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The Yeast GTP-Binding YPT1 Protein and a Mammalian Counterpart Are Associated with the Secretion Machinery

Nava Segev,* Jon Mulholland, and David Botstein
Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

A yeast GTP-binding protein, the *YPT1* gene product, has been found to function early in the secretion pathway. The *ypt1-1* mutation causes a phenotype reminiscent of early secretion-defective mutants, including accumulation of membranes and vesicles as well as a partial defect in secretion and incomplete glycosylation of invertase. Immunofluorescence localization studies using affinity-purified antibody directed against the YPT1 protein showed punctate staining of the cytoplasm of growing yeast cells and very intense staining of small buds, where membrane growth and secretion are most active. The punctate cytoplasmic staining is changed in a mutant (*sec7*) under conditions that cause aberrant Golgi structures to accumulate. The pattern of immunofluorescence obtained when mouse cells were stained with the antibody coincided closely with the pattern observed with wheat germ agglutinin, suggesting that a mammalian counterpart of the yeast YPT1 protein is located in the Golgi apparatus. These results are interpreted as suggesting that GTP-binding proteins may act to direct intracellular vesicle traffic.

Introduction

The secretion process involves the orderly progression of materials from the cytoplasm to the endoplasmic reticulum (ER), through the several levels of the Golgi apparatus and ultimately to the final destination (Palade, 1975). A central problem in understanding cell growth concerns the biosynthesis of the membranous parts of the cells and the sorting of proteins into or through these membranes. It is likely that most, if not all, of the steps in the progression after the insertion into the lumen of the ER occur through the intermediacy of membranous vesicles that form at the surface of the "donor" compartment (e.g., the *trans* Golgi) and fuse specifically with the next "recipient" compartment (e.g., the plasma membrane) with the result that the contents of the vesicles are transported to the recipient compartment (Pfeffer and Rothman, 1987).

One can reasonably ask what might be the basis for the specificity of fusion of vesicles with the correct compartment: fusion of vesicles to membranes is normally restricted to the target membrane, suggesting strongly that there is a system that "labels," through some physical or chemical distinction, the different types of vesicles as well as the different organelle membranes.

* Present address: Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305.

In this paper, we present a candidate gene product for such a system of vesicle labeling, the *YPT1* gene product of yeast (Saccharomyces cerevisiae). The *YPT1* product is a particularly attractive candidate as a component of a system of vesicle labeling because it is a member of the superfamily of GTP-binding proteins that includes the G-proteins and the *ras* proto-oncogenes (Gallwitz et al., 1983; Schmitt et al., 1986). Many members of this family have already been shown to act in the transduction of signals across the plasma membrane (Gilman, 1987).

Previously, we had shown that the *YPT1* gene product is required primarily during the period of the cell cycle when bud growth is maximal (Segev and Botstein, 1987). In Saccharomyces cerevisiae, it has become clear that the cell growth and protein sorting are coupled and occur mainly in the bud (for review, see Schekman and Novick, 1982). We show here that a conditional-lethal mutant (*ypt1-1*) has a phenotype that includes improper membrane growth and a partial defect in protein secretion. Further, the gene product is concentrated in the growing bud in normal cells. Finally, we show, through the use of specific antibodies, that the yeast *YPT1* gene product has a homolog in animal cells that is localized primarily in the Golgi apparatus.

There is precedent for involving a YPT1-like protein very late in the secretion process. In mammalian cells, the effect of GTP and GTP-analogs on exocytosis has led to the suggestion of the existence of two putative G-proteins (Gp and Ge) that are involved in this process (for review, see Burgoyne, 1987). In Saccharomyces cerevisiae, the *SEC4* gene product has recently been shown to be a close homolog of the *YPT1* gene product (Salminen and Novick, 1987) and located on the plasma membrane and the secretory vesicles (P. Novick, personal communication). The *sec4* mutant defect is late in secretion, and mutants accumulate vesicles that would normally fuse with the plasma membrane (Novick et al., 1981).

Our results suggest that *YPT1* is involved in an earlier step in the chain of intracellular transport. We interpret our results as supporting a general role for the G-protein superfamily in regulating intracellular vesicle traffic by serving to label vesicles according to their origin, content, and/or destinations.

Results

Accumulation of Aberrant Membranes in the *ypt1-1* Mutant

We showed previously that the *ypt1-1* mutant cannot undergo mitosis at the nonpermissive temperature. Immunofluorescence microscopy showed aberrant nuclear and microtubular staining within one or two generations after the shift to nonpermissive temperature; these could be a consequence of the mutant's inability to undergo mitosis (Segev and Botstein, 1987). In order to understand the primary defect better, we studied *ypt1-1* mutant cells by electron microscopy. Cells grown at the nominally permis-

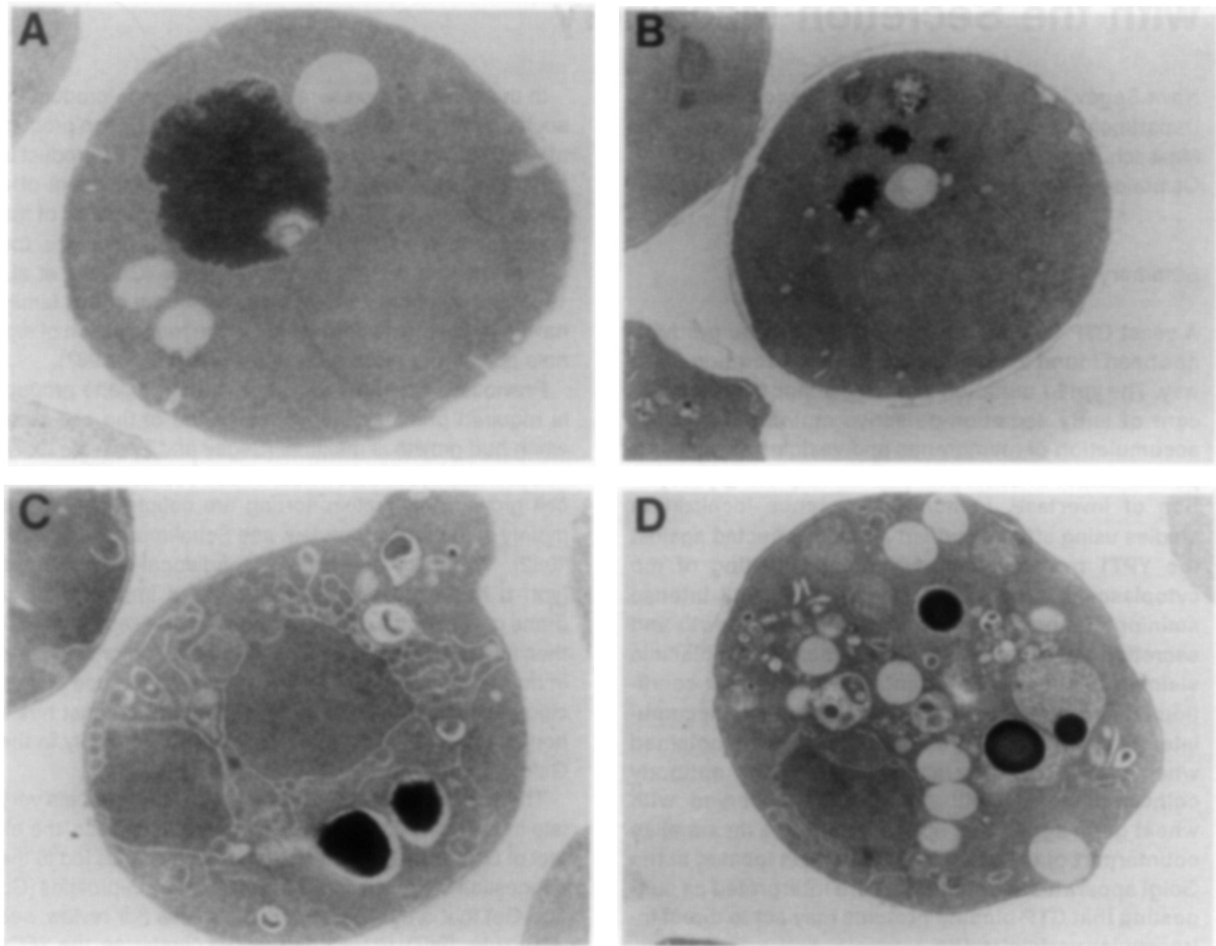

Figure 1. Thin Section Electron Microscopy of Wild-Type and *ypt1-1* Mutant Cells

Cells were grown overnight at 30°C and then shifted to 14°C for various periods of incubation. Cells were processed for electron microscopy, and thin sections were prepared and photographed. (A) Wild-type (DBY1034), 12 hr at 14°C; (B) *ypt1-1* (DBY1803), at 30°C; (C) and (D) *ypt1-1*, 8 hr at 14°C. (Magnification: A, B, C, 20,000×; D, 15,000×)

sive temperature (30°C) were shifted to nonpermissive temperature (14°C) and incubated in growth medium. Samples taken at intervals were prepared for electron microscopy and examined as described in Experimental Procedures.

At the permissive temperature the mutant cells seem relatively normal, although they contain more membranes than wild-type controls; in addition, the vacuole is usually fragmented in the mutants (Figures 1A and 1B). At the nonpermissive temperature, an abundance of aberrant membrane structures of two principal types are seen. The first type looks like long stacks of apparently cylindrical membranes (Figure 1C) that might represent accumulated abnormal endopasmic reticulum (ER) structures. The second structure is an accumulation of membranous vesicles (Figure 1D) that are sometimes reminiscent of "Berkeley bodies," i.e., abnormal structures that accumulate in *sec* mutants that show defects in Golgi functions (Novick et al., 1980).

These changes start appearing after 4 hr of incubation at 14°C (the wild-type generation time is 12 hr at this temperature). Upon longer incubation at the nonpermissive temperature, the aberrant structures appear in more cells and become more severe. The images shown in Figures 1C and 1D are of mutant cells that were incubated for 8 hr at 14°C, at which time virtually all the cells contain significant amounts of these aberrant structures.

The observation of structures resembling the "Berkeley bodies" stimulated us to determine whether lower glucose concentration during growth affects the morphology of these structures, as it does the structure observed with *sec7* mutants (Novick et al., 1981). We found no significant difference in the appearance of the aberrant Golgi-like structures accumulated by *ypt1-1* mutant cells at the nonpermissive temperature (data not shown).

Preliminary immunoelectron microscopy studies suggest that the vesicles we observe in the *ypt1-1* mutant cells at the nonpermissive temperature are indeed abnormal Golgi structures (J. Mulholland and N. Segev, unpublished data). Antibody against a mannan antigen that ac-

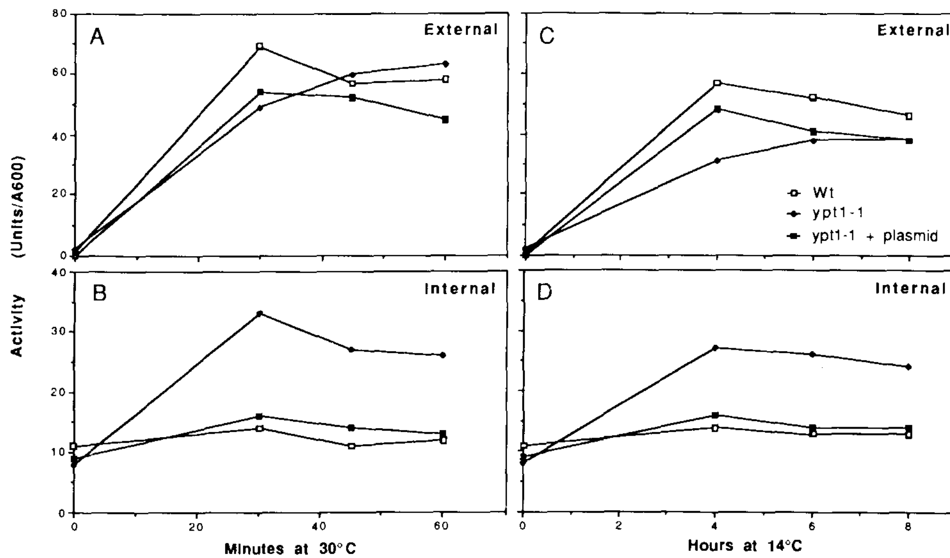

Figure 2. Invertase Secretion and Accumulation

Cells were grown overnight in YEPD medium. At time zero, the cells were shifted to derepression medium (YEP medium containing 0.1% glucose) and incubated either at 30°C (A, B); or at 14°C (C, D). At time points, aliquots were removed and assayed for external and internal pools of invertase. Upper panels show external levels (A, C); bottom panels show internal levels (B, D). The strains used are: wild-type (DBY1034); *ypt1-1* (DBY1803); and *ypt1-1* complemented by *YPT1* on a centromere containing plasmid.

cumulates in aberrant Golgi structures in *sec7* mutants stains specifically the aberrant vesicular structures we see in the *ypt1-1* mutant.

The *ypt1-1* Mutant Is Partially Defective in Invertase Secretion

The accumulation of membranes shown above resembles the phenotypes of several of the *sec* mutants (Novick et al., 1980), and prompted us to look carefully at the possibility that the *ypt1* phenotype might include a frank secretion defect. We studied the secretion of the enzyme invertase, the product of the *SUC2* gene. *SUC2* encodes two transcripts: a constitutively synthesized transcript whose product remains soluble in the cytoplasm and a regulated transcript that is repressed by growth in glucose (Carlson and Botstein, 1982). The protein product of the regulated transcript carries a signal sequence that enables it to be inserted into the lumen of the ER, the first step in its secretion (Carlson et al., 1983). In the ER, the invertase is glycosylated first by attachment of nine carbohydrate units to asparagine residues. This partially glycosylated invertase precursor is transferred to the Golgi, where the carbohydrate cores are extended by addition of many "outer chain" mannose residues (Esmon et al., 1981). The fully glycosylated protein is packaged into small vesicles that, upon fusion with the plasma membrane, release the enclosed invertase, which remains associated with the cell surface, accessible to its substrate.

Secretion of invertase was followed in the *ypt1-1* mutant and wild-type control cells after derepression (by reducing glucose concentration in the medium) either at permissive (30°C) or nonpermissive (14°C) temperature. External invertase was assayed in intact cells; the internal pool was assayed after enzymatic digestion of the cell wall with zymolyase, removal of the wall and any associated invertase, followed by lysis of the resulting spheroplasts.

The results (Figure 2) show that even at permissive temperature, the amount of internal invertase activity is approximately 2.5 times higher in the *ypt1-1* mutant than in wild-type cells, although there is almost a normal amount of invertase secreted to the surface. At the nonpermissive temperature the phenotype is the same, except that there is now a more visible slowness in secretion of invertase to the surface in the mutant.

These results are consistent with a partial defect in secretion, as was observed, for example, in actin-defective mutants (Novick and Botstein, 1985). If the defect is early in secretion, for example at the level of transfer from ER to Golgi, one might expect that the accumulated internal invertase might be incompletely glycosylated. To test this possibility, we examined the mobility of the invertase by immunoblotting after separation of proteins in denaturing SDS-polyacrylamide gels. The invertase from *ypt1-1* cells, derepressed at either 30°C or 14°C, runs faster than invertase from wild-type cells (Figure 3A). In order to show that the altered mobility of the invertase in the mutant was indeed entirely due to differences in the degree of glycosylation and not, for example, to proteolysis, another experiment in which the invertase was deglycosylated with endoglycosidase H was performed. As can be seen in Figure 3B, the invertase made in mutant and wild-type cells at both 14°C and 30°C has the same electrophoretic mobility after deglycosylation.

The invertase secretion phenotype of *ypt1-1* (i.e., partial

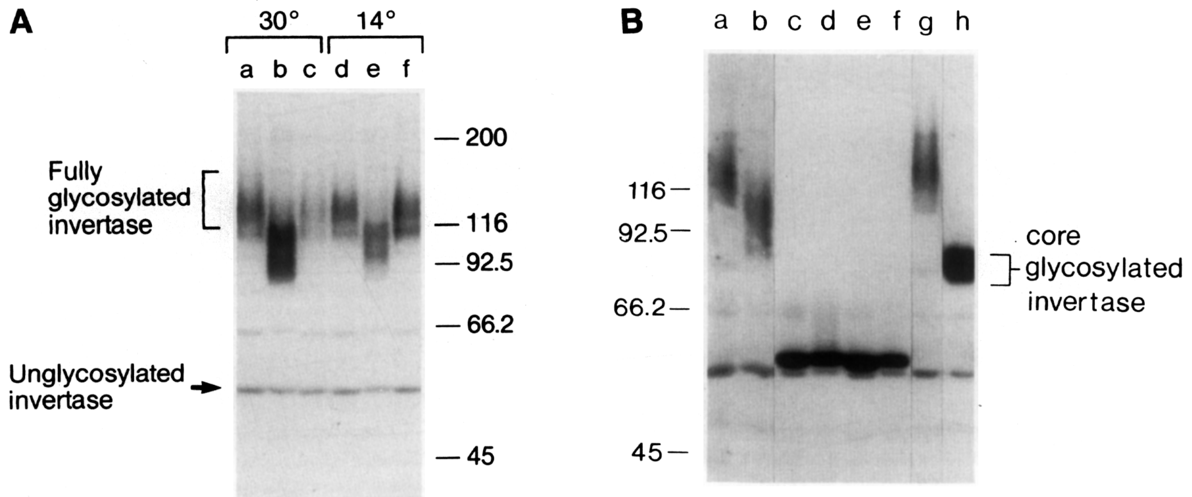

Figure 3. Invertase Analysis on Denaturing Acrylamide Gels

Cells were grown and induced for invertase as described in Figure 2. The cells were incubated either at 30°C for 1 hr or at 14°C for 8 hr. Samples were removed, boiled in sample buffer, treated (or not) with endoglycosidase H, and then analyzed on denaturing acrylamide gel using immunoblotting with anti-invertase antibodies.

(A) Wild-type (DBY1034) cells are compared with *ypt1-1* mutant (DBY1803) cells or with mutant cells also carrying a centromere plasmid expressing the normal YPT1 protein. Lanes a and d contain wild-type; lanes b and e, the mutant; and lanes c and f, the complemented mutant.

(B) The effect of treatment with endo H is measured against standards including wild-type (lane a: DBY1034; lane g: NY13) and the *ypt1-1* mutant (lane b: DBY1803) untreated invertase proteins as well as invertase extracted from *sec18* cells at nonpermissive temperature (lane h: NY431). The treated proteins are in lanes c (DBY1034 grown at 30°C), d (DBY1803 grown at 30°C), e (DBY1034 at 14°C), and f (DBY1803 at 14°C). The figure shows lanes from a single gel with irrelevant samples excised. Lanes a, b, and g were exposed approximately twice as long as were the other lanes.

secretion and incomplete glycosylation) can be completely complemented if the wild-type copy of the *YPT1* gene is present in the mutant cells on a low copy number plasmid (Figure 3A, see also Figure 2). This shows that the secretion phenotype is entirely due to the *ypt1-1* mutation. However, there is very little effect of temperature on the phenotype, a subject to which we return in the Discussion.

Similar degrees of glycosylation were obtained with samples from mutant cells that had been fractionated so that the invertase on the surface was largely removed (data not shown), suggesting that the internal pool of partially secreted invertase has a degree of glycosylation similar to the entire cell's content of invertase. For this reason, and simply from the fact that most of the enzyme is eventually secreted in the mutant, we conclude that virtually all of the invertase in *ypt1-1* cells is incompletely glycosylated. It is also worth noting that the amounts of invertase made by the mutant and wild-type are comparable when estimated in Figure 3B from the lanes containing endo H-treated samples; this observation supports the finding of comparable invertase activity (Figure 2).

Comparison of the mobility of the invertase in the *ypt1-1* mutant with the invertase made by a well-characterized secretion-defective mutant (*sec18*) that accumulates core-glycosylated invertases shows that virtually all of the invertase in the *ypt1-1* mutant migrates more slowly (Figure 3B). Thus, the observed range of molecular size (85–115 kd) characteristic of the *ypt1-1* mutant lies between the size characteristic for wild-type (100–140 kd) and core glycosylated (approximately 80 kd; Esmon et al., 1981). The extent of glycosylation observed is thus consistent with the idea that the *ypt1-1* mutant is defective either in the transfer of material from the endoplasmic reticulum to the Golgi or within the Golgi. Both ideas would be consistent with the finding of accumulation of aberrant membrane structures in the mutant.

Localization of the YPT1 Protein in Yeast Cells

In the hope that localization of the *YPT1* gene product might shed more light on its function, antibodies against a fusion of this protein with the bacterial trpE protein were raised. The fusion protein was purified on polyacrylamide gels and the purified material was used to immunize rabbits; polyclonal antibodies in the rabbit serum were affinity-purified as described in Experimental Procedures. The anti-*YPT1* antibodies were prepared first by collecting protein that failed to bind a *trpE* affinity resin but later bound to a column containing immobilized fusion protein. As controls, antibodies from the same serum that were affinity-purified twice on the *trpE* column were used.

The affinity-purified antisera were characterized by immunoblot analysis (Figure 4), which shows the reaction of the crude serum proteins, the purified anti-*YPT1* antibodies, and the control (anti-*trpE*) antibodies with total yeast and E. coli proteins. Clearly, the purified anti-*YPT1* serum recognizes one size species very strongly and specifically and another three or four species weakly. The strongly reacting band is likely to be the YPT1 protein since it has the right apparent molecular weight (23 kd). In confirmation, it is more abundant in cells that contain the *YPT1* gene on high copy number plasmid than it is in wild-type

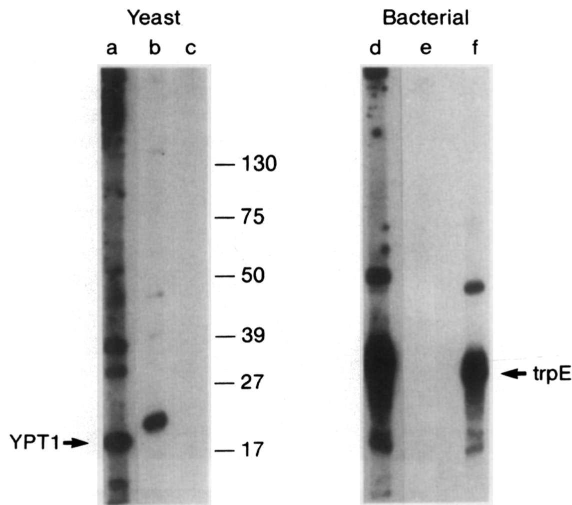

Figure 4. Affinity Purification of Anti-*YPT1* and Anti-*trpE* Antibodies

Protein extracts were prepared from yeast (a, b, c) or bacteria induced for *trpE* (d, e, f). Antibodies used for immunodetection (at dilution of 1:200) are: original anti-*trpE-YPT1* serum (a, d); affinity-purified anti-*YPT1* antibodies (b, e); and affinity-purified anti-bacterial *trpE* antibodies (c, f).

cells (data not shown). It is worth noting that the anti-*YPT1* antibodies do not react with the bacterial trpE protein. The affinity-purified anti-*trpE* antibodies react with the trpE protein in bacterial extracts and not with the YPT1 protein in yeast extracts.

We used these affinity-purified antibodies for immunofluorescence microscopy of growing normal yeast cells. The results (Figure 5) were that the cells stained with antibodies against the YPT1 protein showed a punctate pattern in the cytoplasm. Cells with very small buds showed very intense staining of the buds. This result is consistent with our previous finding that the *YPT1* gene is involved in bud growth (Segev and Botstein, 1987).

In order to test the idea that the punctate staining with anti-*YPT1* antibodies might represent organization of the protein in Golgi-like structures, a mutant (*sec7*; Novick et al., 1981) known to produce aberrant Golgi structures was stained with the antibody. The result (Figure 6) was that the mutant under restrictive conditions displayed apparently aggregated structures; the same strain at permissive temperature showed the punctate pattern observed with wild-type. Other *sec* mutants defective at other points in the secretion pathway were tested (data not shown). One

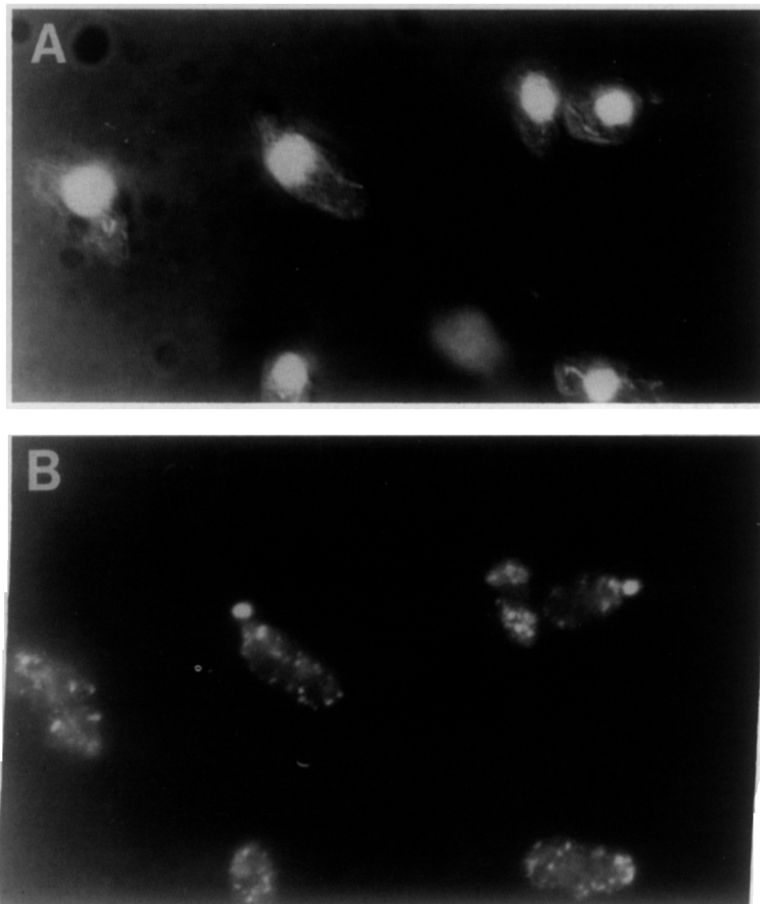

Figure 5. Localization of YPT1 Protein in Yeast Wild-Type Cells

Yeast wild-type cells (JPQP1) were grown at 30°C, fixed, and stained for fluorescence microscopy with DAPI for nuclear staining (A), and with affinity-purified anti-*YPT1* antibodies (at 1:200 dilution) (B). The cell outlines are visible by Nomarski.

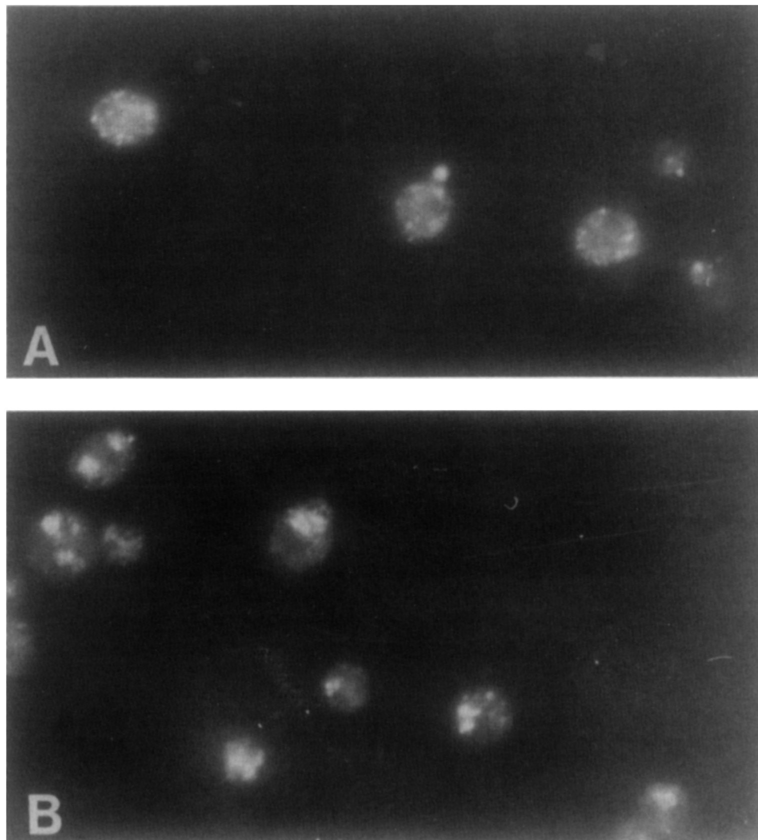

Figure 6. Localization of YPT1 Protein in Yeast *sec7* Cells That Are Defective in Golgi Function and Structure

Mutant (*sec7*) cells (DBY2357) were grown in YEPD medium at 26°C (A); shifted to YEP medium containing 0.1% glucose, and incubated at 37°C for 2 hr (B). The cells were fixed and stained for fluorescence microscopy with affinity-purified anti-*YPT1* antibodies (at 1:200 dilution).

of these (*sec18*) is defective in structure and function of the endoplasmic reticulum, while the other (*sec1*) accumulates secretory vesicles characteristic of the latest stages of the secretion process. In both cases, the pattern of staining with anti-*YPT1* antibody of cells grown at restrictive temperature was not different from the pattern displayed by wild-type. This result shows that the alteration in anti-*YPT1* antibody staining observed with the *sec7* mutant is specific, allowing the suggestion that it is the consequence of changes in the Golgi structures and not the consequence of a secretion defect per se.

It is worth noting the evidence that the YPT1 protein is not associated with the plasma membrane. First, in the immunofluorescence microscopy with affinity-purified anti-*YPT1* antibodies (Figure 5), we did not observe staining of the cell surface. Second, in preliminary cell fractionation experiments, the YPT1 protein did not follow the plasma membrane marker (plasma membrane ATPase: N. Segev, G. Joud, P. Novick, and D. Botstein, unpublished data).

Localization in Mammalian Cells of a Protein Closely Related to YPT1

We used the affinity-purified anti-*YPT1* antibodies characterized above to look for cross-reacting proteins in other eukaryotes. The antibodies reveal one major (approximately 66 kd) and two minor (approximately 23 and 95 kd) species in fission yeast (Schizosaccharomyces pombe). In Xenopus laevis egg protein extracts, the antibody recognized a single species with an apparent molecular weight of 21 kd (data not shown). In mammalian cells, the pattern we observed was more complicated: among the proteins we detected was a 23 kd species that, in preliminary experiments, appears to fractionate with crude Golgi fractions. The data for identification of this protein are not shown because two papers have appeared during the preparation of this paper that describe in some detail mammalian homologs of the yeast *YPT1* gene product (Touchot et al., 1987; Haubruck et al., 1987). The apparently closest homolog has 71% of its residues identical to the yeast YPT1 protein, and it has a similar molecular size (about 23 kd).

We used our affinity-purified anti-*YPT1* antibodies in immunofluorescence microscopy with two mouse fibroblast cell-lines, L cells (Figure 7), and NIH-3T3 (not shown). In these two cell lines, the antibody stains a region adjacent to one side of the nucleus. The control serum (affinity-purified anti-*trpE*) gave only faint uniform background staining (not shown). As an additional control, we successfully blocked staining by preincubating the antibody with trpE-YPT1 fusion protein, but were unable to block with comparable amounts of trpE protein (not shown).

In order to strengthen the supposition that the staining observed in Figure 7B represents Golgi localization, cells were doubly stained with anti-*YPT1* antibodies and a Golgi-specific reagent, wheat germ agglutinin (Virtanen et al., 1980). The antibody was visualized with a fluorescein-conjugated second antibody while the wheat germ agglu-

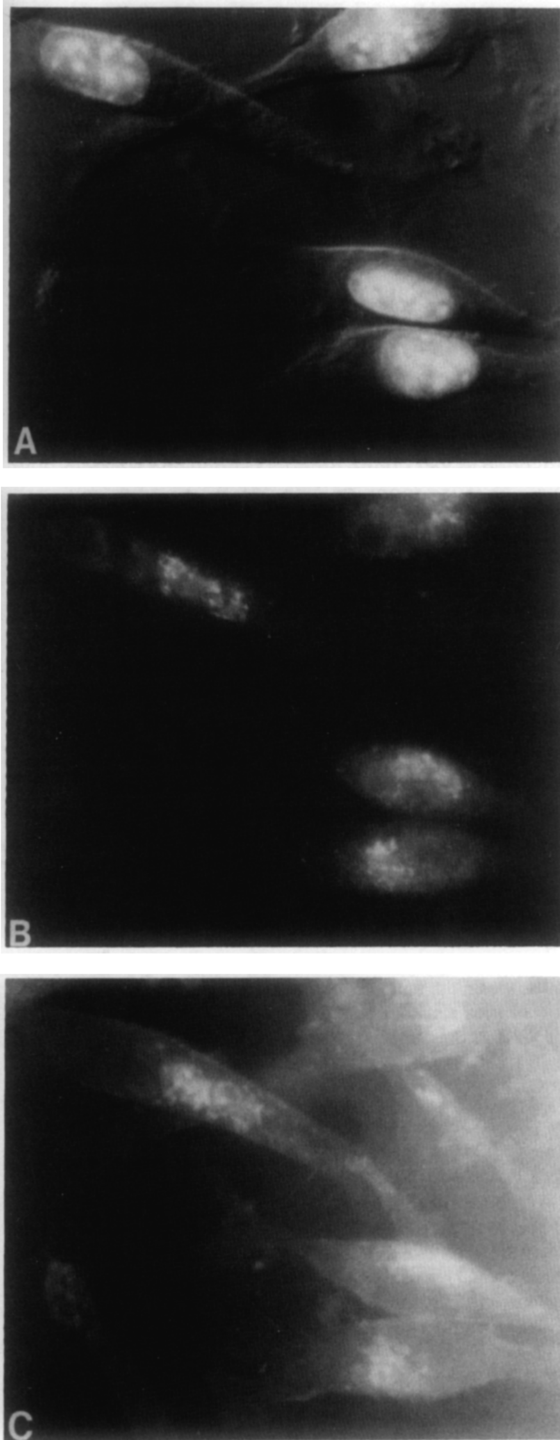

Figure 7. Localization of *YPT1*-Related Protein in Mouse Fibroblast Cells

Mouse L cells were fixed and stained for fluorescence microscopy with DAPI for nuclear staining (A), affinity-purified anti-*YPT1* antibodies (at 1:200 dilution) (B), and wheat germ agglutinin for Golgi staining (C).

tinin was conjugated with rhodamine. As can be seen in comparison of the two images (Figures 7B and 7C), the staining patterns were very similar, but not identical. Since wheat germ agglutinin stains primarily but not exclusively and not completely the Golgi apparatus (Tartakoff and Vassalli, 1983), this result is fully consistent with the idea that a close mammalian relative of the YPT1 protein is present in or in the immediate vicinity of the Golgi apparatus.

Discussion

In this paper, we have presented evidence that the yeast *YPT1* gene product is involved in the coupled processes of bud growth and protein secretion in yeast. The genetic pieces of that evidence are the appearance of obviously aberrant membranous structures soon after shift of the *ypt1-1* mutant to nonpermissive conditions and a partial defect in the secretion and incomplete glycosylation of invertase. The concentration of the gene product in the new buds of growing normal cells and the localization of the gene product and a mammalian relative in what are likely to be Golgi structures completes the evidence now available that links the *YPT1* gene and the secretory apparatus.

The link between the *YPT1* gene and the various mutant phenotypes is made secure by our observation that the entire phenotype of the *ypt1-1* mutant is suppressed by the presence of a low copy plasmid containing only an intact *YPT1* gene. However, despite the fact that the *ypt1-1* mutation is a cold-sensitive lethal mutation, there is little effect of temperature on the secretion phenotype. It must be remembered that by many other criteria the cells do not grow normally even at temperatures that allow colony formation: their growth rate is reduced (2.5 instead of 1.5 hr generation time), they fail to sporulate and respond normally to nutrient limitation (Segev and Botstein, 1987), and they accumulate some aberrant internal membranes even at the nominally permissive temperature. Thus, the secretion defect might be seen as something that occurs at all temperatures, but results in lethality only at lower temperatures. Alternatively, the mutant may be leaky, an idea supported by the finding that null (disruption) mutations are lethal at all temperatures (Segev and Botstein, 1987; Schmidt et al., 1986). Finally, the method of assessment of the secretion defect may have contributed: the temperature was shifted simultaneously with the removal of glucose to start derepression of invertase production. No matter how one interprets the temperature lethality, the fact remains that the secretion defect, along with all the other phenotypes, is the consequence of the mutation in the *YPT1* gene.

The connection between the YPT1 protein in yeast and bud growth is supported as well by previously published evidence from our laboratory that the *ypt1-1* mutant specifically affects the cell cycle at the point of maximum bud growth (Segev and Botstein, 1987). In mammalian cells, the connection between such a protein and secretion rests almost entirely upon our current finding that affinity-purified anti-*YPT1* protein antibodies recognize a mammalian homolog (presumably the one described by Touchot et al., 1987 and Haubruck et al., 1987) that is apparently localized to the Golgi apparatus.

These connections all support the notion that the YPT1 protein plays a role relatively early in the secretion/mem-

brane biosynthesis pathway. Thus we are led to propose a mechanistic role for this protein based on its membership in a large family of proteins known to bind guanine nucleotides and often seen playing a role in signal transduction through membranes (Gilman, 1987; Barbacid, 1987). We suppose that the YPT1 protein and its mammalian counterpart are involved in vesicular traffic as "labels" that signal the vesicles origin, destination, and/or contents. In this way, the major problem posed by the multiplicity of vesicles in the cell bound for disparate destinations might be understood.

Although we cannot yet prove directly this view of the role of YPT1 protein function, there are considerable bits of precedent for this kind of role for G-proteins. Since the G-proteins have generally been regarded as involved only in stimulated signal transduction through the plasma membrane (Styer and Bourne, 1986), most of these precedents are very recent, and most are results involving the plasma membrane itself. In mammalian systems, it has been proposed, on the basis of studies with guanine nucleotides and analogs, that two G-proteins, Gp and Ge, are involved in exocytosis (Burgoyne, 1987). The proposed new G-proteins are thought to be plasma membrane–or granular vesicle–associated (Gompart, 1986). In another connection, Bar-Sagi and Feramisco (1986) reported that microinjection of ras proteins induces membrane ruffling and pinocytosis.

A recent precedent has been reported for involvement of a *YPT1* homolog in a late step of secretion in Saccharomyces cerevisiae. Salminen and Novick (1987) showed that the yeast *SEC4* gene, known to be involved in the fusion of the secretory vesicles with the plasma membrane, is a *ras* homolog.

In each of these previous cases, it had been assumed that the role of the GTP-binding protein was related to the involvment of the plasma membrane. Our results, implicating as they do earlier steps in secretion, suggest instead that the reason for involvment of GTP-binding proteins must be sought elsewhere. Another difference between the cases in mammalian systems and yeast is that it had previously been assumed that the signals transduced by GTP-binding proteins were simulated from the outside, and not constitutive. On the basis of our results, it would seem that the YPT1 protein and its mammalian counterpart must transduce without external stimulus, based on their intracellular localization. Salminen and Novick (1987) also suggested the possibility that the SEC4 protein works constitutively in controlling or aiding vesicle fusion to the plasma membrane.

Two recent in vitro studies support the idea that GTP-binding proteins might be involved at several different and early steps in secretion in animal cells. First, translocation-competent binding of ribosomes to the ER membrane has been reported to require GTP or a nonhydrolyzable analog (Connoly and Gilmore, 1986). Second, GTP analogs were recently shown to inhibit transport between two Golgi compartments (Melancon et al., 1987).

Although the localization of the mammalian YPT1-like protein to the vicinity of the Golgi apparatus is quite clear from our results, the localization of the protein in yeast is less clear. From the functional defect in glycosylation of invertase by the *ypt1-1* mutant, Golgi is indicated. The electron microcopic evidence from the mutant and the preliminary immunolocalization results are consistent with either ER or Golgi or both; the same can be said for the staining with anti-*YPT1* antibodies of the aberrant membranous structures that accumulate in the *sec7* mutant. The observation of intense staining in young buds suggests a structure concentrated in such buds: unfortunately it is not yet known when after bud emergence functional Golgi structures can be found in the bud. The important point here is that all the evidence suggests steps of secretion and membrane growth well before the final fusion with the plasma membrane. Previously we and others observed that microtubule organization is aberrant in the prolonged absence to *YPT1* gene product (Segev and Botstein, 1987; Schmitt et al., 1986). Our current results support the idea suggested previously (Segev and Botstein, 1987), that this effect is secondary to the defect in bud growth. Such a secondary consequence is fully consistent with the dependency in the yeast cell cycle of later morphological changes on earlier events in the cell cycle (Pringle and Hartwell, 1981).

In conclusion, our results are compatible with the view that the YPT1-like GTP-binding proteins may be present at all stages of the secretion pathway in mammalians cells as well as in yeast. The role of these proteins is as yet uncertain, although the idea that different variant proteins might label the membrane they occupy so that vesicle traffic will be orderly is particularly attractive at this time.

Experimental Procedures

Cells, Plasmids, and Growth Conditions

S. cerevisiae strains used are: wild-type, DBY1034; *ypt1-1*, DBY1803 (Segev and Botstein, 1987); *sec7*, DBY2357 (*MATa*, *ura3-52*, *leu2-3*, *leu2-112*, *suc2−*); *sec18-1* and its isogenic wild-type were NY431 and NY13, respectively both are *MATa* and *ura3*–52, obtained from Peter Novick. The wild-type tetraploid strain is JPQP1 (Adams and Pringle, 1984).

Plasmids used are: pRB319, a CEN plasmid that contains the *YPT1* gene, and pRB320, a 2 μm plasmid that contains the *YPT1* gene (Segev and Botstein, 1987). The plasmid that contain the fusion protein trpE-YPT1 is pRB578. This plasmid was constructed for this study by insertion of the 0.765 kb EcoRI–BamHI fragment from pRB301 (Segev and Botstein, 1987), which contains the *YPT1* gene without the first 50 nucleotides, into pRB535, which contains the bacterial *trpE* gene. pRB535 is a pATH3 plasmid that was kindly given to us by Dr. A. Tzagoloff. The E. coli strain that was used for transformation with the fusion plasmid is RR1 (*leu*, *hsdR*, *hsdM*).

Mammalian cell lines used in this study are: mouse fibroblast L cells (from H. Eisen at MIT) and mouse NIH3T3, given to us by Dr. K. Mostov (Whitehead Institute).

Media and growth of yeast cells were described by Sherman et al. (1974).

Thin Section Electron Microscopy

Approximately 2 × 10^8 cells were processed for electron microscopy as described by Novick and Botstein (1985), except for the cell wall removal, which was done by treatment with glusulase diluted 19 times in 41 mM of potassium phosphate, 8 mM sodium citrate buffer (pH 5.8) for 2 hr at 37°C. Sections were cut by diamond knife and stained with lead citrate.

Measurement of Invertase Activity

Cells at an absorbance of 0.2 at 600 nm were harvested, resuspended

in 10 mM sodium azide, and kept at 4°C. External invertase was assayed using intact cells as described by Goldstein and Lampen (1975). Internal invertase activity was measured in spheroplast lysates; 50 μl of the cell suspension (absorbance 600–10) was mixed with 450 μl of solution containing 2.8 M sorbitol, 50 mM potassium phosphate (pH 7.5), 50 mM 2-mercaptoethanol and 50 μg/ml Zymolyase 60,000. After 1 hr at 14°C, the spheroplasts were centrifuged and then lysed by addition of 50 μl of 0.5% Triton X-100. N-ethylmaleimide, 0.2 mM, was added to the second stage of the assay to prevent interference by residual 2-mercaptoethanol. Units of activity are nanomoles of glucose released per minute.

Preparation of Affinity Purified Anti-*YPT1* Antibodies

The trpE-YPT1 fusion protein was prepared from the E. coli strain that contain the plasmid pRB578. The cells were grown and induced for high expression of the fusion protein, and the insoluble fraction was prepared as described by Haarer and Pringle (1987).

For immunization approximately 200–400 μg of the fusion protein band was cut from acrylamide gels, crushed in PBS buffer, mixed with Freund adjuvant (complete adjuvant for the first two injections, and incomplete adjuvant for the third injection), and injected into New Zealand White rabbits. Boosters were given twice, 3 and 5 weeks after primary immunization. Blood collection was begun 12 days after the third booster, and every 1 or 2 weeks thereafter.

Affinity-purified anti-*YPT1* and anti-bacterial *trpE* antibodies were prepared from the crude anti-*trpE-YPT1* antiserum using affinity columns. The bacterial *trpE* column was prepared by coupling of 6 mg of the insoluble fraction, prepared from E. coli containing the *trpE* plasmid (pRB535), with 2 ml of CNBr-activated Sepharose-4B (Pharmacia). Coupling was performed as suggested by the manufacturer, but in the presence of 0.1% SDS. The *trpE-YPT1* fusion column was prepared by coupling 1.85 mg of acrylamide gel–purified fusion protein with 1.5 ml of the same resin.

Affinity purification was done as described by Pfeffer et al. (1983). The anti-*YPT1* antibodies were prepared by first collecting the flow-through that did not bind to the bacterial *trpE* column, and then bound to the tprE-*YPT1* fusion column. This purification was repeated twice. As a control we prepared anti-bacterial *trpE* (non-*YPT1*) antibodies from the same serum, by affinity purifying twice on the bacterial-*trpE* column.

Preparation of Protein Extracts and Immunoblotting

Yeast cells protein extracts were prepared by harvesting approximately 2×10^7 cells, washing in 10 mM sodium azide in 25 mM Tris buffer (pH 7.5), resuspending in Laemmli sample buffer, adding the same volume of glass beads and vortexing for 1 min. The sample was boiled immediately for 2 min, and the supernatant was loaded on the gel. E. coli protein extracts were prepared by boiling the cells in Laemmli sample buffer for 5 min. For invertase blots, with and without prior deglycosylation with endo H, 10 ml of cells at absorbance 600–0.2 were harvested, prepared, and assayed as described by Kaiser et al. (1987).

SDS-polyacrylamide gels (10%) and the Laemmli buffer system (Laemmli, 1970) were used for all protein electrophoresis. Proteins were electroblotted to nitrocellulose and radiographic detection was done by incubation with antibodies and ^{125}I-protein A (Burnette, 1981).

Immunofluorescence Microscopy and Photography

Yeast cells were fixed and stained as described by Kilmartin and Adams (1984).

Mammalian cells were grown on glass slides to approximately 50% confluency. The cells were rinsed with PBS buffer and fixed in 3.7% formaldehyde in the same buffer. After 30 min at room temperature, the cells were washed and then stored in PBS at 4°C. Permeabilization was done by succesive incubations in aceton:water: 50% for 2 min, 100% for 5 min, and 50% for 2 min at −20°C. Indirect immunofluorescence was done as described for yeast cells.

Nuclear staining was done with 4′,6′-diamidino-2-phenylindole (DAPI) (Williamson and Fennel, 1975); 10 min for yeast cells and 5 sec for mammalian cells. Golgi-specific staining was done using rhodamine-conjugated wheat germ agglutinin (WGA) (Molecular Probes) at 50 μg/ml for 10 min. Microscopy and photography procedures were as described by Novick and Botstein (1985).

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