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 *ras*like, 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)[1] by the binding protein curtails 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_s, G_i 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 *ras*like 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, has been clearly implicated in regulating the function of adenylate cyclase in response to nutrient starvation, the functions of the other *ras*like proteins are less clear.

We have focused on the function of one specific *ras*like 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 *YPT1* gene 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 $^{45}Ca^{++}$ 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 the secretory pathway and an accumulation of membrane bounded organelles at the restrictive temperature. Schmitt et al. (1988) have interpreted this phenotype to be a secondary consequence of the alteration in intracellular Ca^{++} concentration, while Segev et al. (1988) argue that the primary role of the Ypt1 protein is 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 Ypt1 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 (Ito 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.

GTP Binding

Lysates were prepared by vortexing cells in the presence of glass beads, followed by addition of SDS to 2% and heating to 100°C for 3 min. Proteins were displayed by electrophoresis on 12% polyacrylamide gels and then transferred to nitrocellulose. GTP-binding proteins were revealed by the method of Lapetina and Reep (1987).

Growth Conditions

Yeast cells used for the preparation of permeabilized yeast cells (PYC); 3,000 *g* supernatant (S3) fractions; high speed, 100,000 *g*, supernatant (HSS); and high speed, 100,000 *g* pellet (HSP) fractions were usually grown in YPD medium (1% yeast extract, 2% bactopeptone, and 2% glucose). Strains containing high copy plasmids were grown in Wickerham's minimal medium under selective conditions; for radiolabeling experiments, sulfate salts were replaced by chloride salts and ammonium sulfate was added to a final concentration of 100 or 25 μM. Cells grown in minimal medium were incubated for approximately one generation at 25°C in YPD medium and then used for the preparation of S3 fractions. The growth medium was changed by harvesting cells at room temperature in a clinical centrifuge. Cell densities were measured in a 1-cm quartz cuvette at 599 nm in a spectrophotometer (4054 UV/Visible Biochrom Ultrospec Plus; LKB Instruments Inc., Bromma, Sweden).

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 [^{32}S]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 translocated into the ER lumen retained within the PYC. 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 PYC. To separate the reaction product from the PYC, 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 Tris-salt buffer (50 mM NaCl, 10 mM Tris (pH 7.5). PYC, 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 *ypt1* and *sec4* mutants with wild type cells, S3 fractions and PYC 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.

Table I. Strain List

NY 15	*MATα, ura3-52, his4-619*
NY 45	*MATα, ura3-52, sec3-2, ypt1-2*
NY 371	*MATα, his4-619*, pNB142 (*SEC4, URA3*, 2 μ)
NY 404	*MATα, his4-619, sec4-8*
NY 431	*MATa, ura3-52, sec18-1*
NY 435*	*MATa, ura 3-52, ypt 1-1*
NY 703	*MATα, ura3-52, ypt1-2*
NY 710	*MATα, ura3-52, ypt1-2*, pNB166 (*YPT1, URA3, CEN4*)
NY 711	*MATα, ura3-52, ypt1-2*, pNB167 (*YPT1, URA3*, 2 μ)
SFNY26-6A	*MATα, his4-619*

* DBY 1803 (*MATa, ura 3-52, his 4-539, lys 2-801*, ypt 1-1) was backcrossed four times to wild type.

In Vivo Labeling and Immunoprecipitation

Cultures grown overnight at 25°C in minimal medium were supplemented with 100 μM ammonium sulfate, 2% glucose, histidine, and uracil. Cells (1 OD_{599} U) were pelleted, resuspended in 0.5 ml of minimal medium supplemented with 25 μM ammonium sulfate, histidine, uracil, and 200 μCi of [^{35}S]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).

Results

Isolation of a New Allele of ypt1

A new allele of *ypt1* 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. After transfer to nitrocellulose, GTP-binding proteins were revealed by incubation of the filter with [α^{32}P]GTP and autoradiography. Typically 5 bands are seen by this procedure (Goud et al., 1988). One strain, NY45, failed to show binding at the position corresponding to the Ypt1 protein (23 kD). Since NY45 carries the *sec3-2* mutation, it was backcrossed to a wild-type strain, and tetrads were analyzed for linkage of the *sec3-2* growth defect and the loss of the Ypt1, GTP-binding band. The two phenotypes were found to be unlinked. Temperature-resistant spores that exhibited a defect in the Ypt1 band were found, as were temperature-sensitive strains that showed normal binding by the Ypt1 band. The new mutation was tentatively defined as *ypt1-2* and was backcrossed two additional times to a wild-type strain. The *ypt1-2* mutation, by itself, confers only a slight growth defect, which is most apparent in single colony growth at 37°C on YPD plates. Therefore, this defect is much more subtle than the previously isolated *ypt1-1* mutation (Segev and Botstein, 1987) that causes very slow growth at intermediate temperatures, 25–30°C (grows at approximately half the rate of wild type at 25°C), and lethality at both higher (37°C) and lower temperatures (14°C).

The identity of the new allele was verified by complementation analysis. The mutant was transformed with plasmids carrying the wild-type allele of *YPT1*. The transformants and the parental strain were evaluated on a GTP-binding blot (Fig. 1). The parental *ypt1-2* strain, NY703, showed no bind-

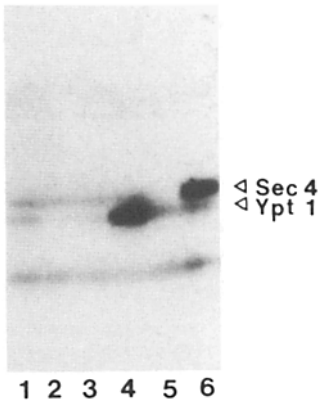

Figure 1. The *ypt1-2* mutant is defective for binding GTP. Lysates prepared from various strains were electrophoresed on an SDS polyacryamide gel. The protein was transferred to nitrocellulose and incubated with α[^{32}P]GTP. Lane *1*, wild type; lane *2*, *ypt1-2* (NY703); lane *3*, *ypt1-2* with *YPT1* on a CEN vector (NY710); lane *4*, *ypt1-2* with *YPT1* on a 2-μm vector (NY711); lane *5*, *sec4-8* (NY404); lane *6*, wild type with *SEC4* on a 2-μm vector (NY371).

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 *ypt1* 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 multi-copy plasmid (Fig. 1, lane *6*).

The ypt1 Mutant Blocks Transport to the Vacuole In Vivo

Conditional lethal mutations in the *ypt1* 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, p1 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 NH_2 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 p1 form of CPY (67 kD).

CPY transport was examined in the conditionally lethal *ypt1-1* allele. Mutant cells were grown at 25°C and then immediately labeled with [^{35}S]sulfate upon a 30-min shift to the restrictive temperature (37°C). Cells were converted to spheroplasts by enzymatic removal of the yeast cell wall, lysed with detergent, and CPY was immunoprecipitated with anti-CPY antibody. As shown in Fig. 2, lane *1*, wild-type cells synthesized the glycosylated, processed, mature form of CPY (61 kD); trace amounts of p1 and p2 CPY were also detected. In contrast, the *ypt1-1* mutant synthesized a form of CPY similar in mobility to the p1 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 *ypt1-1* lysates was slightly higher in apparent molecular mass than was the p1 form in *sec18-1* lysates. The significance of this slight shift is addressed in the Discussion. Although the *ypt1-2* 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 *ypt1* 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 NH_2-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 *ypt1-2* 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

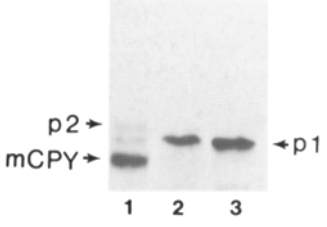

Figure 2. The *ypt1* mutant synthesizes the p1 form of CPY. Newly synthesized CPY was labeled with a 30-min pulse of [^{35}S]methionine at 37°C and was immunoprecipitated with anti-CPY antibody. The solubilized immunoprecipitates were analyzed on a 10% SDS polyacrylamide slab gel: (lane *1*) wild type (NY15); (lane *2*) *ypt1-1* (NY435); (lane *3*) *sec18-1* (NY431).

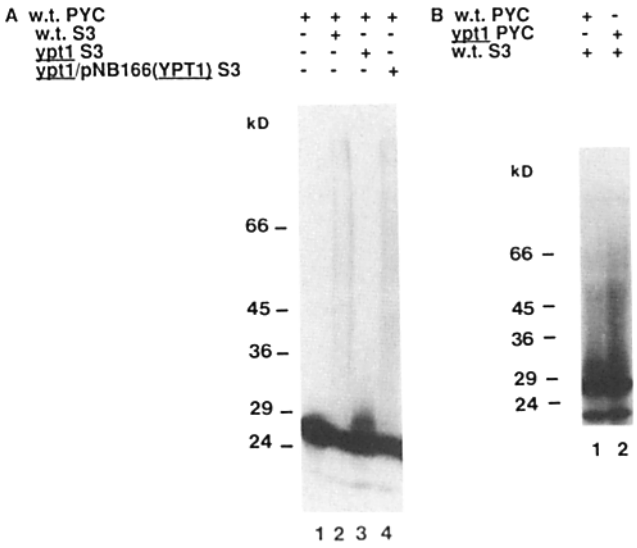

Figure 3. The S3 fraction from the *yptl-2* mutant is defective for transport. *A*, permeabilized yeast cells (60 μg of protein), containing the ER form of α-factor, were incubated in the absence (lane *1*) or presence (lane *2*) of a wild-type (SFNY26-6A) S3 fraction (1.0 mg of protein). The *yptl-2* 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 *YPTI* 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.

migrating just above the 26-kD species at an approximate apparent molecular mass of 28 kD was observed. This band is not always well resolved from the 26 kD form. If the S3 fraction was obtained from a *yptl-2* mutant strain that harbored a low copy number, centromere-based plasmid carrying a wild-type copy of the *YPTI* gene (pNB166), no defect was observed (Fig. 3, lane *4*). This indicated that pNB166 complemented both the transport defect observed in vitro and the GTP-binding defect (Fig. 1), providing additional evidence that NY703 harbored a mutation in the *yptl* gene and established that the *yptl* mutation is responsible for the transport defect. The *yptl-2* mutation did not affect the ability of the permeabilized cells to support transport, since mutant cells were fully functional when assayed in the presence of a wild-type S3 fraction (Fig. 3 *B*, compare lanes *1* and *2*). Thus, a defect in the Yptl protein disrupts transit to the site of extensive glycosylation in the Golgi complex in vitro and this defect is associated with the S3 fraction and not with the PYC.

The Golgi Complex Is Defective in the yptl Mutant

The S3 fraction can be further subfractionated at 100,000 *g* for 1 h to generate HSS and HSP fractions. The soluble factors are provided by the HSS, while the HSP contains the functional Golgi apparatus (Ruohola et al., 1988). Since the function of each compartment can be assayed independently, this has enabled us to test the ability of *yptl* mutant fractions to support transport in vitro.

The HSS obtained from the *yptl-2* mutant was found to support transport when assayed in the presence of wild-type HSP and wild-type PYC (Fig. 4, compare lanes *1* and *4*); 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* lysate (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.

In an earlier report (Ruohola et al., 1988), we demonstrated that, a portion of the 26-kD species and all of the high molecular mass Golgi form of α-factor, reside outside the PYC at the end of the assay. The released 26-kD species is contained within a vesicular reaction intermediate. Release of this intermediate from the permeabilized cells is dependent upon the presence of ATP (our unpublished results). Fig. 3 shows that when transport is blocked in the *yptl-2* mutant, a 28-kD species accumulates. To determine whether this form of α-factor remains within the cells or is released during the reaction, wild-type cells were incubated with a mutant *yptl-2* S3 fraction for 90 min at 20°C. Following this reaction, the permeabilized cells were pelleted in a microfuge, and the forms of pro-α-factor residing in the superna-

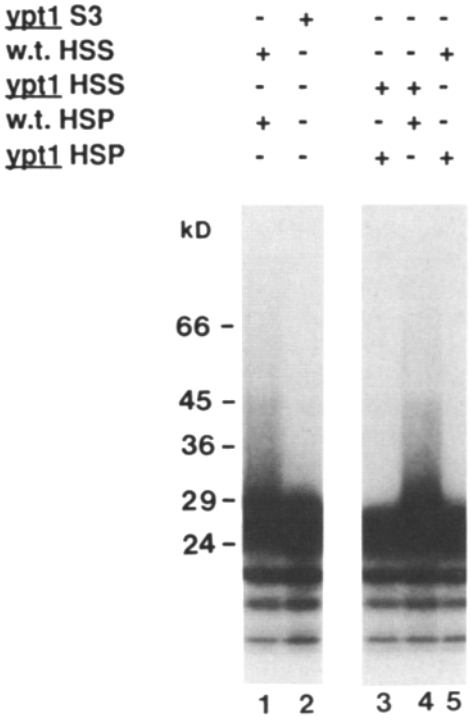

Figure 4. The Golgi complex is defective in the *yptl* mutant. The S3 fraction from a *yptl-2* mutant was defective for transport when assayed with wild-type (SFNY26-6A) PYC (compare lane *1* with lane *2*). This S3 fraction was subfractionated at 100,000 *g* for 1 h to generate HSS and HSP fractions (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.

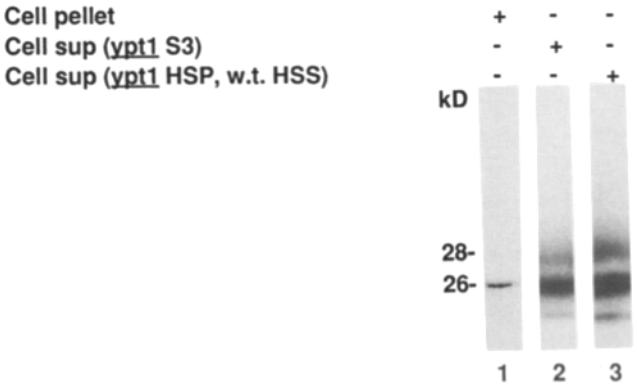

Figure 5. The 28-kD species is found outside the permeabilized cells. Wild-type donor cells were incubated with either a *yptl-2* (NY 703) S3 fraction (lanes *1* and *2*) or with wild-type (SFNY26-6A) cytosol in the presence of a mutant HSP fraction (lane *3*). At the end of the assay, the samples were 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).

tant and remaining in the cell pellet were examined. We observed that most of the 26-kD species and all of the 28-kD form of α-factor were found in the supernatant (Fig. 5, compare lanes *1* and *2*). The formation of the 28-kD species was dependent upon addition of a mutant HSP fraction that contains the *yptl* Golgi complex (Fig. 5, lane *3*). This form of α-factor was not seen when the PYC were incubated with mutant cytosol (not shown). This finding suggests that the 28-kD species is associated with the *yptl* mutant Golgi fraction that was added exogenously to the permeabilized cells during the assay.

Fractionation studies have revealed the existence of a soluble pool of Yptl protein in wild-type cells (Molenaar et al., 1988). Potentially, the wild-type soluble pool could attach to the *yptl* mutant Golgi apparatus upon preincubation in vitro. Fig. 6 (compare lanes *1* and *2* with lanes *3* and *4*) illustrates that the defect in the *yptl-2* Golgi compartment was partially relieved when the mutant HSP was preincubated with a wild-type HSS for 35 min at 20°C in the presence of 1 mM DTT. Restoration of transport was not observed when this incubation was performed with HSS obtained from the *yptl* mutant (not shown).

YPT1 and Ca⁺⁺ Regulation

One proposal regarding the role of the Yptl 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 $CaCl_2$, 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 $^{45}Ca^{++}$. 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

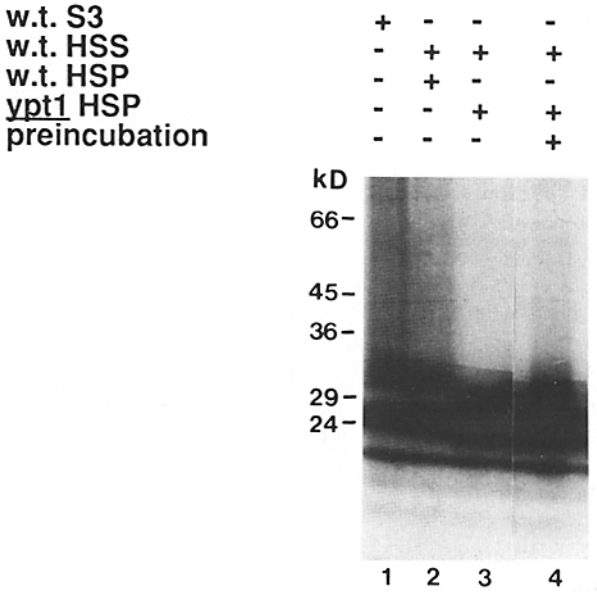

Figure 6. The defect in the *yptl* mutant can be complemented in vitro. Transport was observed when a wild-type (SFNY26-6A) S3 fraction was incubated with wild-type PYC (lane *1*). This S3 fraction was subfractionated into HSS and HSP fractions that were active (lane *2*). No activity was seen (lane *3*) when the wild-type HSS was incubated with the *yptl* HSP (NY703). However, transport was restored if the wild-type HSS was preincubated with the *yptl* HSP for 35 min at 20°C in the presence of 1 mM DTT (lane *4*).

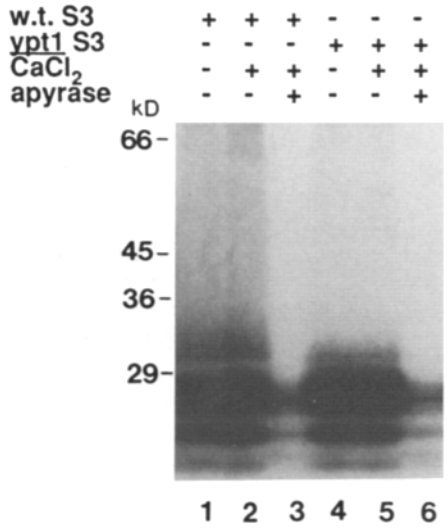

Figure 7. $CACl_2$ does not relieve the transport defect in the *ypt1* mutant. Transport was observed when a wild-type (SFNY26-6A) S3 fraction was incubated with wild-type PYC in the absence (lane *1*) or the presence (lane 2) of added $CaCl_2$ (10 μM), but not in the presence of $CaCl_2$ and apyrase (lane *3*). Transport was not observed when a *ypt1-2* (NY703) S3 fraction was incubated with wild-type PYC in either the absence (lane *4*) or presence (lane *5*) of added $CaCl_2$ (10 μM), or in the presence of $CaCl_2$ and apyrase (lane *6*).

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

Genetic Interactions between ypt1 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 Ypt1 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 *YPT1* 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 *YPT1*. Representative alleles of *sec* and *bet* mutants were transformed with pNB167, a multicopy plasmid carrying *YPT1*. 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 *ypt1-1* 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 *ypt1-1* 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 *ypt1-2* 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 *ypt1-2*, yet in several cases

Table II. Genetic Interactions with YPT1

	Viability of *ypt1-1* double mutants, 25°C	Growth of *ypt1-2* double mutants				Growth of single mutants			
		25°C	30°C	34°C	37°C	25°C	30°C	34°C	37°C
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 *ypt1-2* 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 *ypt1-2* to *sec7-1*, *sec14-3*, *sec21-1*, and *bet1-1*. In the crosses with *sec7-1* and *sec14-3*, the growth of the double mutants failed at 33.5°C, while the *sec7-1* and *sec14-3* single mutants failed to grow at 37°C. In the crosses with *sec21-1* and *bet1-1*, the growth of the double mutants failed at 30°C, while the *sec21-1* and *bet1-1* 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, *ypt1-1* (Segev and Botstein, 1987) and *ypt1-2*. The *ypt1-1* 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 *ypt1-2* 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 *ypt1-1* and *ypt1-2* correlate well with the defects seen in the transport of CPY to the vacuole in vivo: *ypt1-1* exhibits a complete block, while *ypt1-2* exhibits only a slowing of transport. The *ypt1-2* 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 Ypt1 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 *ypt1* 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 Ypt1 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 *ypt1-2* S3 fraction than is converted to the high molecular mass form in the presence of a wild-type S3 fraction. Therefore, the *ypt1-2* 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-Ypt1 antibody in both yeast and mammalian cells suggest that the Ypt1 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 Ypt1 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 *ypt1-1* or *ypt1-2* 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 Ypt1 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 *ypt1-1* allele and not the *ypt1-2* 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 Ypt1 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 *ypt1* 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 *ypt1-2* defect. In total, our results are consistent with, though do not definitively establish, the possibility that the Ypt1 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 fufill 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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