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GTP-binding Ypt1 protein and Ca^{2+} function independently in a cell-free protein transport reaction

(endoplasmic reticulum/Golgi/yeast/*Saccharomyces cerevisiae*)

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ABSTRACT **The 21-kDa GTP-binding Ypt1 protein (Ypt1p) is required for protein transport from the endoplasmic reticulum to the Golgi complex in yeast extracts. Ypt1 antibodies block transport; this inhibition is alleviated by competition with excess purified Ypt1p produced in bacteria. Furthermore, extracts of cells carrying the mutation *ypt1-1* are defective in transport, but transport is restored if a cytosolic fraction from wild-type cells is provided. The *in vitro* transport reaction also requires physiological levels of Ca^{2+}. However, Ypt1p functions independently of Ca^{2+}. First, buffering the free Ca^{2+} at concentrations ranging from 1 nM to 10 μM does not relieve inhibition by Ypt1 antibodies. Second, consumption of a Ca^{2+}-requiring intermediate that accumulates in Ca^{2+}-deficient incubations is not inhibited by anti-Ypt1 antibodies, although completion of transport requires ATP and an *N*-ethylmaleimide-sensitive factor. Thus, Ypt1p and Ca^{2+} are required at distinct steps.**

A central problem in understanding the pathways of protein transport and secretion is how the directionality and accuracy of vesicular traffic is assured. Analysis of mutations in two yeast genes, *SEC4* and *YPT1* (1, 2), suggested that ras-like GTP-binding proteins might function in transport. Cells carrying the mutation *sec4* are deficient in the last step of secretion; mature secretory vesicles accumulate (3). The Sec4 protein is located on the cytoplasmic surface of such vesicles (4). In contrast, cells carrying the mutation *ypt1* accumulate endoplasmic reticulum (ER) and Golgi-like membranes, indicating a defect early in the pathway (2, 5). Immunolocalization studies in yeast and mammalian cells suggest the Ypt1 protein (Ypt1p) is associated with the Golgi complex (2).

The observation of a connection between GTP-binding proteins and secretion stimulated an examination of the effect of GTP analogues on protein transport in cell-free systems. The nonhydrolyzable analog guanosine 5′-[γ-thio]triphosphate (GTP[γS]) blocks vesicular transport within the Golgi complex (6), transport from the ER to the Golgi (7–9), fusion of endocytic vesicles, and recycling of the mannose 6-phosphate receptor (10, 11); however, the inhibition has not been associated with a specific protein target. Transport of the precursor of the secreted mating pheremone α-factor (prepro-α-factor) from the ER to the Golgi has been reconstituted in yeast lysates (7, 8). This reaction is inhibited by GTP[γS]; based on the *in vivo* work, Ypt1p is a candidate target protein.

In addition to its role in secretion, Ypt1p has been implicated in other cellular processes (5, 12, 13). Two observations have suggested a link between *YPT1* and Ca^{2+}. High extracellular concentrations of Ca^{2+} suppress the temperature-sensitive (ts) grown defect of the *ypt1*-ts mutant (5). Furthermore, deletion of *PMR1*, a gene that encodes a Ca^{2+}-ATPase homologue, suppresses lethality of the *ypt1-1* mutation (13).

Here we examine the role of Ypt1p and Ca^{2+} in the yeast ER–Golgi *in vitro* reaction. We find that either a mutation in *YPT1* or an antibody against Ypt1p blocks transport. Further, we show that Ypt1p and Ca^{2+} function independently in transport.

MATERIALS AND METHODS

Cells, Plasmids, and Materials. *Saccharomyces cerevisiae* strains used are: GPY60 (MATα, *ura3-52, trp1, leu2, his4, pep4:URA3*); NSY12 (MATα, *ura3-52, trp1, his4, lys2, leu2, pep4::URA3, ypt1-1*); DBY1034 (*YPT1*); and DBY1803 (*ypt1-1*) (14). Plasmid pRS9 was constructed by ligation of the *Eco*RI–*Bam*HI fragment of *YPT1* from pRB301 (14) into the *Xba* I and *Bam*HI sites of the *trp* promoter in pHTH207-1 (a gift from Dennis Henner) by using a 50-base-pair oligonucleotide that includes the 5′ end of the *YPT1* gene with a *Xba* I site. Ypt1p was purified from *Escherichia coli* cells containing pRS9 by urea extraction of inclusion bodies (15). Anti-TrpE-Ypt1 antiserum and affinity-purified anti-Ypt1 and anti-trpE antibodies were prepared and checked as described (2). Anti-Ypt1 Fab fragments were prepared from anti-trpE-Ypt1 antiserum by using papain coupled to agarose beads (Pierce) according to the manufacturer's instructions. Other materials were as described (7).

Preparation of Microsomes and Pellet from High-Speed Centrifugation. Spheroplasts prepared as described (7) were harvested by centrifugation through a cushion of 0.8 M sucrose/1.5% Ficoll 400/20 mM Hepes, pH 7.4 (10,500 × *g* for 5 min at 4°C), resuspended at 100 OD_{600} units/ml in lysis buffer (100 mM sorbitol/20 mM Hepes, pH 7.4/50 mM KOAc/2 mM EDTA/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride) at 4°C, and homogenized with a motor-driven Potter–Elvejem homogenizer (10 strokes). After centrifugation of the lysate at 3000 × *g* for 10 min, the supernatant was collected and centrifuged at 12,000 × *g* for 10 min. The resulting microsomal pellet was either washed in reaction buffer as described below or further purified by centrifugation on sucrose. Approximately 0.5 ml of microsomes resuspended in a minimal volume of lysis buffer with gentle Dounce homogenization was loaded onto a 2.0-ml sucrose step gradient (1.0 ml each of 1.5 M sucrose and 1.2 M sucrose in lysis buffer) and centrifuged at 100,000 × *g* for 1 hr at 4°C. The microsomes at the 1.2 M/1.5 M interface were collected, washed twice in reaction buffer by centrifugation at 12,000 × *g* for 10 min, and resuspended in the same buffer (3 mg of

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Abbreviations: Ypt1p, Ypt1 protein; ts, temperature sensitive; ER, endoplasmic reticulum; GTP[γS], guanosine 5′-[γ-thio]triphosphate; (α1-6)Man, (α1→6)-linked mannose.

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protein per ml). The "high speed pellet" fraction was prepared from the 12,000 × *g* supernatant by centrifugation at 100,000 × *g* for 1 hr at 4°C. The pellet was washed once in reaction buffer [250 mM sorbitol/20 mM Hepes, pH 6.8/150 mM KOAc/5 mM $Mg(OAc)_2$] by centrifugation at 200,000 × *g* for 30 min at 4°C and resuspended in the same buffer with dounce homogenization (15 mg of protein per ml).

Protein concentrations were determined in the presence of 1% sodium dodecyl sulfate (SDS) by the procedure of Lowry *et al.* (16) using bovine serum albumin as a standard. Samples were either used directly or frozen in liquid N_2 and stored at −80°C. No significant loss of activity was detected over 3 months of storage.

Microsome-Based Transport Reaction. Transport reactions were carried out in two stages. In the first stage, 20 μg of microsomes were mixed with 4 μl of ^{35}S-labeled prepro-α-factor in 50 μM GDP-mannose, 1 mM ATP/40 mM creatine phosphate/200 μg of creatine phosphokinase per ml in a total reaction buffer volume of 12 μl. After incubation for 15 min at 10°C, the reaction mixture was diluted 1:9 with reaction buffer, and microsomes were collected by centrifugation at 12,000 × *g* for 10 min. In the second stage, 10–20 μg of washed microsomes containing core glycosylated pro-α-factor was mixed with 100 μg of cytosol, 20 μg of the high speed pellet, 50 μM GDP-mannose, 1 mM ATP, 40 mM creatine phosphate, and 200 μg of creatine phosphokinase per ml in a total reaction buffer volume of 50 μl and was incubated for 90 min at 20°C. The amounts of cytosol and high speed pellet added were titrated for each preparation to give optimal transport efficiency. The reactions were terminated by addition of 50 μl of 2% SDS, heated for 5 min at 95°C, and precipitated with concanavalin A-Sepharose or with an antiserum specific for (α1→6)-linked mannose [(α1-6)Man] and protein A-Sepharose as described (7).

RESULTS

Ypt1 Antibodies Block Transport. Protein transport from the ER to the Golgi complex has been reconstituted by using broken yeast spheroplasts (7). Transport was measured through the coupled addition within the Golgi complex of outer chain carbohydrate to core glycosylated [^{35}S]methionine-labeled pro-α-factor originating from the ER. We took advantage of the observation that yeast microsomes are competent for transport (8) to develop a transport assay that shared the same basic feature (ATP and temperature dependence; *N*-ethylmaleimide and GTP[γS] sensitivity) as transport with broken spheroplasts but required 1/10th the membrane protein (see *Materials and Methods*).

Antibody inhibition studies were used to investigate the role of Yptlp in the wild-type transport reaction. Affinity-purified Ypt1 and TrpE antibodies were prepared from antiserum raised against a Ypt1-TrpE fusion protein (2). The Ypt1 antibodies completely inhibited a wild-type transport reaction (Fig. 1 *Left*, closed squares), while the control TrpE antibodies had little effect (Fig. 1 *Left*, closed circles). Addition of purified Yptlp made in bacteria, sufficient to saturate the antibody, relieved the inhibition (Fig. 1 *Left*, open squares). To rule out the possibility that inhibition was due to membrane aggregation by the divalent antibodies, the experiment was repeated with monovalent Fab fragments. Anti-Ypt1 Fab fragments inhibited the transport reaction (Fig. 1 *Center*, open symbols). Again, the block was relieved by addition of excess bacterially produced Yptlp (Figure 1 *Center*, closed symbols).

In vivo studies have suggested that Yptlp may function in both ER–Golgi and Golgi–Golgi transport (2, 5). Core glycosylated invertase accumulated at the restrictive temperature in the *ypt1*-ts mutant, whereas partially outer-chain glycosylated invertase accumulated in the *ypt1-1* mutant. That anti-(α1-6)Man antibodies used to quantify the results of transport reactions do not react with glycosylated forms of the pro-α-factor migrating just above the core glycosylated ER form on SDS gels (7). To determine the effect of the Ypt1 antibody on the production of these partially outer-chain glycosylated species, the radioactive pro-α-factor accumulated in an antibody inhibition experiment was displayed by using SDS gel electrophoresis (Fig. 1 *Right*). There was no significant accumulation of partially outer-chain glycosylated species in the presence of the anti-Ypt1 Fab fragments (compare lane 3 to lane 1). Thus, Yptlp is involved in a transport partial reaction that precedes arrival in the first Golgi cisterna capable of outer-chain glycosylation.

***ypt1-1* Mutant Cell Components Are Defective in Transport *in Vitro*.** To investigate further the role of Yptlp in transport *in vitro*, transport reactions were performed with components prepared from *ypt1-1* mutant cells. In the microsome-based transport assay, the microsomes provide a source of translocation-competent ER, but transport in addition re-

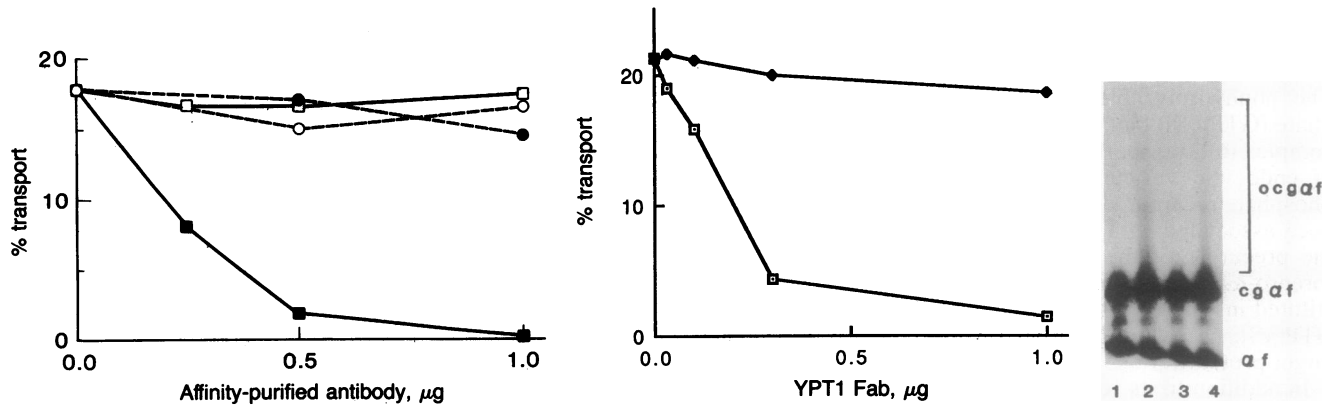

FIG. 1. Anti-Ypt1 antibodies inhibit transport. Standard transport reactions were performed as described with various additions. (*Left*) Increasing concentrations of affinity-purified anti-TrpE (circles) or anti-Ypt1 antibodies (squares) were added to transport reactions containing (open symbols) or not containing (closed symbols) 0.75 μg of purified Yptlp. (*Center*) Increasing concentrations of Fab fragments were added to transport reactions containing (closed symbols) or not containing (open symbols) 0.75 μg of purified Yptlp. (*Right*) Standard transport reactions were supplemented with 1% SDS (reaction 1), 0.5 μg of YPT1 Fab (reaction 3), or 0.5 μg of YPT1 Fab and 1 μg of Yptlp (reaction 4) as indicated. After 90 min at 20°C, 1% SDS was added to reactions 2, 3, and 4. All reactions were then subjected to immunoprecipitation with anti-α-factor antibody, SDS gel electrophoresis, and fluorography as described (7). αf, prepro-α-factor; cg αf, core glycosylated pro-α-factor; ocgαf, outer-chain glycosylated pro-α-factor.

Table 1. Cytosol and high speed pellet (HSP) are required for transport

Addition	Anti-(α1-6)-Man-precipitable cpm, % of maximum
Cytosol (100 μg) + HSP (20 μg)	100
Cytosol (100 μg)	8
HSP (20 μg)	8
Reaction buffer	7

In a two-stage reaction, pro-α-factor-containing microsomes were prepared as described and then incubated with the additions indicated above in 50-μl of reaction buffer containing GDP-mannose and an ATP-regenerating system. Reactions were terminated after 1.5 hr by addition of 2% SDS, and reaction products were analyzed by immunoprecipitation followed by scintillation counting. A typical complete reaction yielded ≈1000 cpm of (α1-6)Man-precipitable cpm, which represented ≈25% of the translocated pro-α-factor.

quires both a cytosol and a high speed pellet fraction (Table 1). Immunoblotting analysis showed that the microsome, cytosol, and high speed pellet fractions contribute 10%, 42%, and 48% of the total Yptlp in a transport reaction. The experiments with mutant fractions contained high speed pellet and microsome fractions from the same *ypt1-1 pep4* strain. Cytosolic fractions were prepared from an isogenic pair of Pep$^+$ strains differing only by the *ypt1-1* mutation. Transport was defective in reactions containing all *ypt1-1* cell components (Fig. 2, closed symbols). Cytosol prepared from the isogenic wild-type strain restored transport (Fig. 2, open symbols). Normal transport also occurred when mutant cytosol and microsomes were incubated with a wild-type high speed pellet fraction (data not shown). Thus, the efficiency of transport appears to reflect the amount of wild-type Yptlp rather than the origin of a particular fraction.

The *in Vitro* Transport Reaction Requires Ca^{2+} and Yptlp Independently. The combination of the mutant and antibody studies argues strongly that Yptlp plays a role in the *in vitro* transport reaction. As a connection has been proposed between Yptlp and Ca^{2+} flux (5), we wanted to determine whether Yptlp functioned through Ca^{2+} *in vitro*. A Ca^{2+} requirement has been demonstrated in a mammalian ER–Golgi transport reaction (9). Transport in semiintact CHO cells was inhibited by 5 mM EGTA and restored when the free Ca^{2+} concentration was adjusted to 0.1 μM. EGTA (5 mM) also inhibited the yeast ER–Golgi transport assay (Table 2, line 1). Transport was only partially restored by Ca^{2+}

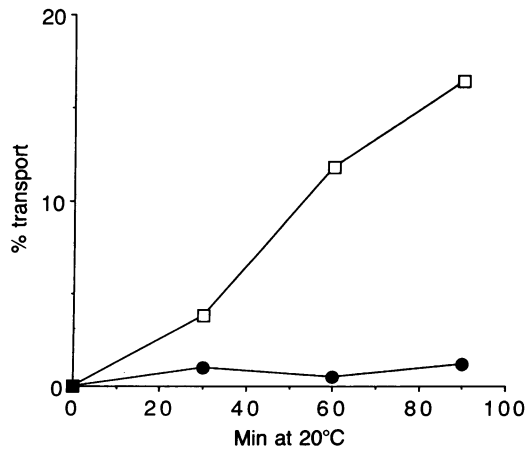

FIG. 2. ypt1-1 mutant cell components block *in vitro* protein transport. Microsomes and high speed pellet fractions were prepared from a *ypt1-1* mutant strain (NSY12). Cytosols were prepared from wild-type (open symbols) (DBY1034) and *ypt1-1* (closed symbols) (DBY1803) cells. *In vitro* reactions were performed as described.

Table 2. Ca^{2+} is required for transport

Exp.	Ca^{2+} Free, nM	Ca^{2+} Total, μM	Mn^{2+} Free, nM	Mn^{2+} Total, μM	(α1-6)Man-precipitable cpm
1	<1	<5	<0.1	<5	90
2	<1	<5	1.5	100	100
3	120	500	<0.1	<5	360
4	120	500	1.7	100	846

Standard transport reactions were supplemented with 5 mM EGTA and the indicated concentrations of Ca^{2+} and Mn^{2+}. The resultant free cation concentrations were estimated by using a modification of a previously described computer program (17). Addition of these concentrations of cations did not affect the pH of the incubations (data not shown). Concanavalin A-precipitable pro-α-factor averaged 5500 cpm per point. A typical reaction without EGTA gave 950 cpm of transported pro-α-factor.

(Table 2, line 3). Mn^{2+} is required by the mannosyltransferases responsible for the outer-chain carbohydrate elongation that is measured in the transport assay (18), and EGTA has a high affinity ($K_d \approx$ pM) for Mn^{2+}. Addition of Mn^{2+} alone did not relieve inhibition by EGTA (Table 2, line 2). Transport was fully restored when an EGTA-treated reaction was supplemented with both cations to yield free concentrations of 120 nM Ca^{2+} and 1.2 nM Mn^{2+} (Table 2, line 4). Transport was optimal at about 100 nM Ca^{2+} (Fig. 3), which is the estimated free intracellular Ca^{2+} concentration in yeast (Hidetoshi Iida, personal communication). At higher concentrations of Ca^{2+}, protein transport was partially inhibited. It is likely that the free Ca^{2+} concentration in reaction buffers not supplemented with EGTA would exceed the inhibitory concentration in the transport reaction. Microsomes contain a potent Ca^{2+}-sequestering pump (A. Antebi and D. Baker, unpublished observations), which may explain why reactions proceed in the absence of EGTA.

It is possible that Yptlp exerts its effect by modulating Ca^{2+} flux *in vitro*. To investigate this possibility, anti-Ypt1 Fab fragments were added to transport reactions having free Ca^{2+} concentrations ranging from 1 nM to 10 μM. The anti-Ypt1 Fab fragments inhibited transport at all Ca^{2+} concentrations tested (Fig. 3, closed symbols). Thus, Yptlp does not function solely by controlling Ca^{2+} levels *in vitro*.

Inhibition by EGTA is reversible in the mammalian ER–Golgi reaction. This was also the case in the yeast transport reaction: transport resumed when Ca^{2+} was added back to an EGTA-treated reaction (Fig. 4, column 7). This allowed us to order the requirements for Ca^{2+} and Ypt1 *in vitro*. Standard transport reactions containing EGTA and Mn^{2+} either were supplemented immediately with Ca^{2+} (Fig. 4, columns 2–6) or were preincubated for 60 min at 20°C and then supplemented with Ca^{2+}. To compare the requirements of transport from the ER and transport from the Ca^{2+}-requiring intermediate, aliquots of both preincubated and freshly prepared reaction mixtures were treated with either the ATP scavenger apyrase, the alkylating agent *N*-ethylmaleimide, the anti-Ypt1 Fab, or the guanine nucleotide analogue GTP[γS]. As previously reported, all of the treatments blocked transport from the ER to the Golgi (Fig. 4, columns 3–6). Transport from the Ca^{2+}-requiring intermediate was ATP dependent (Fig. 4, column 11), cytosol dependent (M. Rexach, personal communication), and *N*-ethylmaleimide sensitive (Fig. 4, column 8). These results, coupled with the fact that the outer-chain mannosyltransferases require Mn^{2+} and not Ca^{2+} as a cofactor, suggest that the Ca^{2+}-dependent step is a transport partial reaction rather than glycosylation. By analogy with results obtained in the mammalian transport reaction, we suspect that the *N*-ethylmaleimide-sensitive factor is Sec18 protein, the yeast homologue of this mammalian factor (19, 20). The reactions that had been preincubated for 60 min

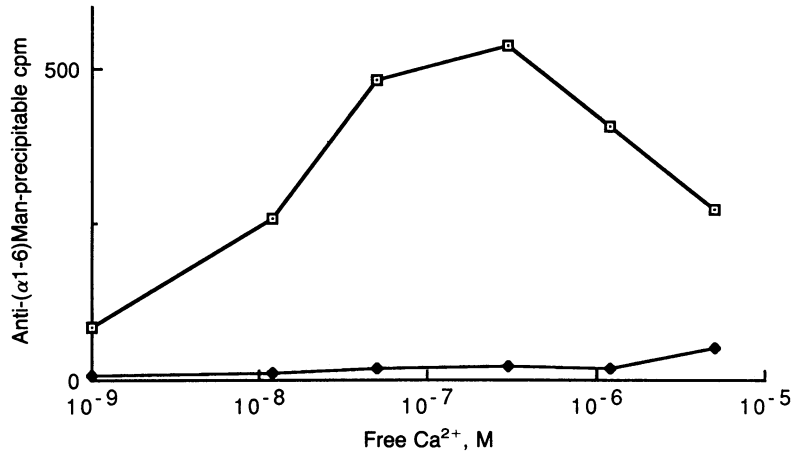

FIG. 3. Ca^{2+} does not relieve inhibition by anti-Ypt1 antibodies. Standard transport reactions were performed in the presence of an EGTA/Ca^{2+}/Mn^{2+} buffer to yield the indicated free Ca^{2+} concentration and 1–9 nM free Mn^{2+}. The buffers were adjusted to pH 6.8 with KOH prior to addition to the reactions. Variation of Mn^{2+} concentration in this range did not affect transport (data not shown). The reactions were further supplemented with 0.5 μg of anti-Ypt1 Fab fragments (closed symbols) or a buffer control (open symbols).

in the absence of Ca^{2+} no longer were inhibited by the anti-Ypt1 Fab fragments or by GTP[γS] (Fig. 4, columns 9 and 10). Therefore, transport partial reaction(s) sensitive to the Ypt1 antibodies and GTP[γS] either precedes the Ca^{2+}-requiring step or occurs on an independent pathway.

DISCUSSION

We have demonstrated that Ypt1p is required for protein transport *in vitro*. *ypt1-1* mutant cell components are defective in transport, and Ypt1 antibodies inhibit transport in wild-type extracts. These results support the recent proposal, based on analysis of the ypt1-1 mutant phenotype *in vivo*, that Ypt1p is involved in early stages of the yeast secretory pathway (2). The pleiotropic effects of *ypt1* mutations *in vivo* left open the possibility that the transport defect was a secondary consequence of some perhaps unknown primary lesion. The biochemical data presented in this paper argue that Ypt1p is directly involved in transport.

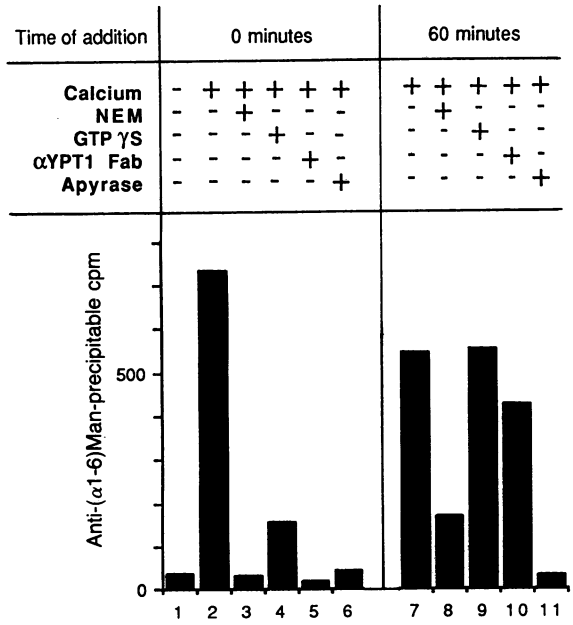

FIG. 4. Anti-Ypt1 Fab fragments block transport before the Ca^{2+}-requiring step. Standard transport reactions were performed in the presence of 5 mM EGTA and 100 μM Mn^{2+}. After either 0 or 60 min of incubation at 20°C, the reactions were supplemented with *N*-ethylmaleimide (NEM) (to 10 mM), apyrase (2.5 units), GTP[γS] (100 μM), or anti-Ypt1 Fab fragments (0.5 μg) as indicated. After 10 min at 4°C, dithiothreitol (to 20 mM) was added to the *N*-ethylmaleimide-containing reaction mixture. Ca^{2+} (500 μM) was then added, and all reactions were incubated for an additional 90 min at 20°C. The final free Ca^{2+} and Mn^{2+} concentrations were 120 nM and 1.7 nM, respectively.

The yeast transport reaction requires about 100 nM free Ca^{2+}. The overall Ca^{2+} dependence of the reaction is similar to that of mammalian ER–Golgi transport, providing further evidence that the mechanisms underlying transport have been highly conserved through evolution. However, inhibition at greater than physiological Ca^{2+} concentrations is less pronounced in the yeast reaction: 1 μM free Ca^{2+} causes 75% inhibition of the mammalian reaction and only 20% inhibition of the yeast reaction. This is consistent with the proposal that the transient increase in free Ca^{2+} is responsible for the inhibition of transport during mitosis in mammalian cells (9). As transport is not blocked during mitosis in yeast (21), such a Ca^{2+} "shut off" mechanism would not be expected in the yeast transport reaction.

Ypt1p functions independently of Ca^{2+} in transport. Ca^{2+} cannot suppress inhibition of Ypt1p function in transport, and Ypt1p and Ca^{2+} are required in different transport partial reactions. This does not exclude the possibility that Ypt1p regulates Ca^{2+} flux in addition to playing a direct role in transport.

Wild-type Ypt1p in either the cytosol or high speed pellet fractions can restore transport activity to a *ypt1-1* mutant cell reaction. One interpretation of this result is that the functional form of Ypt1p may cycle between the cytosol and a membrane target. Hence, complementation by the cytosolic fraction may require that the soluble Ypt1p be recruited to a functional binding site as proposed for Sec4 protein (22). Alternatively, the soluble fraction may contain particles or small membrane fragments that are functionally equivalent to the form that sediments along with membranes. Preliminary sizing column analysis suggests that the active form of Ypt1p in soluble fractions may be associated with a large protein complex or a small vesicle.

Two other genes that encode small GTP-binding proteins have recently been implicated in early steps of protein transport in yeast. *SAR1* was identified as a multicopy suppressor of a *sec12* mutation (23); mutants defective in a gene encoding ADP-ribosylation factor (*ARF1*) have a ypt1-like phenotype (T. Stearns, M. C. Willingham, D. Botstein, and R. A. Kahn, unpublished data). It is possible that each of

these GTP-binding proteins plays a role in a separate subreaction in early protein transport.

As discussed previously (2, 7, 24), GTP-binding proteins involved in transport could function in a manner similar to either the signal-transducing guanine nucleotide-binding proteins (G proteins) or the elongation factors of protein synthesis. We would like briefly to elaborate on the analogy to protein synthesis. Protein synthesis and protein transport share the common requirements of unidirectionality, speed, and accuracy. Bourne (24) emphasized the possible role of GTP hydrolysis in assuring unidirectionality; other features may also be involved. Peptide bond formation from aminoacylated tRNAs has a ΔG of -7 kcal and thus will proceed unidirectionally in the absence of GTP and elongation factor Tu (25). However, GTP-binding proteins greatly increase the rate and accuracy of protein synthesis. The rate of polypeptide chain elongation is increased over 100 fold by elongation factors Tu and G (26). By coupling GTP hydrolysis to a slow recycling step, a GTPase could similarly increase the rate of protein transport. Elongation factor Tu also increases the fidelity of protein synthesis through a "kinetic proofreading" mechanism in which the aminoacyl-tRNA–codon interaction is tested before and after GTP hydrolysis (27). GTP-binding proteins could increase the accuracy of protein sorting through a similar proofreading mechanism. Thus, GTP-binding proteins may guarantee the specificity of the interaction between secretory vesicles and acceptor components. These hypotheses might now be closer to being tested through the study of the role of Ypt1p in the *in vitro* transport reaction.

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