The Ypt1 GTPase Is Essential for the First Two Steps of the Yeast Secretory Pathway

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Abstract. Small GTPases of the rab family are involved in the regulation of vesicular transport. The restricted distribution of each of these proteins in mammalian cells has led to the suggestion that different rab proteins act at different steps of transport (Pryer, N. K., L. J. Wuestehube, and R. Scheckman. 1992. *Annu Rev. Biochem.* 61:471–516; Zerial, M., and H. Stenmark. 1993. *Curr. Opin. Cell Biol.* 5:613–620). However, in this report we show that the Ypt1-GTPase, a member of the rab family, is essential for more than one step of the yeast secretory pathway. We determined the secretory defect conferred by a novel *ypt1* mutation by comparing the processing of several transported glycoproteins in wild-type and mutant cells. The *ypt1-A136D* mutant has a change in an amino acid that is conserved among rab GTPases. This mutation leads to a rapid and tight secretory block upon a shift to the restrictive temperature, and allows for the identification of the specific steps in the secretory pathway that directly require Ypt1 protein (Ypt1p). The *ypt1-A136D* mutant exhibits tight blocks in two secretory steps, ER to *cis*-Golgi and *cis*- to *medial*-Golgi, but later steps are unaffected. Thus, it is unlikely that Ypt1p functions as the sole determinant of fusion specificity. Our results are more consistent with a role for Ypt1/rab proteins in determining the directionality or fidelity of protein sorting.

TRANSPORT of proteins in eukaryotic cells involves their orderly progression through a series of membranous compartments (Palade, 1975). The different steps of this transport pathway appear to be mediated by vesicles that bud from one compartment and fuse with the next (Pfeffer and Rothman, 1987). The mechanisms that control directionality and specificity of the different steps are not known. A large number of GTPases belonging to the rab/Ypt1/Sec4 family have been shown to play a role in vesicular trafficking in yeast and mammalian cells. Because different rab proteins have distinct subcellular distributions, it has been suggested that each rab protein acts at a specific step in transport through the exocytic, endocytic, or transcytotic pathway (Pfeffer, 1992; Ferro-Novick and Novick, 1993; Zerial and Stenmark, 1993). Current models for the determination of vesicle targeting specificity propose that a vesicle-associated rab protein interacts with a vesicle-associated SNAP receptor (v-SNARE) specificity component (Novick and Brennwald, 1993; Rothman, 1994). Such a mechanism would imply that a given rab protein acts at only one step rather than at multiple steps of transport, a question that has not been tested experimentally. Resolution of this issue will help elucidate the functions of rab proteins.

Two alternative models have been proposed for the mechanism of action of rab GTPases in vesicular transport. First, they might serve as specificity determinants for each step, either alone or in conjunction with the SNAREs (Novick and Brennwald, 1993; Zerial and Stenmark, 1993; Rothman, 1994). Alternatively, rab proteins might regulate the assembly of docking and/or fusion complexes that link vesicles with their target membranes (Sogaard et al., 1994). An argument against the first model was the finding that an artificial rab protein can function in more than one step of the yeast secretory pathway. Thus, a Ypt1p-Sec4p chimera was shown to function as both Sec4p and a minimal Ypt1p (it can complement a *ypt1*-deletion, but the cells are heat and cold sensitive; Brennwald and Novick, 1992; Dunn et al., 1993). The bifunctionality of this artificial GTPase might mean that rab proteins do not, by themselves, function as specificity determinants (Ferro-Novick and Jahn, 1994). However, it is conceivable that Ypt1p and Sec4p do function as specificity factors, but the specificity-bearing domains are in different portions of the two proteins, so that the artificial fusion protein contains both domains. We therefore did a complementary experiment by testing whether a single rab GTPase functions in vivo at more than one step in the secretory pathway.

The Ypt1 GTPase has been demonstrated to function

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1. *Abbreviations used in this paper*: EF-Tu, elongation factor Tu; GAP, GTPase activating protein; SD, synthetic dextrose; v-SNARE, vesicle-associated SNAP receptor; YPD, yeast extract, peptone, and dextrose.

early in the yeast secretory pathway, and this protein has been localized to the yeast ER and Golgi complex (Segev et al., 1988; Preuss et al., 1992). Although Ypt1p appears to function after vesicle formation in the targeting of vesicles to an acceptor compartment (Rexach et al., 1991; Segev, 1991), it is not clear whether Ypt1p functions at more than one transport step. The role of Ypt1p in the first step of the pathway, ER to Golgi, is well established since antibodies against Ypt1p completely inhibit this step in a cell-free system (Baker et al., 1990). However, in vivo and in vitro studies of *ypt1* mutants have been inconclusive, suggesting that Ypt1p might act in ER to Golgi transport or in transport between early Golgi compartments (Schmitt et al., 1988; Segev et al., 1988; Bacon et al., 1989). Although the mutant studies implicated Ypt1p in each of these steps, Ypt1p did not appear to be required because the secretory blocks were not tight. These results were inconclusive for two reasons. First, the three *ypt1* mutants used in previous studies (*ypt1-1*, *ypt1-2*, and *ypt^{ts}*) exhibit severe secretory defects even under permissive conditions, and/or the blocks are either leaky or slow to take effect. These factors complicate the analysis due to possible indirect effects that are unrelated to the specific processes controlled by Ypt1p. Second, previous studies of the role of Ypt1p in glycoprotein transport did not examine the nature of the carbohydrate modifications, which are indicative of passage through particular secretory compartments. Rather, the analyses were based on the electrophoretic mobility of the marker proteins, and, as we discuss in this report, such analyses can be misleading. We wished to address two questions concerning the role of Ypt1p in the yeast secretory pathway: first, is there a primary intra-Golgi defect in *ypt1* mutant cells? Second, if there is such a defect, which of the intra-Golgi steps are affected?

In this study, we determined the steps in which Ypt1p functions in vivo using a novel *ypt1* mutant whose phenotype does not exhibit the complications discussed above. This mutant, *ypt1-A136D*, exhibits a rapid and tight block of secretion upon shifting to the nonpermissive temperature. We determined the nature of the secretory block by monitoring the specific modifications acquired by transported glycoproteins in these mutant cells. Our results demonstrated that the *ypt1-A136D* mutation confers a block in the first two steps of the secretory pathway (i.e., ER to *cis*-Golgi and *cis*- to *medial*-Golgi). Thus, it appears that rab GTPases do not define the specificity of membrane trafficking, but instead perform an essential regulatory function in vesicular transport.

Materials and Methods

Cells, Mutants, and Plasmids

The *ypt1-1* allele was amplified by PCR from genomic DNA using the upstream primer 5′-GGGCC CGCAT GCGCA CCAGT TTTGA GGAGG-3′ and the downstream primer 5′-GGGCC CGGAT CCGAT AAGAA AGAAT G-3′. PCR products were inserted into pGEM3Zf(−) (Promega Corp., Madison, WI), and 20 insert-containing clones were pooled and sequenced. The *ypt1-A136D* allele was made by site-directed mutagenesis using the method of Kunkel (Kunkel, 1987) in the *Escherichia coli* phagemid vector pRS300 that contained the 767-bp fragment from EcoRI to BamHI of *YPT1* inserted into pIBI31 (International Biotechnologies, Inc., New Haven, CT). The mutagenic oligonucleotide was: 5′-GTG GAA TAT GAC GTC GAC AAG GAA TTT GCG GAC-3′. DNA modifying enzymes were from Boehringer Mannheim Corp. (Indianapolis, IN). The mutation was confirmed by sequencing with the Sequenase sequencing kit (United States Biochemical Corp., Cleveland, OH). The *ypt1-A136D* mutation was targeted to the chromosome as previously described (Segev and Botstein, 1987). Two pairs of *Saccharomyces cerevisiae* strains were used in this study: (*a*) wild-type, DBY1034; and *ypt1-T40K*, DBY1803 (Segev and Botstein, 1987); (*b*) wild-type, NSY160: *MATα his4-539(am) lys2-801(am) ura3-52*; and *ypt1-A136D*, NSY161: *MATα his4-539(am) ura3-52* (this study). Yeast strains were grown on yeast extract, peptone and dextrose (YPD) or synthetic dextrose (SD) medium containing the appropriate nutritional supplements (Sherman et al., 1979).

Cell Labeling and Immunoprecipitation

Yeast cells were grown to mid-logarithmic phase SD medium containing the appropriate nutritional supplements. Before labeling cells were pelleted in a microcentrifuge at 200 *g* for 1 min and resuspended in fresh media containing 1 mg/ml BSA to a final concentration between 2 and 10 OD_{600} units/ml. Tran^{35}S-label was added to a final concentration of 50 μCi/OD_{600} unit. Labeling was terminated by the addition of a 1/10 volume of 20 mM cysteine, 50 mM methionine, or in the case of temperature shift experiments the cells were spun (200 *g* for 1 min) and resuspended in prewarmed (42°C) medium containing 2 mM cysteine, 5 mM methionine, and 1 mg/ml BSA. For the immunoprecipitation of α-factor and carboxypeptidase Y (CPY) the chase was terminated by the addition of TCA to 5% and cells were processed as previously described (Graham and Emr, 1991). Invertase was immunoprecipitated as previously described (Franzusoff and Schekman, 1989). CPY and invertase were resolved on 8% SDS-PAGE gels and α-factor was resolved on 13% gels (Laemmli, 1970). For spheroplasting, sodium azide (to 5 mM final concentration) was added to the cells that were then pelleted in a microfuge at 150 *g* for 5 min at 4°C. The cells were then resuspended in spheroplasting buffer (50 mM potassium phosphate, 1.4 M sorbitol, 5 mM sodium azide, and 5 mM β-mercaptoethanol) to a concentration of 1 OD_{600} unit/100 μl, and Zymolyase 100T was added to 10 μg/OD_{600} unit of cells. Spheroplasting was carried out for 30 min at 37°C, after which time the cells were pelleted in a microfuge at 150 *g* for 5 min at 4°C, washed twice in spheroplasting buffer and lysed by the addition of 2× Laemmli buffer (Laemmli, 1970). Zymolyase 100T and Tran^{35}S-label were obtained from ICN (Irvine, Ca); all other chemicals were from Sigma Chemical Co. (St. Louis, MO). Antibody to CPY was from Dr. T. Stevens, antibody to invertase was obtained from Dr. D. Botstein (Stanford Univ., Stanford, CA), antibody to α-factor was from Dr. T. Graham (Vanderbilt Univ., Nashville, TN), and antisera to α-1,6–mannose linkages and α-1,3–mannose linkages were from Dr. R. Scheckman (Univ. of California, Berkeley, CA) and Dr. A. Franzusoff (Univ. of Colorado, Boulder, CO), respectively.

Results

To determine the secretory steps for which Ypt1p is needed we employed phenotypic analysis of two *ypt1* mutations, and analyzed different marker proteins to distinguish between all assayable steps of the yeast secretory pathway. The recessive mutant allele *ypt1-1* (Segev and Botstein, 1987; Segev et al., 1988; Bacon et al., 1989; Brennwald and Novick, 1992) has been shown to contain a single point mutation, *T40K*, in a residue that is highly conserved among the ras-related protein family and is within the putative effector domain of Ypt1p. The second and more informative mutation is *ypt1-A136D*, a novel mutation that is analogous to the *sec4-8* mutation (Salminen and Novick, 1987). It has been suggested that the mutant Sec4-8 protein is defective in the interaction with its nucleotide exchange factor (Moya et al., 1993).

The analysis of the secretory defects exhibited by the *ypt1* mutants was based on the ability to assay the different steps of the yeast secretory pathway, which has been studied extensively both genetically and biochemically (Pryer et al., 1992). The compartmental organization of the Golgi

complex has been characterized primarily by using two mutants, *sec18* and *sec7*, and by cell fractionation (Cunningham and Wickner, 1989; Franzusoff and Schekman, 1989; Graham and Emr, 1991). Three functionally distinct Golgi compartments have been defined that contain, from cis to trans: α-1,6–mannosyltransferases, α-1,3–mannosyltransferases, and the Kex2 protease (Fig. 1). Using pulse–chase experiments we followed the processing of three proteins that traverse the secretory pathway: the secreted proteins invertase and α-factor, and the vacuolar protein CPY.

The ypt1-T40K Mutation Confers Multiple Secretory and Glycosylation Defects

The *ypt1-T40K* mutant has been used in previous studies aimed at determining the role of Ypt1p in the yeast secretory pathway. Mutant cells display a tight cold sensitivity and a less severe heat sensitivity for growth (Segev and Botstein, 1987; Segev et al., 1988; Bacon et al., 1989). In vivo and in vitro experiments suggested that the *ypt1-T40K* mutant has a transport defect between ER and Golgi and/or between Golgi cisternae. The Golgi defect was revealed by the appearance of an underglycosylated faster-migrating form of CPY and invertase in the mutant cells, as compared to wild-type control cells (Segev et al., 1988; Bacon et al., 1989; Rossi et al., 1991; Brennwald and Novick, 1992; and data not shown). However, a more detailed analysis, using mannose linkage-specific antibodies, indicates that the phenotype is much more complex.

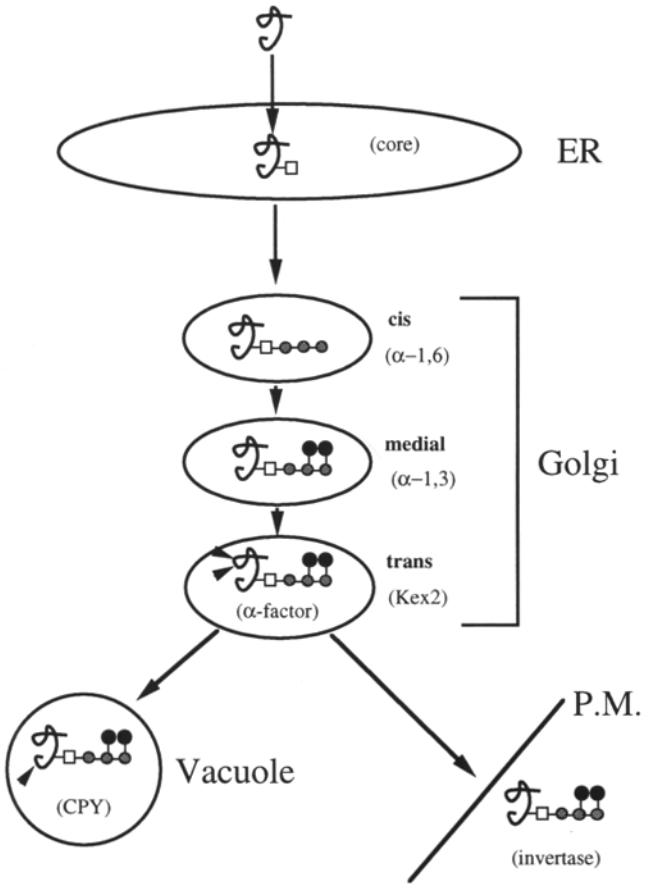

Figure 1. Model for the organization of the yeast secretory pathway. Illustrated are steps in the yeast secretory pathway that were analyzed in this study. Transport through the ER is determined by immunoprecipitation with antibodies against protein markers that undergo an increase in molecular mass upon core-glycosylation (*boxes*). Transport through the *cis*- and *medial*-Golgi compartments is determined by a second immunoprecipitation using mannose linkage-specific antibodies that recognize the α-1,6 (*cis*) (*open circles*) and α-1,3 (*medial*) (*closed circles*) linkages. Transport through the *trans*-Golgi can be measured for α-factor, since this protein has a cleavage site for the Kex2 protease (*arrowheads*). Transport across the plasma membrane (*P.M.*) is analyzed by determining internal and external invertase. Transport to the vacuole is detected by assaying for the proteolytic maturation of CPY (*arrowhead*).

To determine the Golgi compartment(s) through which the underglycosylated form of invertase had transited in the mutant cells, we analyzed the carbohydrate modification of this form. Transport of invertase was analyzed in *ypt1-T40K* mutant cells after a shift to 37°C. Immunoprecipitation with α-1,3–mannose specific antibodies showed that about half of the underglycosylated invertase present in the mutant cells contained this modification, although the molecular weight of the α-1,3–modified invertase was lower in these cells than in wild-type cells (data not shown). Thus, the underglycosylated invertase in the mutant cells has transited through both the *cis*- and *medial*-Golgi compartments. In addition, the fact that this α-1,3–modified form accumulates intracellularly (see above), demonstrates a partial transport defect also in later steps of the pathway, beyond the *medial*-Golgi. Under the conditions of this experiment, in *ypt1-T40K* mutant cells each assayable step is defective, whereas wild-type cells modify all of the invertase to the *medial*-Golgi α-1,3–modified form and secrete it. *ypt1-T40K* mutant cells exhibit two additional phenotypes: first, a defect in carbohydrate elongation, as evinced by the high electrophoretic mobility of the α-1,3–modified invertase; second, secretion of the *cis*-Golgi (α-1,6–modified) form of invertase (data not shown). The likeliest interpretation is that these phenotypes, which are not exhibited by another *ypt1* mutation (see below), result from indirect effects of the *ypt1-T40K* mutation on the secretory machinery. These effects may be due to the partial inhibition of secretion that is observed even when the cells are grown at the permissive temperature (Segev et al., 1988). However, it is possible that some of these additional phenotypes are specific to effector domain mutations.

In summary, this study illustrates two reasons why previous in vivo and in vitro analyses of *ypt1-T40K* and other *ypt1* mutant stains (see Introduction) are not sufficient for establishing a role for Ypt1p in intra-Golgi transport. First, the nature of the defect can not be determined only by examining changes in the electrophoretic mobility of secretory proteins. Second, all the mutations used to date fail to exhibit a rapid and tight secretory block that occurs when the cells are shifted to the restrictive conditions. Therefore, indirect effects on secretory compartments upstream or downstream of the Ypt1p-dependent steps cannot be ruled out. To address this question more directly we sought to identify other *ypt1* mutations that do not have a secretory defect when grown under permissive conditions and that exhibit a tighter and more rapid secretory block when shifted to nonpermissive conditions.

The ypt1-A136D Mutation Confers a Rapid and Tight Block of ER to cis-Golgi and cis- to medial-Golgi Transport Steps

ypt1-A136D is a recessive mutation that confers a very tight block of growth at 37°C without any defect at the permissive temperature of 26°C (Fig. 2). We used pulse–chase experiments to compare the processing at 26°C of the oligosaccharide side chains of two marker proteins in mutant cells versus wild-type cells (Fig. 3). During the pulse, a brief delay in processing is apparent in mutant cells compared to wild-type cells, as seen by the appearance of somewhat greater amounts of the immature forms of invertase (core-glycosylated, Fig. 3, *bottom*) and α-factor (core and α1-6; see Fig. 5, chase time 0). In addition, invertase isolated from mutant cells migrates faster (underglycosylated) than that isolated from wild-type cells. However, both markers are processed efficiently during the chase, despite a slight slowing in the movement of invertase through the secretory pathway (Fig. 3, *bottom*, chase time 10 min). Thus, *ypt1-A136D* mutant cells exhibit a very minor secretion defect at the permissive temperature. The *ypt1-A136D* mutant was tested for the rapid onset of a secretory defect by labeling the cells immediately after a shift to 37°C and determining the processing of invertase using immunoprecipitation. Under these conditions, mutant cells accumulate only the ER form of invertase (Fig. 4), reflecting a tight block of the first transport step at which Ypt1p is essential: ER to *cis*-Golgi. The secretory block exhibited by the *ypt1-A136D* mutant cells after the shift to the restrictive temperature is immediate and tight. This property makes the *ypt1-A136D* mutant suitable for physiological studies.

By exploiting the ability to rapidly inactivate Ypt1p, the different secretory compartments were marked with radiolabeled transport intermediates of the marker proteins using a brief pulse under permissive conditions followed by a shift to the nonpermissive temperature. This procedure is necessary for testing whether there are blocks in steps of the secretory pathway downstream of the ER to *cis*-Golgi step. In addition, this procedure minimizes the indirect effects on the secretory compartments themselves (Graham and Emr, 1991). We followed the transport of three different glycoproteins: the secreted proteins α-factor and invertase and the vacuolar protein CPY. Wild-type and mutant cells were labeled at the permissive temperature (26°C) for 7 min, and then shifted to the nonpermissive temperature (37°C) and chased for 10 or 30 min. α-factor was immunoprecipitated from the cells, and then it was subjected to a second immunoprecipitation using antibodies specific for α-1,6 or α-1,3 mannose. In wild-type and *ypt1-A136D* mutant cells the various secretory compartments were loaded with different forms of α-factor during the pulse, as seen by the presence of the core, α-1,6–modified, α-1,3–modified, and mature forms (Fig. 5, chase time 0). In wild-type cells, even after a short chase, all of these forms were converted to the mature form. *ypt1-A136D* mutant cells when shifted to 37°C exhibited a nearly complete block in the chase of both the ER (core) and the *cis*-Golgi (α-1,6) forms of α-factor. In contrast, the *medial*-Golgi (α-1,3) form, which is cleaved in the *trans*-Golgi to produce mature α-factor peptide, disappears (Fig. 5). Thus, *ypt1-A136D* mutant cells exhibit a block in the first two steps of α-factor transport, ER to *cis*-Golgi and *cis*- to *medial*-Golgi. The medial (α-1,3) and trans (Kex2) Golgi compartments appear to function normally in these mutant cells for the processing of α-factor.

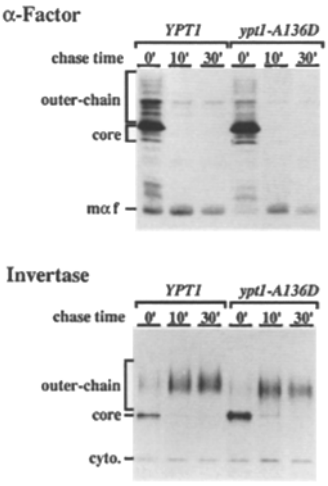

Figure 3. Processing of α-factor and invertase by *ypt1-A136D* mutant cells at the permissive temperature. Wild-type and mutant cells were grown at 26°C and labeled for 8 min. The label was chased for the indicated times. After cell lysis α-factor (*top*), and invertase (*bottom*) were immunoprecipitated and analyzed by gel electrophoresis. Positions of cytoplasmic (for invertase), ER (core), Golgi (outer-chain), and mature (for α-factor) forms are noted in the left margin.

Invertase processing was analyzed in *ypt1-A136D* mutant cells to confirm the secretory defect described above using a different marker and to examine the last transport step, between the *trans*-Golgi and the cell surface, which was not visualized by examining α-factor. We can distinguish between invertase that resides in the later Golgi compartments (*medial* and *trans*) and secreted invertase by determining the levels of intracellular and external invertase. Wild-type and mutant cells were labeled at the permissive temperature (26°C) for 7 min, and then shifted to the nonpermissive temperature (37°C) and chased for

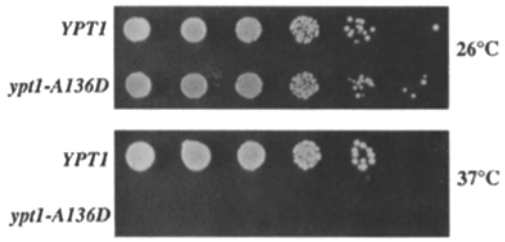

Figure 2. The *ypt1-A136D* mutant exhibits a tight block in cell growth at 37°C. Wild-type (NSY160) and *ypt1A136D* (NSY161) mutant cells (10-fold serial dilutions from left to right) were grown on YPD plates at 26 and 37°C.

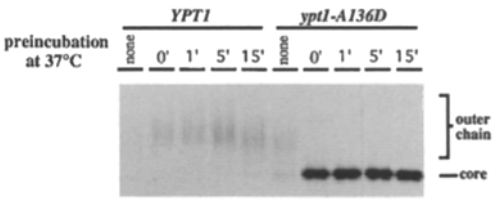

Figure 4. Rapid and tight block in invertase secretion in *ypt1-A136D* cells at 37°C. Wild-type (*left*) and *ypt1-A136D* mutant (*right*) cells were grown at 26°C. Cells were shifted to 37°C for the indicated times and labeled for 30 min. Invertase processing was analyzed as in Fig. 3.

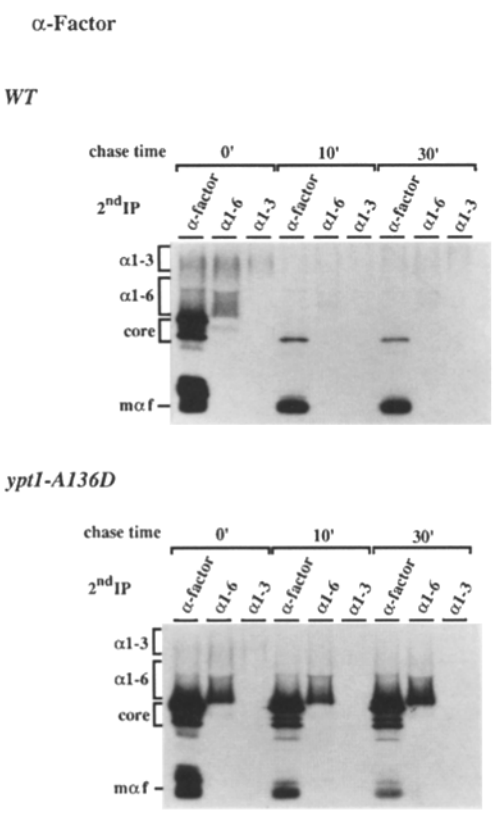

Figure 5. The *ypt1-A136D* mutant exhibits a tight block of the first two transport steps of α-factor. Wild-type (*top*) and *ypt1-A136D* mutant (*bottom*) cells were labeled for 7 min at 26°C. Cells were shifted to 37°C and chased for the times indicated. The first immunoprecipitation was done with anti–α-factor antibodies. The second immunoprecipitation, with antisera to α-factor, α-1,6–mannose linkages or α-1,3–mannose linkages, is shown in the figure. Positions of ER (core), *cis*-Golgi (α-1,6), *medial*-Golgi (α-1,3), and mature α-factor forms are noted in the left margin.

10 or 30 min. Spheroplasts were separated from periplasm and medium, invertase was immunoprecipitated, and then it was subjected to a second immunoprecipitation using antibodies specific for α-1,6 or α-1,3 mannose. In wild-type and *ypt1-A136D* mutant cells the various secretory compartments were loaded with different forms of invertase during the pulse, as seen by the presence of the core, α-1,6– and α-1,3–modified forms (Fig. 6, chase time 0). In both wild-type and mutant strains a small amount of invertase was secreted during the pulse. All of the secreted invertase was fully glycosylated, as seen by the equally efficient immunoprecipitation by all of the antibodies. After a 10 min chase of wild-type cells, there was no internal invertase, and all the forms that were present during the pulse were converted to the mature, α-1,3–glycosylated form and secreted. *ypt1-A136D* mutant cells shifted to 37°C exhibited an essentially complete block in the chase of the ER (core) and *cis*-Golgi (α-1,6) forms of invertase even after a 30-min chase. In contrast, the level of the *medial*-Golgi form (internal α-1,3) was substantially reduced, and most of this species was secreted (external α-1,3 form; Fig. 6). This analysis confirms that there is a primary block in the secretion of invertase in the first two steps of the pathway, but not downstream of the *medial*-Golgi.

Transport to the vacuole was analyzed by following the processing of CPY (Fig. 7). The purpose of this experiment was to determine whether there was a defect in CPY processing beyond the two early steps of the secretory pathway, en route to the vacuole. We could not distinguish between the ER and cis-Golgi forms of CPY because they both migrate as the p1 form (CPY receives only limited mannose extension). The secretory compartments were loaded at the permissive temperature with the labeled forms of CPY (ER, Golgi, and vacuolar), and then shifted to 37°C for the chase. In wild-type cells all of the CPY was converted to the mature form after 30 min of chase. In *ypt1-A136D* mutant cells that were chased at the nonpermissive temperature, the ER and *cis*-Golgi (p1) forms were not transported while the *medial*-Golgi (p2) form was converted to the mature vacuolar form. A block in the *medial*- to *trans*-Golgi step can be resolved in *sec18* mutant cells, in which some of the p2 form is not chased (Fig. 7, and Graham and Emr, 1991). Thus, transport from the *medial*-Golgi compartment through the *trans*-Golgi to the vacuole is not blocked in *ypt1-A136D* mutant cells. Taken together with the α-factor and invertase data, these results clearly indicate that the two first steps of the secretory pathway are blocked in *ypt1-A136D* mutant cells while subsequent steps occur normally.

Discussion

In this study we show that the *ypt1-A136D* mutation efficiently blocks the first two steps of the yeast secretory pathway and has little or no effect on later steps. This conclusion is supported by results with the dominant mutation *ypt1-N121I*, which also confers a block, although partial, in the same two steps (G. Jedd and N. Segev, unpublished results). These two mutations might both interfere with Ypt1p function in a similar way, namely its ability to cycle from the GDP- to the GTP-bound form. Thus, the analogous mutation to *ypt1-A136D* in *SEC4* has been suggested to encode a protein that is defective in the interaction with its nucleotide exchange factor (Moya et al., 1993). Similarly, Ypt1-N121I protein exerts its dominance by interfering with nucleotide exchange of wild-type Ypt1p (Jones et al., 1995). The fact that different conditions are used for the expression of these two mutant phenotypes (temperature shift for *ypt1-A136D*, and galactose induction for *ypt1-N121I*) further supports the conclusion that these two steps of the secretory pathway are primary execution points of the Ypt1p. A third mutation, *ypt1-T40K*, confers additional secretion and glycosylation defects in later steps of the secretory pathway. However, this mutation confers a partial secretory block even at the permissive temperature, and therefore it is likely that these additional phenotypes result from indirect effects on the constituents or the organization of later secretory compartments. Thus, the simplest interpretation of the collected data is that Ypt1 GTPase is required only for the first two steps of the secretory pathway.

Previous in vivo studies have suggested the possibility that an individual rab protein might function in more than one step of the secretory pathway. However, none of these experiments provided conclusive evidence that a given rab protein is needed for more than one step. Analyses of *ypt1*

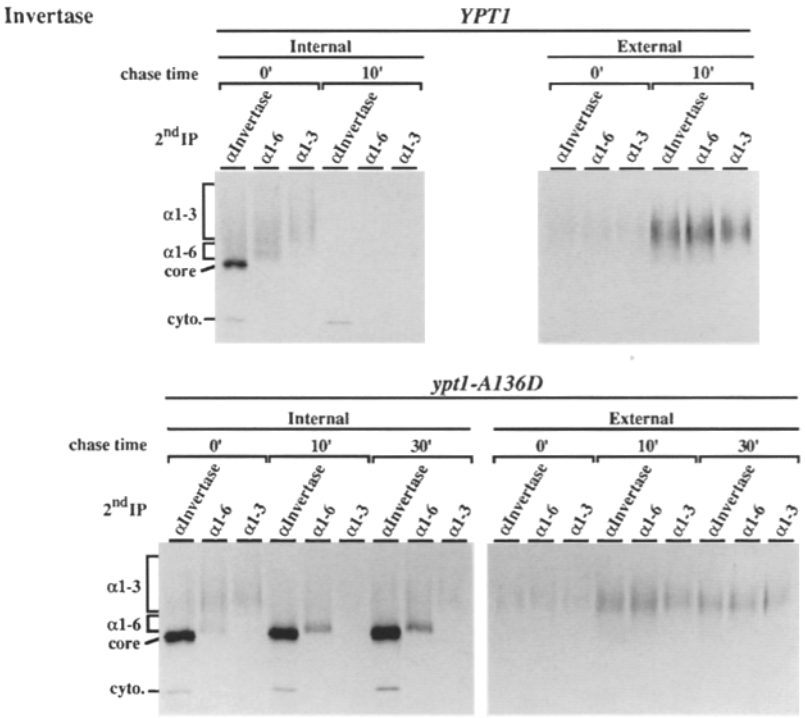

Figure 6. The *ypt1-A136D* mutant exhibits a tight block of the first two transport steps of invertase. Wild-type (*top*) and *ypt1-A136D* mutant (*bottom*) cells were labeled for 7 min at 26°C. Cells were shifted to 37°C and chased for the times indicated. Internal (within spheroplasts) invertase was separated from external (in periplasm and media) invertase. The first immunoprecipitation was done with anti-invertase antibodies. The second immunoprecipitation, with antisera to invertase, α-1,6–mannose linkages and α-1,3–mannose linkages, is shown in the figure. Positions of cytoplasmic, ER (core), *cis*-Golgi (α-1,6), and *medial*-Golgi (α-1,3) forms are noted in the left margin.

mutants suggested an intra-Golgi defect in addition to the ER-to-Golgi block (Ferro-Novick and Novick, 1993). However, the mutations used in those studies display defects in growth and secretion under permissive conditions and/or exhibit slow and leaky secretion blocks under nonpermissive conditions (see Results). Studies using the rab1a-S25N dominant mutation in mammalian cells revealed a clear block in ER to Golgi transport and a possible intra-Golgi transport block (Nuoffer et al., 1994). Because this rab1a dominant mutation triggers disassembly of the Golgi complex at times earlier than those used for showing the transport blocks, it is difficult to draw firm conclusions about an intra-Golgi role for this rab protein (Wilson et al., 1994). In a mammalian cell-free system, anti rab1b-antibodies block ER to Golgi transport and partially block intra-Golgi transport (Plutner et al., 1991). However, the block in the second step is only partial. Thus, the present study is the first to show clearly that a single rab GTPase is necessary for two steps of the secretory pathway.

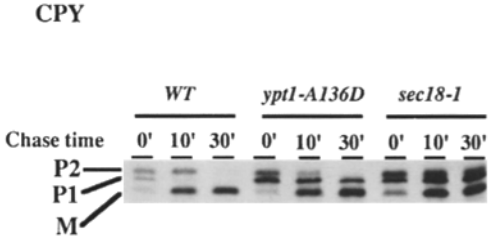

Figure 7. CPY transport in ypt1-A136D mutant cells. Wild-type, *ypt1-A136D,* and *sec18* mutant cells were labeled for 15 min at 26°C, shifted to 37°C and chased for the times indicated. Immunoprecipitation was done with anti-CPY antibodies. Positions of the ER (*p1*), Golgi (*p2*), and vacuolar (*m*) forms are indicated in the left margin. Immunoprecipitation and gel electrophoresis were as described for Fig. 3.

The novel mutation that we describe in this paper, *ypt1-A136D*, confers a very tight and rapid secretory block when the cells are switched to the non-permissive temperature, but shows no effect on growth and a minimal effect on secretion at the permissive temperature. The Ypt1-A136D protein is present in mutant cells in lower amounts than wild-type Ypt1p, and its quantity does not change significantly even after one hour of incubation at the restrictive temperature (data not shown). Thus, this mutation probably encodes a protein that undergoes a temperature-sensitive change in function rather than in abundance. Such a mutation is ideal for physiological experiments, since it is possible to study the effect of the mutation immediately after the shift to the restrictive condition, avoiding any indirect effects on the secretory compartments themselves. The A136D mutation lies in a region of Ypt1p that is conserved in all rab proteins. The analogous mutation in Sec4p, G147D, also confers a tight and rapid block in secretion (G. Jedd and N. Segev, unpublished results). Another advantage of the *ypt1-A136D* and *sec4-G147D* mutations is that they are recessive. The use of dominant-interfering mutations for determining a role of a specific rab protein in vivo (Nuoffer et al., 1994) is complicated by the possibility that the dominant mutant proteins might sequester factors needed for other rab proteins. Thus, introducing mutations analogous to *ypt1-A136D* might be a useful tool for determining the functions of other Ypt/rab proteins in yeast.

Another gene, *SEC23*, has been shown to function in the same two steps of the yeast secretory pathway as *YPT1* (Graham and Emr, 1991). *SEC23* encodes a GTPase activating protein (GAP) that stimulates GTP hydrolysis by Sar1p, a GTPase belonging to another sub-family that functions in the formation of vesicles (Yoshihisa et al., 1993). It is therefore possible that the first two steps of the secretory pathway are regulated by the same pair of GTPases,

Sar1p for vesicle budding and Ypt1p for vesicle targeting. To date, no protein has been shown to function in one of these two steps and not in the other. Thus, the specificity determinants that distinguish the first two steps of the secretory pathway have yet to be identified; possible candidates are the SNAREs (Rothman, 1994; Banfield et al., 1995).

Recently it has been suggested that the enzymes that catalyze the initiation and the elongation of α-1,6–mannose chains reside in two different Golgi compartments. This suggestion is based on the observation that a marker protein that recycles from the *cis*-Golgi to the ER contains α-1,6–modifications, but lacks elongated α-1,6–mannose residues (Gaynor et al., 1994). Cell fractionation experiments are needed to test this idea. If it is correct, our results are consistent with a requirement for Ypt1p function for transport from the ER through two *cis*-Golgi compartments (α-1,6–initiation and α-1,6–elongation) to the *medial*-Golgi, since elongated α-1,6–modified forms of both α-factor and invertase accumulate in the *ypt1-A136D* mutant cells after a shift to the nonpermissive temperature (Figs. 5 and 6). In other words, Ypt1p may be required for the first three transport steps of the secretory pathway.

The present study sheds new light on the mechanism of action of the rab GTPases. In mammalian cells, different rab proteins have been localized to distinct intracellular compartments, and certain rab proteins are known to be restricted to differentiated cell types. However, as mentioned above, there is in vitro evidence in mammalian cells consistent with a role for a single rab protein at two steps in the secretory pathway (Plutner et al., 1991). Our data provide further support for the idea that a given rab protein can act at more than one vesicular transport step, and they argue against the possibility that rab proteins serve as specificity determinants for vesicular targeting, as suggested by some current models (Pfeffer, 1992; Ferro-Novick and Novick, 1993; Zerial and Stenmark, 1993). In these models a given rab protein is proposed to interact with a specific v-SNARE, to promote its interaction with the cognate t-SNARE in the target membrane. We suggest that rab proteins might either promote an efficient interaction of specificity determinants, presumably SNARE proteins (Sogaard et al., 1994), or else serve in a proofreading mechanism for SNARE interactions without themselves being part of the specificity determining system, in a role analogous to that of the elongation factor Tu (EF-Tu) GTPase in protein translation (Bourne, 1988; Baker et al., 1990). Alternatively, rab proteins might have a function in defining the identity of individual membrane-bound compartments. This hypothesis would account for the involvement of Ypt1p in interactions of the *cis*-Golgi with neighboring compartments of the secretory pathway. Thus, even if Ypt1p had a single site of action within the *cis*-Golgi, it would still be required for two distinct transport steps, since its loss would affect both fusion with, and budding from the *cis*-Golgi compartment.

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