C-Terminal Truncation of α-COP Affects Functioning of Secretory Organelles and Calcium Homeostasis in *Hansenula polymorpha*

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In eukaryotic cells, COPI vesicles retrieve resident proteins to the endoplasmic reticulum and mediate intra-Golgi transport. Here, we studied the *Hansenula polymorpha* homologue of the *Saccharomyces cerevisiae* *RET1* gene, encoding α-COP, a subunit of the COPI protein complex. *H. polymorpha* *ret1* mutants, which expressed truncated α-COP lacking more than 300 C-terminal amino acids, manifested an enhanced ability to secrete human urokinase-type plasminogen activator (uPA) and an inability to grow with a shortage of Ca\(^{2+}\) ions, whereas a lack of α-COP expression was lethal. The α-COP defect also caused alteration of intracellular traffic of the glycosylphosphatidylinositol-anchored protein Gas1p, secretion of abnormal uPA forms, and reductions in the levels of Pmr1p, a Golgi Ca\(^{2+}\)-ATPase. Overexpression of Pmr1p suppressed some *ret1* mutant phenotypes, namely, Ca\(^{2+}\) dependence and enhanced uPA secretion. The role of COPI-dependent vesicular transport in cellular Ca\(^{2+}\) homeostasis is discussed.

In eukaryotic cells, secretory proteins synthesized at the endoplasmic reticulum (ER) pass through multiple distinct membrane-bound organelles comprising the secretory pathway. Transport of proteins and lipids between membrane compartments of the early secretory system is mediated by COPI- and COPII-coated vesicles that capture cargo, bud from the donor membrane, and then target, dock, and fuse with an appropriate acceptor compartment (40). Both the COPI and COPII protein coat complexes employ a GTP switch mechanism for coating and uncoating. The COPII complex mediates selective protein export from the ER, while COPI-coated vesicles retrieve resident proteins to the ER and mediate intra-Golgi transport (8, 38).

The Golgi apparatus is composed of distinct cisternal regions, namely, the cis, medial, trans-Golgi, and trans-Golgi-network regions (13). Secretory cargo proteins passing through the Golgi apparatus are modified by the unique sets of resident enzymes of different subcompartments. Two alternative models for the origin of the Golgi subcompartments suggest different roles for COPI vesicles in secretory cargo transport and the function of the Golgi apparatus. In the stable-compartments model, anterograde COPI vesicles carry secretory cargo forward and exclude resident Golgi proteins. The cisternal-mutation model, entire cisternae carry secretory cargo forward, and retrograde COPI vesicles recycle resident Golgi proteins to younger cisternae. Thus, the distinction between the two models centers on the contents of COPI vesicles and the directionality of their transport. However, recent experimental data indicate that intra-Golgi COPI traffic has a bidirectional character (for reviews, see references 23 and 38). Moreover, the dynamics of cisternal progression and vesicular traffic are different in higher eukaryotes versus yeasts. Progression of the tightly stacked Golgi cisternae of animal cells occurs at a significantly lower rate than the traffic of most cargoes through the secretory pathway. COPI vesicles mediating cargo transport in the anterograde direction provide “fast-track” secretion. In contrast to animal cells, cisternal maturation provides a fast track for anterograde transport in budding yeast (for a review, see reference 38).

The COPI coat consists of the small ras-like GTPase, ARF, and coatomer, a protein complex of seven subunits: α, β, β′, γ, δ, ε, and ζ. Genetic and biochemical evidence shows that COPI in the yeast *Saccharomyces cerevisiae* and mammalian COPI have very similar fractionation properties and subunit compositions. The genes for yeast COPI proteins were identified in screening for mutants with defects in the secretion of proteins (sec mutants) or in protein retrieval from the Golgi apparatus to the ER (ret mutants). Each of the COPI genes is essential for viability, except for SEC28, encoding ε-COP, which functions as a structural component of coatomer, stabilizing α-COP and thus the whole coatomer at elevated temperatures (16). Yeast ARF is encoded by at least three genes, two of which, *ARF1* and *ARF2*, provide essential, overlapping functions in secretory traffic (20).

Based on the sequence analysis, three tentative domains have been identified in α-COP. The highly conserved N-terminal domain contains six WD40 repeats (33). Mutations in this domain (*ret1-1*, *ret1-2*, *ret1-4*, and *ret1-5*) cause a strong defect in the ER retrieval of dilysine-tagged proteins but no anterograde transport defect, except for the *ret1-1* mutation, which specifically disrupts the transport of glycosylphosphatidylinositol (GPI)-anchored proteins to the Golgi apparatus (33, 46). This domain is also homologous to the N-terminal WD domain of β′-COP. The middle regions of these proteins also show similarity, while their C-terminal domains (~400 C-terminal residues in the case of α-COP) are unique (18). Point mutation in the C-terminal part of α-COP in the *ret1-3* mutant leads to structural alterations of coatomer, α-COP...
degradation, and defects in forward transport at restrictive temperatures (16, 18).

Traffic through the secretory organelles obliges cargo to be properly sorted and modified, and the essential role in the quality control of newly synthesized proteins belongs to the ER quality control (ERQC) system. ERQC is a complex sorting system that separates proteins according to their folding and maturation status. Properly folded proteins rapidly move via the ER exit sites and intermediate compartment to the cis-Golgi compartment and beyond, while misfolded or unassembled proteins either aggregate or degrade. In most cases, ER-associated degradation (ERAD) involves translocation of misfolded proteins to the cytosol, ubiquitination, and degradation by proteasomes (14). Selective retrieval of misfolded proteins from the intermediate compartment or the cis-Golgi compartment by retrograde transport to the ER has also been reported (25). It is known that both ERQC and ERAD depend on the concentration of Ca$^{2+}$ in the ER (22, 35, 50), though in yeast the mechanism of sequestration of Ca$^{2+}$ into the ER remains to be studied.

Although most of the genetic data on the secretion process accumulated so far came from studies of the baker’s yeast S. cerevisiae, full elaboration of the structure and functioning of the eukaryotic secretory organelles requires their comparative analyses in different organisms. Such analyses have been restrained by the lack of model organisms suitable for genetic studies. Development of the genetic system of the methylotrophic yeast Hansenula polymorpha, which was primarily highlighted as an efficient host for the production of heterologous proteins, made this organism convenient for basic research (for a review, see reference 21). This stimulated its use in studies of the secretion pathway (3, 4, 29, 30). In the present work we isolated and characterized the H. polymorpha homologue of the S. cerevisiae RET1 gene, encoding α-COP, a subunit of the COPI protein complex. Study of the H. polymorpha ret1 mutants revealed a relationship between COPI vesicular transport and Ca$^{2+}$ homeostasis in yeast.

**MATERIALS AND METHODS**

**Plasmids, yeast strains, and genetic techniques.** Plasmids used in this study are listed in Table 1. To distinguish the S. cerevisiae and H. polymorpha genes and proteins, they are designated when necessary by Sc or Hp, respectively. Plasmid p27OPU8 was recovered from the H. polymorpha CBS4732 genomic library by complementation of the ret1-27 mutation. To construct the H. polymorpha genomic library, the 5- to 10-kb fraction of DNA fragments obtained by partial digestion of H. polymorpha chromosomal DNA with SacI was ligated with the SauI-digested AMipSL1 vector (5). Two base pairs of the cohesive DNA ends was preliminarily filled in with Klenow fragment. Transformation of Escherichia coli with the ligation mix gave rise to more than 2 × 10$^6$ colonies. Library DNA was prepared from a pool of E. coli transformants and used for H. polymorpha transformation (3). Plasmid p27OPU8 was produced by insertion of the 4.4-kb Kpn1-Mlu fragment (the HpRET1 gene lacking the 5’ part of the open reading frame [ORF]) of the p27OPU8 plasmid into the MluI-HindIII sites of the AMipSL1 shuttle vector. To allow ligation, the Kpn1 and HindIII overhangs were removed by the Klenow enzyme. The pAM409 plasmid was obtained as diagrammed in Fig. 1 and described in the legend. The pCOP1BE plasmid was constructed by insertion of the 1.9-kb BglII-EcI136II fragment of the HpRET1 gene from p27OPU8 into the BamHI-EcI136II sites of the pJ1282 plasmid. To construct the pMCOP1-L plasmid, the 5’ part of the HpRET1 gene (the 882-bp Expl-BglII fragment of the p27OPU8 plasmid) was fused with the MOX promoter (the 0.9-kb SnuI-ScaI fragment of pMMU [5]) and inserted together with the ScLEU2 selectable marker (the 1.6-kb BsrGI-ScaI fragment of pJ282) into the SnuI-ScaI sites of the pUK21 plasmid (49). The pH5 plasmid was constructed by insertion of the 1.3-kb NotI-EcoRV fragment of pJCHLX (45) into the NotI and Smal sites of the pE1 plasmid (29).

Derivatives of the H. polymorpha strains CBS4732 (ATCC 34438) and DL-1 (ATCC 26012) used in this study are listed in Table 2. The H. polymorpha ret1-27 mutant strain 2dMA56 was obtained as described in Results. Strain 2d-C was generated by integration of the pl27KM plasmid, possessing a portion of the HpRET1 gene, into the ret2-7 mutant locus of strain 2dMA56, a procedure that restored the wild-type RET1 gene. Strain 2d-L was obtained by integration of the empty vector AMipL1 into strain 2dMA56. Strains 2d-P2 and 2d-P10, possessing 1 and ca. 10 extra copies, respectively, of the PMR1 gene, integrated into unidentified loci, were obtained by transformation of strain 2dMA56 with the HindIII-digested plasmid pH5. Corresponding transformants were studied by Southern blot analysis, and the copy numbers of the integrated PMR1 gene were determined by comparing the intensity of PMR1-specific bands in dilution series.

Modifications of the RET1 locus by integration of a plasmid sequence via homologous recombination were carried out in two derivatives of the DL-1 strain, DLU and DL-LA. These strains were used due to the higher efficiency of homologous recombination compared to that in strains derived from CBS4732 (2). To introduce the ret1-27 mutation into the RET1 locus of strain DLU, plasmid pAM409 carrying the 5’-end-truncated portion of the ret1-27 mutant allele was digested with Eco47III (see Fig. 1) and transformed into this strain. Homologous recombination with chromosomal RET1 should produce two copies of this gene, one of which is inactive, lacking the promoter and 5’-end region of the RET1 coding sequence, and one of which carries the ret1-27 mutation. Such strains must express the ret1-27 phenotypes. In fact, some of the transformants obtained had mutant phenotypes, namely, slow growth on yeast extract-peptone-dextrose YEED, high halo-forming ability on fibrin-containing medium, and sensitivity to EGTA. To obtain strain DLU-C with the 5’-terminal deletion of RET1 (ret1-DC), DLU was transformed with the pCOP1BE plasmid carrying the BglII-EcI136II DNA fragment, corresponding to the internal part of the RET1 ORF (Fig. 1). Prior to transformation, this plasmid was digested with Eco47III in this fragment to stimulate homologous recombination with the chromosomal gene via single crossover. Such recombination should generate two truncated copies of the RET1 gene. One of these must not be expressed, due to the

| Table 1. Plasmids used in this study |

| Plasmid | Characteristics | Yeast marker | Source or reference |
|---------|----------------|--------------|---------------------|
| AMipL1  | H. polymorpha shuttle vector carrying HARS36 | HpLEU2 | This study |
| p27OPU8 | H. polymorpha shuttle vector carrying HARS36 and a fragment of chromosomal DNA with the HpRET1 wild-type allele | ScLEU2 | This study |
| pL27KM  | Plasmid harboring 5’-end-truncated HpRET1 | HpLEU2 | This study |
| pAM409  | Plasmid carrying the 5’-end-truncated H. polymorpha ret1-27 allele | HpLEU2 | This study |
| pCOP1BE | Plasmid carrying the fragment of chromosomal DNA corresponding to the internal part of the HpRET1 ORF | ScLEU2 | This study |
| pMCOP1-L | Plasmid carrying the 5’-end fragment of HpRET1 under the control of the MOX promoter | ScLEU2 | This study |
| pH5     | Plasmid carrying HpPMRI | HpLEU2 | This study |
| pJJ282  | S. cerevisiae shuttle vector | ScLEU2 | 28 |
absence of the promoter and 5′-end region of the RET1 ORF, whereas expression of the other would result in a protein lacking the last 305 amino acids. Several transformants were selected, and Southern blot analysis has shown that they had disruptions of RET1 by the anticipated plasmid integration (data not shown). To obtain strain DL-LA/MC with methanol-regulatable expression of COP (inducible by methanol and repressible by glucose) (24), strain DL1-LA was transformed with plasmid pMCOP1-L carrying the 3′ deletion allele of RET1 under the control of the MOX promoter. Prior to transformation, the plasmid was digested with HindIII inside the RET1 sequence. Integration of the plasmid into the chromosomal RET1 gene via single crossover generates the RET1 gene fused to the MOX promoter and the 3′-truncated copy of RET1, encoding a protein which lacks 901 C-terminal amino acids. The integration event was confirmed by PCR for some of these transformants (data not shown).

H. polymorpha cells were grown at 37°C in the complex medium YEPD or FIG. 1. Strategy of cloning of the genomic DNA fragment possessing the ret1-27 mutation. Strain 2dMA6 was transformed with plasmid p27KM carrying the 5′-end-truncated H. polymorpha RET1 gene. Prior to transformation, the plasmid was digested with Eco47III inside the RET1 ORF. Some of the transformants, which grew faster than others, manifested the wild-type phenotype, i.e., ability to grow in the presence of EGTA and inability to overproduce uPA. Since the plasmid used for transformation possessed only a portion of RET1, the wild-type phenotype could arise only by recombination of the plasmid with the chromosomal mutant allele. This localized the mutation downstream of the Eco47III site. Genomic DNA from one such integrant was isolated and digested with BamHI, which produced a fragment containing the vector sequence with a portion of the mutant allele. Self-ligation of this fragment resulted in the circular plasmid pAM409, which was then recovered by E. coli transformation. Restriction sites: Es, EspI; K, KpnI; B, BglII; E, Eco47III; Ec, Ecl136II; Ba, BamHI. Open bars, the chromosomal RET1 gene; hatched bars, plasmid copy of the RET1 gene; filled bars, the plasmid amp′ and HpLEU2 genes. Asterisk indicates position of the ret1-27 mutation.

TABLE 2. H. polymorpha strains used in this study

| Strain   | Genotype*                        | Original strain | Source or reference |
|----------|----------------------------------|-----------------|---------------------|
| 8V       | leu2                             | CBS4732         | 1                   |
| 27-8V    | leu2 ret1-27                     | CBS4732         | 3                   |
| 1B/SM/L  | max::uPA leu2 ade2 [HpLEU2]      | CBS4732         | 3                   |
| 2dMA6    | max::uPA leu2 ret1-27            | CBS4732         | This study          |
| 2d-L     | max::uPA leu2 ret1-27 [HpLEU2]   | CBS4732         | This study          |
| 2d-C     | max::uPA leu2 ret1-27 [HpLEU2 HpRET1] | CBS4732     | This study          |
| 2d-P1    | max::uPA leu2 ret1-27 [HpLEU2 HpPMR1] | CBS4732     | This study          |
| 2d-P10   | max::uPA leu2 ret1-27 ×10×[HpLEU2 HpPMR1] | CBS4732     | This study          |
| DLU      | max::uPA leu2                    | DL-1            | 4                   |
| DLU-L    | max::uPA leu2 [ScLEU2]           | DL-1            | This study          |
| DLU-C/A  | max::uPA leu2 ret1 [ScLEU2]      | DL-1            | This study          |
| DL1-LA   | leu2 ade2 ret1 [ScLEU2 MOX:RET1] | DL-1            | 5                   |
| DL1-LA/MC| leu2 ade2 ret1 [ScLEU2 MOX:RET1] | DL-1            | This study          |

* Square brackets indicate plasmid genes integrated into the genome. [HpLEU2 HpPMR1] and ×10×[HpLEU2 HpPMR1], genes of the pH5 plasmid integrated in 1 and approximately 10 copies, respectively. max::uPA, the uPA expression cassette inserted into the chromosomal MOX locus; MOX:RET1, the HpRET1 gene fused to the MOX promoter. For details, see Materials and Methods.
YEPG (1% yeast extract, 2% peptone, and 2% glucose or glycerol, respectively) or in one of the synthetic complete media SC-D, SC-M, and SC-G (0.67% yeast nitrogen base without amino acids, supplemented with 2% glucose, methanol, or glycerol, respectively). The EGTA-containing medium was prepared as described elsewhere (41). For induction of uronidase-elsewhere nitrogen activator (uPA) expression, overnight cultures grown in liquid YEPD containing 0.3 M NaCl were sixfold diluted with induction medium [IM, comprising 1% yeast extract, 3% peptone, 25 mM NH₄HPO₄, 25 mM (NH₄)₂HPO₄, 0.1 M NaCl, 0.3% glycerol, and 1% methanol] and incubated with vigorous agitation for 70 h. Strains of H. polymorpha were crossed, and hybrids were sporulated on a maltose-containing medium. Procedures for dissecting the asci of H. polymorpha hybrids have been described elsewhere (10). H. polymorpha was transformed according to the modified lithium acetate method (10). The S. cerevisiae strain RSY372 (MATa ura3-52 leu2-3,112 sec18-1) was grown at 27°C in YEPD.

uPA activity assay and zymography. A qualitative test for the ability of yeast transformants to secrete uPA was performed by examination of their capacity to create halos during growth on fibrin-containing medium as described previously (7). Fibrinolytic activity was measured by using a fibrin plate assay (7). The electrophoretic mobility of nondenatured uPA was studied as follows. Synthesis of uPA was induced as described above, aliquots of the culture medium were treated with endo-β-N-acetylgalactosaminidase H (endo H) and mixed with sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 0.5% sodium dodecyl sulfate [SDS], 0.002% bromphenol blue), and proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (32). After electrophoresis, the gels were washed with 1% Triton X-100, transferred onto fibrin plates, and incubated at 37°C for 14 to 22 h. The amounts of uPA in culture medium were normalized to total cellular protein (26).

Immunoblotting of Gas1p, Pmr1p, and uPA. To study Gas1p maturation, the overnight YEPD culture of H. polymorpha was diluted in fresh medium and grown to an optical density of 0.8 at 600 nm. The S. cerevisiae strain for 10 min and lysates were centrifuged again at 10,000 ×g for 1 min. Aliquots of lysates were mixed with sample buffer (30 mM Tris-HCl [pH 7.5]) and 150 mM NaCl). Cell lysates were prepared by vortexing with glass beads in TBS buffer containing 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and a protein inhibitor cocktail (Complete; Boehringer Mannheim). Glass beads and cell debris were removed by centrifugation at 8,000 ×g for 1 min. Aliquots of lysates were mixed with sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.002% bromphenol blue) and boiled, and proteins were separated by SDS–8% PAGE followed by immunoblotting with the anti-Gas1p antibody. Protein concentrations in cell lysates were determined by the method of Bradford (12).

Analysis of uPA aggregation and comparison of uPA contents in culture supernatants of different strains have been described previously (4). To study uPA aggregation, cell debris was removed from lysates by centrifugation at 300 ×g for 10 min and lysates were centrifuged again at 10,000 ×g for 10 min. Pellets were dissolved in a fivefold volume of the starting lysate. The pellet and supernatant fractions obtained were analyzed by Western blotting. Pmr1p levels among spores of nine tetrads isolated, suggesting that these phenotypes are attributable to a single mutation.

Cloning and sequencing of the H. polymorpha RET1 gene. The gene corresponding to the uPA supersecretion mutation was isolated by complementation of the inability of the mutant to grow on an EGTA-containing medium. Strain 2dMA56 carrying the uPA supersecretion mutation was transformed with the H. polymorpha genomic library, and transformants were selected on an EGTA-containing medium. One of the EGTA-resistant transformants lost the ability to overproduce extracellular uPA—a result from a single mutation, the autonomous uPA-expression vector pESS1 was lost and the resulting Leu– derivative of this strain was crossed with the Ade– Leu+ strain 1B/SM/L (Table 2). This strain produced uPA due to the uPA expression cassette replacing the MOX gene, which allowed monitoring of the ability to secrete this enzyme among uPA-negative meiotic progeny of the diploid. The resulting diploid was sporulated, and a mox-negative segregant exhibiting poor growth on YEPD, improved uPA secretion, and inability to grow on an EGTA-containing medium was selected and designated 2dMA56. This segregant was then crossed with strain 1B/SM/L, the resulting diploid was sporulated, and tetrads were dissected. All mutant phenotypes segregated 2:2. No recombinants were observed among spores of nine tetrads isolated, suggesting that these phenotypes are attributable to a single mutation.

RESULTS

An H. polymorpha mutant with enhanced ability to produce extracellular uPA. The H. polymorpha mutant studied here was isolated in a screen for mutants that were able to overproduce extracellular uPA (3). The growth of one such mutant, 27-8V, depended on the presence of Ca2+ in the culture medium: this mutant did not grow on synthetic medium prepared without the addition of Ca2+ and supplemented with 25 mM EGTA. Besides, this mutant grew slowly on a rich medium (YEPD). To investigate whether the mutant phenotypes of strain 27-8V—poor growth, dependence on Ca2+, and overproduction of extracellular uPA—resulted from a single mutation, the autonomous uPA-expression vector pESS1 was lost and the resulting Leu– derivative of this strain was crossed with the Ade– Leu+ strain 1B/SM/L (Table 2). This strain produced uPA due to the uPA expression cassette replacing the MOX gene, which allowed monitoring of the ability to secrete this enzyme among uPA-negative meiotic progeny of the diploid. The resulting diploid was sporulated, and a mox-negative segregant exhibiting poor growth on YEPD, improved uPA secretion, and inability to grow on an EGTA-containing medium was selected and designated 2dMA56. This segregant was then crossed with strain 1B/SM/L, the resulting diploid was sporulated, and tetrads were dissected. All mutant phenotypes segregated 2:2. No recombinants were observed among spores of nine tetrads isolated, suggesting that these phenotypes are attributable to a single mutation.

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ing from 61 to 74%. The highest similarity was shown by \textit{S. cerevisiae}/H9251-COP, whose amino acid sequence was 58% identical and 74% similar to that of the deduced \textit{H. polymorpha} protein (Fig. 2). In \textit{S. cerevisiae} this protein is encoded by the \textit{RET1} gene; therefore, we used the same designation for the corresponding gene of \textit{H. polymorpha}.

Like that of \textit{S. cerevisiae}, the sequence of \textit{H. polymorpha}/H9251-COP can be divided into three domains (33). As in \textit{S. cerevisiae}, the N-terminal region of this protein possesses six WD repeats (Fig. 2). In addition, the N-terminal and middle domains (residues 1 to 780) show similarity to the corresponding regions of \textit{S. cerevisiae}/H9252/H11032-COP, while the C-terminal parts of these proteins are unique (data not shown). The N-terminal part of \textit{H. polymorpha}/H9251-COP (residues 1 to 333) and the sequence between residues 487 and 785 in the middle domain appear to be conserved, since they are 78 and 73% homologous, respectively, to the corresponding regions of human/H9251-COP. The homology for the entire protein is 64%.

C-terminal truncation of \(\alpha\)-COP is not lethal but results in mutant phenotypes in \textit{H. polymorpha} strains of different origin. The \textit{H. polymorpha} genomic DNA fragment possessing the uPA supersecretion mutation, designated ret1-27, was isolated as shown in Fig. 1. Sequencing of this DNA fragment revealed a single nucleotide substitution, which led to appearance of the TGA nonsense codon instead of the TGG codon for W181. Recombination of the \textit{RET1} fragment possessing this nucleotide substitution with chromosomal \textit{RET1} (\textit{ret1-27/C}) (see Materials and Methods) generated transformants with phenotype characteristics for strain 2dMA56. This finding proved that this mutation was responsible for the mutant phenotypes. In addition, it showed that the \textit{ret1-27} mutation caused the same phenotypes in \textit{H. polymorpha} strains of different origin.

The \textit{S. cerevisiae} \textit{RET1} gene is essential for life (33), and deletion of 170 C-terminal amino acids of \textit{H. polymorpha} is lethal. However, overexpression of such a C-terminally truncated protein can rescue the \textit{RET1} disruptant (18). The characterization of the mutant \textit{RET1} allele performed here suggested that the life-essential function of \textit{H. polymorpha} \(\alpha\)-COP was not affected by deletion of the last 389 amino acids. However, it was also possible that the nonsense mutation was not lethal due to read-through of the TGA codon by natural tRNA, a phenomenon described in different organisms, including yeast (for a review, see reference 9). The latter possibility, however, was rejected by construction of the strain with the 3' terminal deletion of the \textit{RET1} gene (\textit{ret1-27/C}) (see Materials and Methods). Like the \textit{ret1-27} mutant, the \textit{ret1-27/C} strain was unable to grow on the EGTA-containing medium and manifested the uPA supersecretion phenotype.

The \textit{H. polymorpha} \textit{RET1} gene is essential for life. The data obtained demonstrate that in contrast to that of \textit{S. cerevisiae}, the C-terminal region of \textit{H. polymorpha} \(\alpha\)-COP is dispensable. This raised the question of whether the \textit{H. polymorpha} \textit{RET1} gene is essential for life. To address this question, strain DLI-1A/MC, carrying the \textit{RET1} gene under the control of the MOX
promoter, was constructed by transformation of DL1-LA with plasmid pMCOP1-L (see Materials and Methods). Approximately 70% of transformants, selected on minimal medium containing methanol as a sole carbon source, were not able to grow in the presence of glucose. Several of these integrants were transformed with a plasmid containing the wild-type HpRET1 gene. The transformants thus obtained demonstrated the ability to grow on glucose-containing medium, whereas transformants obtained with a control vector could not grow in the presence of glucose. Taken together, these observations strongly suggested that H. polymorpha α-COP is essential for viability.

A defect in α-COP causes secretion of abnormal forms of human uPA. Our recent studies suggested that uPA is poorly secreted by H. polymorpha cells because the milieu in the yeast ER is unfavorable for its proper folding. Misfolded uPA is retained in the ER, where it aggregates or is eliminated via ERAD (4). Fractionation of cell lysates revealed that the ret1-27 mutation significantly decreased the degree of uPA aggregation (Fig. 3A).

The properties of uPA secreted into the culture medium by the wild-type strain and the ret1-27 mutant were different, as revealed by PAGE in nondenaturing conditions. Prior to electrophoresis, samples were treated with endo H, removing the N-linked oligosaccharide chain, which otherwise could influence the electrophoretic mobility of uPA. uPA secreted by the wild-type cells migrated as two bands. The band with the lower mobility corresponded to the full-length “50-kDa” forms, consisting of single- or two-chain uPA (for a review, see reference 34), while the faster-migrating band appeared due to proteolysis of the N-terminal domains, which were not required for the fibrinolytic activity. In contrast to the wild-type strain, the ret1-27 mutant secreted additional uPA species, revealed as a slowly migrating smear (Fig. 3B). This was not due to incomplete removal of the N-glycoside chain, since a similar smear was also observed for the mutant uPA-Q102 variant, lacking the N-glycosylation site (data not shown). Remarkably, the activities of secreted uPA forms migrating as the 50-kDa band appeared to be similar for the mutant and wild-type strains, though total fibrinolytic activity in the culture medium, as well as the amount of uPA revealed by Western blotting (data not shown), was approximately ninefold higher for the mutant strain. This indicated that the difference in uPA productivity between the ret1-27 and wild-type strains depended substantially on secretion of the abnormal uPA species. We suggested that such abnormal uPA forms corresponded to the improperly folded and probably aggregated uPA, which is normally retained in the ER. The defect of retention of misfolded uPA within the ER of the ret1-27 mutant could be a reason for the decrease in the levels of its intracellular aggregation. It is noteworthy that the zymogram analysis underestimated uPA activity in slowly migrating smears because uPA does not efficiently lyse the fibrin gel if not concentrated in a single spot (our unpublished data), and besides, such abnormal uPA forms may diffuse inefficiently from the polyacrylamide gel to the fibrin gel.

α-COP is involved in the transport of a GPI-anchored protein, Gas1p, in H. polymorpha. It is known that in S. cerevisiae, mutations affecting α-COP can block the transport of GPI-anchored proteins to the Golgi apparatus (46). In this work we have investigated the intracellular transport of the H. polymorpha GPI-anchored protein Gas1p. Transport of this protein can be monitored by studying conversion of the 105-kDa core-glycosylated ER-specific form to the mature 125-kDa form, which is formed only upon arrival in the medial-Golgi compartment (27). Western blot analysis of cell lysates with an antibody against S. cerevisiae Gas1p revealed the appearance of the 105-kDa form of this protein in the ret1-27 mutant 2d-L, whereas in the wild-type strain 2d-C, only the mature 125-kDa form of Gas1p was detected (Fig. 4). These data indicated that the ret1-27 mutation affected delivery of Gas1p to the Golgi apparatus. Blocking vesicular transport in S. cerevisiae resulted in a similar effect: the 105-kDa form of Gas1p appeared in the lysate of the sec18-1 temperature-sensitive mutant after 1 h of incubation at 37°C (Fig. 4). These observations suggested that,
as in S. cerevisiae, in H. polymorpha α-COP is involved in the transport of Gas1p from the ER to the Golgi apparatus.

**Interplay between α-COP and Pmr1p in H. polymorpha.** Three mutations in yeasts are known to simultaneously cause Ca\(^{2+}\)-dependent growth and supersecretion of uPA. The first is the S. cerevisiae pmr1 mutation, which affects a Golgi Ca\(^{2+}\)-ATPase (6, 41). The second is the S. cerevisiae ssu21 mutation, presumably influencing Ca\(^{2+}\) transport between the cytoplasm and vacuoles (37). The third is the H. polymorpha ret1-27 mutation described here. It is noteworthy that both the ret1-27 (Fig. 5) and ret1-ΔC (data not shown) mutations caused at least a fourfold decrease in Pmr1p levels. The amount of Pmr1p was decreased at the posttranscriptional level, since semiquantitative RT-PCR revealed no decrease in PMR1 mRNA levels (data not shown).

Availability of the H. polymorpha homologue of the S. cerevisiae PMR1 gene (29) allowed further investigation of the interrelation between the H. polymorpha RET1 and PMR1 genes. It was found that extra copies of the PMR1 gene, isolated from strain DL-1, were able to suppress some ret1-27 mutant phenotypes: Ca\(^{2+}\) dependence, uPA supersecretion (Fig. 5), and decreased intracellular uPA aggregation (Fig. 3). Remarkably, suppression of Ca\(^{2+}\) dependence and uPA supersecretion was not complete. No noticeable suppression of other mutant phenotypes, namely, poor growth on YEPD and the defect in Gas1p maturation, was detected.

**DISCUSSION**

COPI-coated vesicles were first identified by electron microscopy of the mammalian Golgi membranes treated with cytosol and ATP (36). Progress in the study of the COPI coat, based initially on biochemical analyses in mammals, was further supported by convergent genetic and biochemical data obtained in the yeast S. cerevisiae (for a review, see reference 20). However, with the completion of the S. cerevisiae genome sequencing project, and with efforts to sequence other genomes under way, the analysis of newly identified homologous components of the secretory pathway is of increasing importance for the understanding of their functions. Indeed, although the H. polymorpha and S. cerevisiae α-COPs are highly homologous, mutations of the same protein regions had different effects. Analysis of such effects may help to clarify the functional significance of α-COP and the whole COPI complex. For example, while S. cerevisiae α-COP without 170 C-terminal amino acids was able to support cell viability only upon overexpression (18), the H. polymorpha ret1 mutant expressing α-COP lacking more than 300 C-terminal amino acids was viable, though it manifested some mutant phenotypes. The C-terminal part of S. cerevisiae α-COP is probably involved in interaction with ε-COP, which is necessary to stabilize α-COP and the coatomer complex (16). In contrast, it is unlikely that C-terminal truncation destabilized H. polymorpha α-COP, but it disrupted the delivery of Gas1p to the Golgi apparatus, the effect revealed for the S. cerevisiae ret1-1 mutant with an altered α-COP N-terminal WD40 domain (33, 46).

In H. polymorpha an α-COP defect enhanced secretion of the heterologous protein uPA, a trait which had not been revealed previously for S. cerevisiae mutants defective in COPI protein transport. uPA is poorly secreted by yeasts because of retention in the ER, which in turn affects uPA folding and
leads to the formation of high-molecular-weight aggregates (4). Mutations in the *H. polymorpha RET1* gene caused secretion of abnormal forms of uPA into the culture medium, indicating a role for COPI in the retention of misfolded proteins in the ER. Another *ret1* mutant phenotype, which had never been observed for *S. cerevisiae* mutants defective in COPI transport, was Ca\(^{2+}\) dependence, an effect which linked COPI functions to Ca\(^{2+}\) homeostasis. This relationship was further supported by the observation that Ca\(^{2+}\) dependence could be suppressed by overexpression of Pmr1p, the medial-Golgi ion pump, which supplies the yeast secretory pathway with Ca\(^{2+}\) ions (17, 44).

Ca\(^{2+}\) has been shown to be implicated in different processes that take place in compartments of the secretory pathway including the ER. For example, ER-related processes such as retention of resident luminal proteins (11), export of secretory proteins (for a review, see reference 43), protein folding and degradation (22, 35, 50), and the association of the ER chaperone BiP with misfolded proteins (47) have been shown to depend on Ca\(^{2+}\). Study of *S. cerevisiae pmr1* mutants has revealed an important role for Ca\(^{2+}\) of the yeast secretory organelles in uPA secretion (41). Furthermore, in *S. cerevisiae*, mutations in the *PMR1* gene improved uPA secretion and caused a defect in ERAD (17), indicating that they influenced the ER-hosted processes. This may indicate a direct involvement of the yeast Golgi Ca\(^{2+}\) pump, Pmr1p, in supplying the ER with Ca\(^{2+}\) ions. Indeed, unlike mammalian cells, yeasts do not have an ER-localized Ca\(^{2+}\) ion pump, and it remains uncertain how Ca\(^{2+}\) enters the early secretory compartments. It is possible that the early secretory compartments of the yeast cell can be provided with the ions pumped into the medial-Golgi compartment by means of vesicles mediating retrograde transport to the ER. Although retrieval of proteins and lipids from the early Golgi compartments to the ER takes place in all eukaryotic cells, higher eukaryotes still require the ER-localized Ca\(^{2+}\) pump. Therefore, it is possible that the flux of ions in the retrograde direction between compartments of the Golgi apparatus of higher eukaryotes is not efficient enough to ensure a sufficient supply of Ca\(^{2+}\) ions to the early Golgi compartments and the ER. Indeed, there is evidence that, unlike their transport in yeasts, secretory cargo in animal cells are transported in the anterograde direction through the stack of relatively stable cisternae of the Golgi apparatus via COPI vesicles (for a review, see reference 38). Thus, the retrograde vesicle flow in this case is mostly destined to recycle lipids and resident Golgi proteins, which otherwise are drained from the Golgi apparatus by the anterograde COPI vesicles. Rapid progression of the yeast Golgi cisternae requires more intensive COPI vesicular retrieval of the Golgi resident components, thus providing the early secretory compartments with a sufficient supply of Ca\(^{2+}\) ions. On the other hand, retrograde transport may satisfy the requirements of the yeast ER for Ca\(^{2+}\) ions, because yeasts accumulate Ca\(^{2+}\) mostly in the vacuole, and therefore the demand of the ER for Ca\(^{2+}\) is not as high as that in mammals, where the ER is the major reservoir of Ca\(^{2+}\) ions (for reviews, see references 15 and 43).

The amount of Pmr1p was significantly decreased in *H. polymorpha ret1* mutants. This effect was a probable reason for their inability to grow in the presence of low levels of Ca\(^{2+}\) ions, since the Ca\(^{2+}\) dependence of the *ret1*-27 mutant was relieved by the extra copies of *PMRI*. There are some data suggesting that COPI transport plays an important role in maintenance of the Golgi structure and distribution of the resident Golgi enzymes between its subcompartments. For example, in *S. cerevisiae* the sec21-3 (*γ*-COP) mutation altered the distribution of the cis-Golgi protein Och1p, while the distribution of the medial- and trans-Golgi protein Mnn1p appeared to be unaffected (19). Alteration of the glycoside-elongating α-1,6-mannosyltransferase activity in the sec21-3 and ret2-1 (*δ*-COP) mutants could also reflect mislocalization of the corresponding enzyme complex (19). Besides, impairment of COPI transport altered distribution of another cis-Golgi protein, Mmn9p, causing its accumulation in vacuoles (48). Although Pmr1p has been shown to be a medial-Golgi protein in *S. cerevisiae* (17, 44), the decrease in its amount in *H. polymorpha ret1* mutants can also be explained by a defect in COPI-dependent retrieval to the residential compartment, which may lead to degradation of Pmr1p in vacuoles.

The effect of the *H. polymorpha ret1* mutations on uPA secretion cannot be explained solely by the shortage of Ca\(^{2+}\) ions in the secretory organelles due to decreases in Pmr1p levels. Indeed, overexpression of Pmr1p did not completely suppress the supersecretion phenotype of the *ret1*-27 mutant, though it restored uPA aggregation. Besides, the *H. polymorpha pmr1* mutant also was able to supersecrete uPA; however, unlike *ret1* mutants, it did not produce the abnormal slowly migrating uPA species (data not shown). Therefore, we suggest that disturbance of the retrieval of the components of the ERQC machinery in *ret1* mutants may allow misfolded uPA molecules to escape retention in the ER, resulting in their secretion.

The defect in Gas1p transport from the ER to the Golgi apparatus observed in the *ret1* mutant probably did not depend on Ca\(^{2+}\) influx, since, in contrast to Ca\(^{2+}\) dependence, it was not suppressed by Pmr1p overexpression. It is likely that, like the *S. cerevisiae ret1-1* mutation, the *H. polymorpha ret1*-27 mutation disrupted the transport of Gas1p to the Golgi complex due to a defect in the retrieval from the Golgi apparatus of specific factors that are required in yeasts for the transport of GPI-anchored proteins from the ER to the Golgi apparatus (46).

To conclude, the observations made in this work have uncovered the role of the retrograde protein transport machinery in Ca\(^{2+}\) homeostasis in the yeast cell. The availability of various *S. cerevisiae* COPI mutants may help to elucidate whether the phenotypes of the *H. polymorpha ret1* mutants studied here are unique and may facilitate further study of the role of retrograde transport in the redistribution of Ca\(^{2+}\) between the organelles of the yeast secretory pathway and in the maintenance of their structure and function.

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