1 and Ca++ Regulation

One proposal regarding the role of the Ypt1 protein is that it functions to regulate intracellular calcium levels. By this proposal, the effects of yptl mutations on protein transport are a secondary consequence of altered intracellular levels of calcium. The in vitro transport assay offers a critical test of this hypothesis. Because the cytoplasmic space is readily accessible during the assay, we can determine whether the yptl transport defect can be overcome by the direct addition of calcium to the reaction mixture. The assay was performed using wild-type permeabilized cells and either a wild-type, yptl-1, or yptl-2 mutant S3 fraction. As was previously shown, (Fig. 3 A, lane 3 and Fig. 4, lane 2), the mutant S3 fractions are defective. The addition of CaCl2, to a final added concentration of 10 μM, did not restore function to the defective fractions, nor did it lower the efficiency of the wild-type reaction (Fig. 7, lanes 1, 2, 4, and 5). To verify that this level of Ca++ did not interfere with the assay, the reaction was performed in the presence of apyrase, an enzyme that hydrolyses ATP. Since the addition of Ca++ did not bypass the requirement for ATP (Fig. 7, lanes 3 and 6), the reaction seen in the presence of increased Ca++ is authentic.

The use of calcium/EGTA buffers allows a more accurate definition of the free calcium concentration. Unfortunately EGTA causes an inhibition of the assay that cannot be overcome by the addition of calcium (not shown). This may reflect the chelation of a trace divalent cation required for the assay. A likely candidate is manganese, since this ion is required for the activity of the mannosyl transferases that add outer chain carbohydrate (Nakajima and Ballou, 1975). The ER and mitochondria can take up Ca++, and thus, when Ca++ is added to the transport assay, they can act as Ca++ sinks. To try to assess the free Ca++ concentration during the reaction, a mock assay was performed with the addition of several concentrations of 45Ca++. Following the reaction, the mix was centrifuged at 100,000 g for 1 h and the supernatant was counted. Based on this data we can estimate that the addition of 50 μM Ca++ to the reaction mixture containing an S3 fraction from the yptl-2 mutant, results in a free Ca++ concentration of at least 6 μM, and the addition of 10 μM Ca++ results in a free concentration of at least 0.9 μM. The true level of free Ca++ may be somewhat higher as a result of the use of calcium/EGTA buffers. Unfortunately, EGTA causes an inhibition of the assay that cannot be overcome by the addition of calcium (not shown). This may reflect the chelation of a trace divalent cation required for the assay. A likely candidate is manganese, since this ion is required for the activity of the mannosyl transferases that add outer chain carbohydrate (Nakajima and Ballou, 1975). The ER and mitochondria can take up Ca++, and thus, when Ca++ is added to the transport assay, they can act as Ca++ sinks. To try to assess the free Ca++ concentration during the reaction, a mock assay was performed with the addition of several concentrations of 45Ca++. Following the reaction, the mix was centrifuged at 100,000 g for 1 h and the supernatant was counted. Based on this data we can estimate that the addition of 50 μM Ca++ to the reaction mixture containing an S3 fraction from the yptl-2 mutant, results in a free Ca++ concentration of at least 6 μM, and the addition of 10 μM Ca++ results in a free concentration of at least 0.9 μM. The true level of free Ca++ may be somewhat higher as a result.
Figure 7. CaCl₂ does not relieve the transport defect in the yptl mutant. Transport was observed when a wild-type (SFNY26-6A) S3 fraction was incubated with wild-type PYC in the absence (lane 1) or the presence (lane 2) of added CaCl₂ (10 μM), but not in the presence of CaCl₂ and apyrase (lane 3). Transport was not observed when a ypt/-2 (NY703) S3 fraction was incubated with wild-type PYC in either the absence (lane 4) or presence (lane 5) of added CaCl₂ (10 μM), or in the presence of CaCl₂ and apyrase (lane 6).

of Ca²⁺ present in the S3 fraction as well as Ca²⁺ contaminating the added water. Despite this level of free Ca²⁺, the mutant reaction mixture remains inactive.

Genetic Interactions between yptl and Early-blocked Secretory Mutants

Previous studies have shown a strong genetic interaction between the SEC4 gene and a number of the other SEC genes required at a late stage of the secretory pathway (Salminen and Novick, 1987). Interaction with SEC4 has been detected in two ways. It was first observed that introducing a second wild-type copy of SEC4 into a subset of vesicle accumulating sec mutants partially suppressed their growth defect. It was subsequently shown that double mutants carrying both the sec4-8 mutation as well as a temperature-sensitive mutation in one of the interacting genes were inviable at all temperatures, although the single mutants grew well at 25°C. The same set of mutants that were partially suppressed by transformation with a plasmid carrying the SEC4 gene, were lethal when combined with sec4-8. This set of mutants was restricted to vesicle accumulators, mutants blocked at other stages of the secretory pathway showed no interaction.

The similarity in structure between the Sec4 and Yptl proteins suggests that they may function by a similar mechanism, but at two distinct stages of the secretory pathway. For this reason, one might expect to see interaction of YPTl with a subset of the mutants blocked in transport to or through the Golgi complex. We have screened for suppression of the growth defect of mutants blocked at or before the Golgi apparatus by overexpression of YPTl. Representative alleles of sec and bet mutants were transformed with pNBl67, a multicopy plasmid carrying YPTl. Only a very slight improvement in the growth of the sec21-1 strain was seen upon transformation. No change in the growth properties of the other mutants was detected.

We next looked for lethality of double mutants. A yptl-1 strain was crossed to representatives of each of the complementation groups of ER-blocked (sec12, sec13, sec16, sec17, sec18, sec20, sec21, sec22, sec23, bet1, and bet2) and Golgi-blocked (sec7 and sec14) mutants (Novick et al., 1980; Newman and Ferro-Novick, 1987). Analysis of the tetrads revealed that in crosses to seven mutants (sec7-1, sec12-4, sec21-1, sec22-3, sec23-1, bet1-1, and bet2-1), a large number of spores failed to give rise to viable colonies. The pattern of inviability suggested that in these cases it was the double mutants that were inviable (see Table II). The significance of this result was somewhat diminished by the fact that the yptl-1 single mutant grew quite slowly at 25°C. In other words, lethality may be caused by the additive effects of two unrelated partial growth defects rather than by a specific, functional interaction of two gene products. We therefore repeated this series of crosses using the yptl-2 allele. This allele shows no detectable growth defect at 25°C, and is only slightly impaired at 37°C. Lethality of double mutants was not observed in crosses with yptl-2, yet in several cases

| Table II. Genetic Interactions with YPTl |
|-----------------------------------------|
| Viability of yptl-1 double mutants, 25°C | Growth of yptl-2 double mutants | Growth of single mutants |
|-----------------------------------------|---------------------------------|------------------------|
| sec7-1 | Inviable | + | + | - | - | + | + | - | - |
| sec12-4 | Inviable | + | - | - | - | + | - | - | - |
| sec14-3 | Viable | + | + | - | - | + | + | - | - |
| sec13-1 | Viable | + | - | - | - | + | - | - | - |
| sec16-2 | Viable | + | - | - | - | + | - | - | - |
| sec17-1 | Viable | + | + | - | - | + | - | - | - |
| sec18-1 | Viable | + | + | - | - | + | - | - | - |
| sec20-1 | Viable | + | + | - | - | + | - | - | - |
| sec21-1 | Inviable | + | - | - | - | + | - | - | - |
| sec22-3 | Inviable | + | - | - | - | + | - | - | - |
| sec23-1 | Inviable | + | - | - | - | + | - | - | - |
| bet1-1 | Inviable | + | - | - | - | + | - | - | - |
| bet2-1 | Inviable | +/- | - | - | - | + | - | - | - |
growth of the double mutants failed at temperatures significantly reduced from the threshold temperature of the parental strain. Particularly dramatic was the result of the cross between yptl-2 and bet2-1. Both of these mutations confer little if any growth defect at 25°C, and yet the double mutants were nearly inviable at this temperature. Weaker effects were seen in the crosses of yptl-2 to sec7-1, sec34-3, sec21-1, and betl-1. In the crosses with sec7-1 and sec34-3, the growth of the double mutants failed at 33.5°C, while the sec7-1 and sec34-3 single mutants failed to grow at 37°C. In the crosses with sec21-1 and betl-1, the growth of the double mutants failed at 30°C, while the sec21-1 and betl-1 single mutants were able to grow at 30°C, though not at 36°C.

**Discussion**

We have used an in vitro assay (Ruohola et al., 1988) to characterize the role of the **YPT1** gene product in intracellular protein transport in yeast. For our analysis, we have employed two different mutant alleles, yptl-1 (Segev and Botstein, 1987) and yptl-2. The yptl-1 allele is a conditional lethal allele that grows poorly at all temperatures (Segev et al., 1988). A phenotype observed in vitro with this allele could conceivably be an indirect consequence of its slow growth. In contrast, the yptl-2 allele grows well at all temperatures; however, a small but perceptible reduction in growth is observed on YPD plates at 37°C. The growth defects of yptl-1 and yptl-2 correlate well with the defects seen in the transport of CPY to the vacuole in vivo: yptl-1 exhibits a complete block, while yptl-2 exhibits only a slowing of transport. The yptl-2 allele is useful for in vitro studies since this mutant displays a dramatic block in transport in vitro (Fig. 3 A), and yet is not conditional lethal for growth. The finding that a particular mutation affects protein transport to a greater degree in vitro than in vivo suggests that the reaction catalyzed by the gene product is relatively more rate limiting in vitro.

Several lines of evidence suggest that Yptl is required for transport between an early stage and a later stage of the Golgi apparatus. The form of pro-α-factor that accumulates in a yptl mutant in vitro is intermediate in molecular mass between the 26-kD core glycosylated form and the high molecular weight form that accumulates in the Golgi apparatus. Formation of this 28-kD species is dependent upon addition of the mutant HSP fraction to the permeabilized cells. This finding suggests that the 26-kD form of α-factor may be transported to another membrane compartment for conversion to the 28-kD form. Since it is known that the HSP fraction contains the functional Golgi apparatus in this reaction (Ruohola et al., 1988), a likely candidate is the **cis** compartment of the Golgi apparatus. The 28-kD species is found outside the permeabilized cells after the reaction, consistent with the hypothesis that pro-α-factor is transported to a compartment that is added exogenously to the cells. The 28-kD form of pro-α-factor may represent an intermediate in the normal transport reaction since we have observed this species in wild type at early times of transport. It is depleted as the reaction continues on to yield the high molecular mass form (Bacon and Ferro-Novick, unpublished results). Our studies do not exclude the possibility that Yptl is also required for transport from the ER to the Golgi apparatus. Significantly less of the 26-kD form of pro-α-factor is converted to the 28-kD form in the presence of a yptl-2 S3 fraction than is converted to the high molecular mass form in the presence of a wild-type S3 fraction. Therefore, the yptl-2 defect may lead to a partial block in transport from the ER to the Golgi apparatus in addition to a block in transport through the Golgi apparatus.

Although the nature of the modification leading to formation of the 28-kD form is not known, the addition of several mannose residues to each of the three cores of the 26-kD form of pro-α-factor could explain the shift in molecular mass. The same modification could explain the slight shift in molecular mass seen in CPY relative to the ER form; however, the larger size of the polypeptide would make the increase in molecular mass more difficult to detect. A more apparent shift from the core glycosylated form is seen in the case of invertase (Segev et al., 1988). Invertase contains 9–10 core oligosaccharide units; the addition of several mannose residues to each core should be readily observed for this protein.

Immunofluorescence studies with anti-Yptl antibody in both yeast and mammalian cells suggest that the Yptl protein and its mammalian homologue are primarily associated with the Golgi apparatus (Segev et al., 1988), although fractionation studies indicate that a soluble pool may exist as well (Molenaar et al., 1988). Our in vitro findings (Fig. 4) establish that a defect in the Yptl protein leads to a loss of Golgi function without affecting the permeabilized cells or the soluble factors required for transport. This defect is observed with either the yptl-1 or yptl-2 allele. Our findings are therefore consistent with the immunofluorescence localization studies.

Strong genetic interactions have been demonstrated between the **SEC4** gene and a number of the other genes required for vesicular transport from the Golgi complex to the cell surface (Salminen and Novick, 1987). Because of the close structural similarity of the Sec4 protein with the Yptl protein, we have screened for analogous genetic interactions between **YPT1** and genes required for early stages of transport. In general, **YPT1** does not display as strong a pattern of genetic interaction as does **SEC4**. Overexpression of **YPT1** does not lead to strong suppression of the growth defects resulting from mutations in early stages of the secretory pathway, and lethality of double mutants is only seen with the yptl-1 allele and not the yptl-2 allele. Nonetheless, the genetic interactions observed may signify a functional interaction of the gene products. The strongest effect is seen with bet2-1, a mutant blocked in transport from the ER to the Golgi apparatus. Since Yptl is required for transport through the Golgi apparatus, the interactions seen may reflect the involvement of the **BET2** gene product at this stage of the pathway in addition to its demonstrated role in transport from the ER to the Golgi apparatus. Alternatively, the genetic interaction may reflect the involvement of the **YPT1** gene product in transport from the ER in addition to its role in transport through the Golgi apparatus.

Our data suggests that the transport defect in the yptl mutants is probably not a consequence of a failure to correctly regulate intracellular calcium levels. Addition of calcium to the in vitro assay reaction does not bypass the yptl-2 defect. In total, our results are consistent with, though do not definitively establish, the possibility that the Yptl protein plays a direct role in the control of vesicular traffic in the Golgi apparatus.
Earlier studies have shown that the nonhydrolyzable analogue, GTPγS, is a potent inhibitor of vesicular transport through the Golgi apparatus (Melançon et al., 1987). The mammalian homologue of Ypt1 is a possible target of GTPγS action. Like other GTP-binding proteins, Ypt1 must undergo a cycle of binding and hydrolyzing GTP to fulfill its function (Bourne, 1988). GTPγS would prevent Ypt1 from returning to its GDP-bound conformation, and therefore block the cycle. Further work will be required to understand the mechanism by which Ypt1 functions to mediate transport and the role that GTP binding and hydrolysis play in this mechanism.

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