Yol082p, a Novel CVT Protein Involved in the Selective Targeting of Aminopeptidase I to the Yeast Vacuole*

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In the yeast *Saccharomyces cerevisiae* the vacuolar hydrolase

leucine aminopeptidase I (API) is synthesized in the cytoplasm as a precursor (pAPI) (1) and delivered to the vacuole by one of two alternative routes that operate under distinct physiological conditions: the cytoplasm to vacuole targeting (Cvt), in nutrient-rich conditions, and the autophagy (Apg) pathway, under starvation conditions (2). The Cvt pathway is constitutive and biosynthetic, while autophagy is nonselective and degradative and is induced to survive periods of nutrient limitation (3). However, the two pathways share many molecular components and both involve sequestration by double-membrane saccular structures of unknown origin that capture the load, close into vesicles, and then fuse with the vacuole (4). A major difference between these pathways appears to be the size and content of the transport vesicles. The Cvt vesicles exclude cytoplasm and are smaller than autophagosomes that engulf bulk cytoplasm and even organelles (2). Strikingly, despite all these differences, targeting of API to the vacuole is specific and saturable, both in vegetative growth conditions and under nitrogen deprivation (3), although the molecular details of its selective recognition and capture remain essentially unknown.

Previous studies have shown that pAPI recognition by the transport machinery involves its prepro-amino extension (5, 6) and cytoplasmic chaperones of the Ssa family (7, 8). Furthermore, the amino extension is necessary and sufficient to target the reporter protein GFP to the vacuole (9). In this study we report that Yol082p, a protein shown to interact physically with pAPI in a two-hybrid screening performed with the whole yeast genome (10), mediates API loading into transport vesicles and targeting to the vacuole. We also show that Yol082p interacts with API by a process that does not only involve the prepro-amino extension but also the mature part of the API protein. Yol082p is distributed between the cytoplasm and distinct round mobile structures.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Media—**The *S. cerevisiae* strains used in this work, BY4741 (MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ yol082w::kanMX4), and its isogenic strain deleted for *YOL082w* (MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ yol082::kanMX4), were obtained from Euroscarf (European *S. cerevisiae* Archive for Functional Analysis). Disruption of *APG12* in the BY4741 background was produced by one-step targeted integration (11) with a heterologous *HIS3* marker flanked by 46-base pair target guide sequences (12). The control strain carrying a *PEP4* gene disruption was constructed by integration of a *pep4Δ: HIS3* disruption cassette, gift of Dr. H. Wolf, in the *PEP4* locus of strain W303–1B (MAT a ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100) as described in Ref. 9. In

1 The abbreviations used are: API, yeast vacuolar leucine aminopeptidase I; pAPI, API precursor; mAPI, mature API; Apg, autophagy; Cvt, cytoplasm to vacuole targeting; GFP, green fluorescent protein; YR, Yol082p round structure; PCR, polymerase chain reaction; ECL, enhanced chemiluminescence; Pipes, 1,4-piperazinediethanesulfonic acid; CPY, carboxypeptidase Y; PAGE, polyacrylamide gel electrophoresis.
all experiments yeast cells were grown at 30 °C in synthetic minimal medium: 0.67% yeast nitrogen base without amino acids, 2% glucose (SD), 2% raffinose (SRaf), or 2% galactose (SGal), and the appropriate auxotropic requirements added at 100 μg/mL. Synthetic minimal medium containing 2% glucose and 0.17% yeast nitrogen base without amino acids, 2% sodium sulfate (SD–N), was used for nitrogen starvation experiments.

Plasmid Constructions—The plasmid pGAL1-YOL082 expressing the YOL082w gene under the control of the inducible GALI promoter in the yPES2.0 vector (Invitrogen) was constructed as follows: DNA of the YOL082 coding region containing universal termini with EcoRI and PvuII sites at the 5′ and an SmaI site at the 3′ end was amplified by PCR using Universal Yeast open reading frame primer and a YOL082 open reading frame primer. The PCR product was then cloned into SmaI-digested pGFP-N-FUS (13) generating an in frame fusion. The plasmid pYOL082DAC32-GFP expressing a truncated version of Yol082p lacking 32 residues in its carboxy terminus was created by cloning an EcoRI-ClaI fragment of TOPO-YOL082 (residues 1–383 of Yol082p) into pGFP-C-FUS (13) creating an in frame COOH-terminal fusion. Plasmid pYOL082DAC32 was derived from pGFP-C-FUS containing a SmaI-StuI restriction fragment containing the GFP coding region. The resulting plasmids, pGALI-YOL082, pGFP-YOL082, pYOL082DAC32-GFP, and pYOL082AC32, were used to transform the BY4741, Δyol082 and Δgpl12 strains using the lithium acetate procedure (14).

The bait plasmid encoding prey API fused in frame to the Gal4p DNA-binding domain was constructed as follows: the API open reading frame was amplified from pYOL082 expressing a truncated version of Yol082p lacking 32 residues in its carboxy terminus by PCR using Universal Yeast open reading frame primer and a forward primer, 5′-GGTACCCGAGAAGGCTGAGG-3′, which incorporated an SmaI site, and a reverse primer, 5′-CAAGATCTGACGCTGATCTAGCTAGCC-3′, which incorporated a SfiI site. The SmaI-SfiI fragment was then cloned into pbG9T (CLONTECH) to create pbG9T-pAPI (amino acids 1–507). The API region encoding the prepro-amino extension (amino acids 1–44) was cloned using the same forward primer and a reverse primer, 5′-GTCGAGTCGAGGCGAAGCTG-3′, creating in frame fusion. The resulting plasmid pYOL082AC32 was sequenced confirming in frame fusion at the SmaI restriction site.

YOL082 plasmid were grown in low methionine-SD medium, and 15 OD660 units of yeast cells were collected, subjected to trichloroacetic acid precipitation, resolved by 8% SDS-PAGE, and studied by autoradiography. Protein Transport from Cytoplasm to Vacuole—To biochemically determine the subcellular distribution of the GFP-Yol082p, wild-type and Δyol082 cells transformed with the pGFP-YOL082 plasmid were grown in low methionine-SD medium, and 15 OD660 units of yeast cells were collected, subjected to trichloroacetic acid precipitation, resolved by 8% SDS-PAGE, and stained by the ECL technique.

To examine the survival of the different yeast strains under nitrogen starvation conditions, cells were grown to 1 OD660 in SD medium, washed once with distilled water, and resuspended in 1 OD660 in SD–N. At minute intervals aliquots were removed, and the appropriate dilutions were plated in triplicate on YPD (1% yeast extract, 2% peptone, and 2% glucose) plates.

Analysis of API Dodecamers by Sedimentation Velocity Centrifugation—Wild-type and Δyol082 cells grown in SD medium overnight to 1 OD660 were harvested and crude cell extracts prepared with glass beads as described above. The extracts were fractionated by rate-velocity sedimentation in a glycerol gradient (20–50%) (17) and the gradient fractions scrutinized for API by Western blot using the anti-API antibody and the ECL technique.

Protease Protection Assays—Wild-type and Δyol082 cells grown to 1 OD660 in SD medium were harvested and spheroplasted by treatment with Zymolyase 20 T (United States Biological). Briefly, cells were incubated in 0.1 M Tris-OH buffer, pH 9.4, containing 20 mM dithiothreitol for 20 min at 30 °C, collected, and treated with 0.2 mg/mL zymolase in 1.2 mM sorbitol, 20 mM KH2PO4, pH 7.4, for 30 min at 30 °C. Spheroplasts were adjusted to 15 OD660/mL with SL medium (1 mM sorbitol, 1% glucose, 1% proline, 0.17% YNB without amino acids and ammonium sulfate, with the appropriate auxotropic requirements) (18) and 1 ml preincubated for 5 min at 30 °C and metabolically labeled for 10 min using 100 μCi of [35S]methionine (Amersham Pharmacia Biotech) per OD660. Labeled spheroplasts were then diluted 10-fold in SL medium containing 8 mM methionine, 4 mM cysteine, and 0.2% yeast extract and chased for 2 min or 2 h. Chases were stopped by incubation for 2 min with 10 mM Na2S and the spheroplasts harvested and washed with SL medium. Lysates from spheroplasts were prepared by osmotic lysis in 1 ml of 200 mM sorbitol, 5 mM MgCl2, 20 mM Pipes, pH 6.8 (lysis buffer) for 20 min at 4 °C. Unlysates (spheroplasts) were removed by centrifugation for 2 min at 500 × g. Lysates were treated for 4 °C for 30 min with 50 μg/mL proteinase K (Life Technologies, Inc.) in the absence and presence of 0.2% Triton X-100. The proteolytic digests were stopped with a mixture of protease inhibitors (5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and pepstatin, aprotinin, and leupeptin at 1 μg/mL each). Protein was then precipitated with cold 10% trichloroacetic acid, using 100 μg/mL bovine serum albumin as carrier, washed with acetone, and the API in the resuspended pellets immuno-precipitated as described previously (7), resolved by 10% SDS-PAGE, and analyzed by Western blot with anti-API antibody (CLONTECH).
disogenic wild-type strain BY4741. The results showed that in

Growth of the PJ69-4 strain transformed with the indicated constructs, on SD-Ade-Trp-Leu + 5 mM 3-aminotriazole. For clarity, pGBT9 and pACT2 are named with the acronyms pBD (binding domain) and pAD (activating domain), respectively. None of the empty vectors supported growth when co-transformed with any of the constructs. Growth was scored after 3 days incubation at 30 °C. The β-galactosidase activity (milliunits/mg of protein) was measured in crude extracts, and the digits indicate the mean of the levels measured in three independent transformants.

A comprehensive analysis of protein-protein interactions of the yeast proteome using the two-hybrid system (10). The Yo1082 protein has a predicted molecular mass of 47.5 kDa and shows significant homology, 33% identity, to another S. cerevisiae putative protein of unknown function, referred to as Yo1033p. No other homologues were found in the data bases. Analysis of the predicted sequence of Yo1082 suggests that it is a hydrophilic protein with no predictable transmembrane domains. The null mutant of YOL082w, which as described was viable (20), showed wild-type growth rate in SD medium with a doubling time of 150 min for both wild-type and Δyol082 strains, indicating that Yo1082 function is not required for vegetative growth. To further characterize its interaction with API, Yo1082p was fused in frame with the Gal4p activation domain carried in the pACT2 vector and its interaction with the prepro-amino extension of API, pAPI and mAPI, separately cloned into the bait plasmid pGBT9, studied using the two-hybrid system. Yo1082p did not interact with the prepro domain, but interacted with mAPI, as shown by the ability of the co-transformed cells to grow on selective plates (Fig. 1). The interaction with mAPI was enhanced by the presence of the amino extension as evidenced by its stronger interaction with pAPI (Fig. 1). The specificity of the above interactions was confirmed by the observation that none of the constructions co-transformed with the empty vectors allowed growth in the restrictive medium.

Yo1082p Is Involved in Vacuolar API Import/Processing—

The interaction of API with Yo1082p led us to study whether it was involved in the transport of API to the vacuole. To test this, we first compared the vacuolar processing of API in a yeast strain carrying a deletion of the YOL082w gene with that in its isogenic wild-type strain BY4741. The results showed that in Δyol082 cells the majority of API is in its precursor form pAPI (Fig. 2A), both in cells vegetatively growing or starved of nitrogen for 2 h. In contrast, in the BY4741 strain, the entire API detected was in the processed form, mAPI. Furthermore, CPY, a vacuolar protease that is transported to the vacuole through the secretory pathway, was correctly processed in wild-type and mutant cells, indicating normal functioning of the secretory pathway in these strains. These results indicated that Yo1082p was required for transport of pAPI to the vacuole and that this requirement was not bypassed by the 2-h period of nitrogen starvation. The involvement of Yo1082p in the transport of pAPI to the vacuole was then directly tested by studying the conversion of pAPI into mAPI in Δyol082 cells transformed with a multicopy plasmid (pYES2.0), under the control of the GAL1 promoter (pGAL1-YOL082), were grown to logarithmic phase in SRAf medium and then transferred to SGal. Cell samples were taken at the indicated times of induction with galactose, extracted, and their blots probed with anti-API antibody. Also shown are immunoblots of crude extracts from mutant cells transformed with empty pYES2.0 and induced for 6 h in SGal and from wild-type cells transformed with pGAL1-YOL082 and induced for 0 and 6 h in SGal.

FIG. 1. Two-hybrid interaction between API and Yo1082p. Growth of the PJ69-4 strain transformed with the indicated constructs, on SD-Ade-Trp-Leu + 5 mM 3-aminotriazole. For clarity, pGBT9 and pACT2 are named with the acronyms pBD (binding domain) and pAD (activating domain), respectively. None of the empty vectors supported growth when co-transformed with any of the constructs. Growth was scored after 3 days incubation at 30 °C. The β-galactosidase activity (milliunits/mg of protein) was measured in crude extracts, and the digits indicate the mean of the levels measured in three independent transformants.

FIG. 2. Vacuolar processing of API in Δyol082 cells. A, cells of the wild-type (wt) and Δyol082 mutant strains were grown to logarithmic phase in SD medium and either collected or starved for 2 h in SD (−N) before harvesting. Crude extracts were resolved by SDS-PAGE and their blots probed with anti-API, anti-prepro-API, and anti-CPY antibodies. B, cells of the mutant strain Δyol082 expressing Yo1082p from a multicopy plasmid (pYES2.0), under the control of the GAL1 promoter (pGAL1-YOL082), were grown to logarithmic phase in SRAf medium and then transferred to SGal. Cell samples were taken at the indicated times of induction with galactose, extracted, and their blots probed with anti-API antibody. Also shown are immunoblots of crude extracts from mutant cells transformed with empty pYES2.0 and induced for 6 h in SGal and from wild-type cells transformed with pGAL1-YOL082 and induced for 0 and 6 h in SGal.
autophagocytosis. During autophagocytosis, bulk cytoplasm and organelles are packaged into double-membrane vesicles, termed autophagosomes, for further delivery to the vacuole. Under these conditions the majority of pAPI is targeted to the vacuole by autophagosomes (Apg pathway), but the maturation kinetics of pAPI is indistinguishable from that recorded under growing conditions (3). This is in contrast to the slow degradation of cytoplasmic markers (3), thus suggesting API transport by the Apg pathway remains selective. Because the Cvt and the Apg pathways share many of their molecular components (3, 22), we, therefore, investigated the role of Yol082p in the transport of pAPI to the vacuole by the Apg pathway.

One of the distinctive phenotypes of the apg mutants is their reduced viability during starvation. We, therefore, compared the viability of wild-type and \( \Delta yol082p \) cells in SD(\( \sim N \)) medium with that of a \( \Delta \text{apg12} \) mutant strain, a mutant defective in autophagocytosis and sensitive to nitrogen starvation (23). We observed no loss of viability in the wild-type and \( \Delta yol082p \) cells during 10 days of nitrogen starvation, while \( \Delta \text{apg12} \) cells progressively lost viability during the incubation (Fig. 3). We interpreted this difference as an indication that Yol082p was not essential for autophagy. This result was somewhat surprising, since, as described above (see Fig. 2A), we found that API was not processed when the mutant cells were starved of nitrogen for 2 h. Therefore, we studied the effect of extending the nitrogen starvation period on the processing of pAPI. For this purpose, \( \Delta yol082p \) cells were starved of nitrogen for 12, 14, 16, 18, and 24 h and the conversion of pAPI into mAPI analyzed and compared with that in wild-type and \( \Delta \text{apg12} \) cells. It was observed that the extension of the starvation period caused the slow conversion of pAPI into mAPI, with as much as 15% of the precursor being processed in the first 12 h and 35% in 24 h. This observation was in contrast to the inability of the \( \Delta \text{apg12} \) cells to process pAPI under the same conditions (Fig. 4). We interpreted these results as evidence that under prolonged nitrogen starvation conditions pAPI was passively transported to the vacuole through the autophagy pathway.

pAPI Remains in the Cytoplasm of \( \Delta yol082p \) Cells—To characterize the transport step blocked in \( \Delta yol082p \) cells, we first examined the assembly of pAPI into dodecamers, an event that precedes its wrapping by the saccular structures and its targeting to the vacuole (17). For this purpose, the assembly state of pAPI in \( \Delta yol082p \) cells was studied by examining its mobility in glycerol gradients (Fig. 5). We observed that pAPI migrated as the pAPI dodecamers extracted from wild-type cells. It was, therefore, concluded that Yol082p was not involved in API oligomerization.

To study whether pAPI was captured by cvt vesicles in \( \Delta yol082p \) cells, we compared its recovery with membrane fractions prepared by differential centrifugation of wild-type and \( \Delta yol082p \) cells on Ficoll gradients (Fig. 6). For this purpose, osmotic lysates obtained from spheroplasts prepared from \( \Delta yol082p \) cells were centrifuged at 5,000 \( \times \) g and the resulting pellets subjected to flotation in Ficoll as described under “Experimental Procedures.” Upon flotation on Ficoll, membrane-associated proteins are recovered with the light-density fractions, soluble proteins with the denser ones, and large protein complexes with the pellet (18). As expected, most of the API extracted from the wild-type cells floated with the membranes (F1), whereas the majority of the pAPI extracted from \( \Delta yol082p \) cells, which remained as pAPI, was recovered with the pellet (P7) and a small percentage (10%) with the membranes. On the other hand, under these same conditions the CPY extracted from \( \Delta yol082p \) cells was recovered with the membranes, a result consistent with its expected ability to reach the vacuole.

The floating of a small part of pAPI with the membranes from \( \Delta yol082p \) cells led us to study whether it was sequestered within vesicles. For this purpose, spheroplasts prepared from wild-type and \( \Delta yol082p \) cells were pulse-labeled for 10 min with \(^{35}\)S)methionine/cysteine and chased for 2 min or 2 h in medium without radioactivity. Then, the lysates from spheroplasts were incubated at 4 °C for 30 min without or with 50 \( \mu \)g/ml proteinase K in the absence or presence of Triton X-100, prior to immunoprecipitation with the anti-API antibody. It was observed that pAPI remained fully accessible to the protease in the absence of detergent, during the 2-h chase period.
Yol082p is not involved in the assembly of pAPI into dodecamers. Extracts from wild-type and Δyol082 cells were fractionated by sedimentation velocity centrifugation using a 20–50% glycerol gradient and the distribution of the pAPI species studied by Western blot on the gradient fractions using the anti-API antibody. Molecular mass standards were ovalbumin (Ovo, 45 kDa), catalase (Cat, 240 kDa), apoferritin (Apo, 450 kDa), and thyroglobulin (Thy, 669 kDa).

GFP-Yol082p Rescues the Conversion of pAPI into mAPI in Δyol082 Cells—To further learn about the role of Yol082p in API transport and its cellular distribution, we constructed a fusion protein in which the GFP was fused in frame to the amino end of Yol082p. Next, GFP-Yol082p was expressed in the Δyol082 mutant cells and its ability to convert pAPI into mAPI studied. The production of mAPI species in these cells indicated that GFP-Yol082p was functional in vivo (Fig. 5). The complementation of the defect in pAPI processing was, however, not observed when GFP was fused to the carboxyl end of Yol082ΔC32p, a mutant protein developed by deleting the last 32 residues of Yol082p. Moreover, when expressed in wild-type cells Yol082ΔC32p-GFP inhibited the processing of pAPI. These results and the inability of Yol082ΔC32p to complement the defective processing of pAPI in Δyol082cells supported the idea that Yol082ΔC32p and Yol082ΔC32p-GFP were inactive, but correctly localized.

GFP-Yol082p Accumulates in Round, Mobile Cytoplasmic Structures—To further examine whether Yol082p was required for the association of API with membranes, we studied the flotation of the API contained in extracts from Δyol082 cells transformed with pGFP-YOL082 on Ficoll gradients (Fig. 6). The recovery of API as mAPI with the membrane-enriched fractions (F1) indicated that GFP-Yol082p was able to rescue the association of API with membranes as well as its processing in the vacuole. This result, together with the recovery of the API extracted from untransformed Δyol082 cells with the pellet fraction (F7), as pAPI, strongly suggested that Yol082p plays a critical role in the wrapping of pAPI by membranes.

Fig. 5. Yol082p is not involved in the assembly of pAPI into dodecamers. Extracts from wild-type and Δyol082 cells were fractionated by sedimentation velocity centrifugation using a 20–50% glycerol gradient and the distribution of the pAPI species studied by Western blot on the gradient fractions using the anti-API antibody. Molecular mass standards were ovalbumin (Ovo, 45 kDa), catalase (Cat, 240 kDa), apoferritin (Apo, 450 kDa), and thyroglobulin (Thy, 669 kDa).

Fig. 6. Loss of API from the vacuole-rich fraction obtained from Δyol082 Cells is restored by GFP-Yol082p. Spheroplasts from wild-type cells, and from Δyol082 cells transformed or not with pGFP-YOL082, were lysed osmotically, precleared, centrifuged for 10 min at 4 °C at 5,000 × g, and the pellets (P5) further fractionated by differential centrifugation on a Ficoll step gradient as described under “Experimental Procedures.” An aliquot from F5, the gradient fractions (F1–F6) and the gradient pellet (F7) were resolved by SDS-PAGE and analyzed by Western blot, first with the anti-API antibody and then, after stripping the blots, with the anti-CPY antibodies.

Fig. 7. pAPI remains protease-accessible in Δyol082 extracts. Spheroplasts from cells of the wild-type and the Δyol082 mutant strain were metabolically labeled for 10 min using [35S]methionine/cysteine and then chased for either 2 min or 2 h. After their disruption by osmotic shock the resulting lysates were incubated at 4 °C for 30 min without or with 50 μg/ml proteinase K, in the absence or presence of 0.2% Triton X-100. Radiolabeled API protein was immunoprecipitated using the anti-API antibody conjugated to protein A-Sepharose and the precipitates resolved by SDS-PAGE and analyzed by autoradiography.
tures), with an average size between 0.13 and 0.27 μm. These structures were not detected when wild-type or mutant cells were transformed with the empty plasmid pGFP-N-FUS. Furthermore, incubation of the cells for 2 h in SD(-N) medium to activate the autophagy pathway resulted in loss of the YR structures from most of the cells. On the contrary, when the study was repeated in Δapg12 cells, with impeded autophagocytosis, a general decrease in cytoplasmic staining was observed, whereas the YR structures were clearly visible in all the cells. Interestingly, incubation of these cells for 2 h in SD(-N) medium did not result in the loss of the YR structures as observed before in wild-type and Δyol082 cells. The effect of the inactivation of the autophagy pathway on the distribution of GFP-Yol082p led us to study the distribution of the Yol082ΔC32p-GFP mutant protein that, as described above, was unable to complement the defect in the vacuolar targeting and processing of pAPI. The expression of Yol082ΔC32p-GFP in wild-type cells provoked a dramatic shift in the distribution of the protein, as shown both by the disappearance of the cytoplasmic staining and the increase in size of many YR structures. Again, as observed in Δapg12 cells the incubation of these cells in SD(-N) medium did not change the pattern of Yol082ΔC32p-GFP distribution.

Analysis of the localization of the YR structures at different time intervals by time-lapse confocal microscopy (Fig. 10) showed that they were highly mobile, as shown both by their lateral displacement and their continuous popping on and off from the plane under focus. On average a 0.27-μm YR structure was found to move at a rate of 1 nm/s.

### Table

| Medium | Strain | Plasmid |
|--------|--------|---------|
|        | wt     | pGFP-YOL082 |
|        | Δyol082| pGFP-YOL082 |
|        | Δapg12 | pGFP-YOL082 |

**Fig. 8.** GFP-Yol082p, but not Yol082ΔC32p-GFP or Yol082ΔC32p, complements the defect in the vacuolar import and processing of pAPI produced by disruption of YOL082. Cells of the wild-type and the mutant Δyol082 strains, transformed with either pGFP-YOL082, pYOL082ΔC32-GFP, or pYOL082ΔC32, were grown to logarithmic phase in SD medium and blots from their crude extracts probed with the anti-API antibody.

**Fig. 9.** Cellular distribution of Yol082p. Changes in distribution of Yol082p between the cytoplasmic pool and YR structures produced by growing conditions and the inhibition of the transport activity are shown. Wild-type, Δapg12, and Δyol082 strains transformed with pGFP-YOL082 or truncated pYOL082ΔC32-GFP were grown in SD medium to 1.2 OD600 and aliquots further incubated for 2 h in SD(-N) medium, as indicated in the panels. The cellular distributions of both fusion proteins were studied by fluorescence microscopy on air-dried cell smears. Insets are 2.7-fold magnification of the areas next to the white arrows. Bars: 6.64 and 2.3 μm.
cell smears. The 0.4-

D

m plane cutting through the cells at 0.8

m. 

FIG. 10. YR structures are mobile. Δyol082 cells transformed with pGFP-YOL082 were grown in SD medium to 1.2 OD 660 and the localization of the YR structures studied by confocal microscopy on air-dried cell smears. The 0.4-μm plane cutting through the cells at 0.8 μm from the plane of their attachment to the glass slide was photographed at intervals of 2.4 min (panels 1–6). The colored digits in the cell sketch at the bottom panel mark the positions of individual YR structures at time intervals of 2.4 min. Bar, 0.91 μm.

DISCUSSION

In this study we have shown that Yol082p, identified in a whole-genome analysis of protein-protein interactions as an API-interacting protein (10), is required for vacuolar targeting and conversion of pAPI into mAPI, both in vegetative growth and under short term nitrogen starvation conditions.

The interaction of Yol082p with pAPI and mAPI, but not with the prepro-amino extension of the precursor, is particularly interesting given the role of the latter in the transport of pAPI to the vacuole and the observation that it is necessary and sufficient for the transport of the reporter protein GFP from the cytoplasm to the vacuole (9). This observation suggests that transport of API to the vacuole requires additional transport determinants localized in the mature part, outside its amino extension. In addition, the stronger two-hybrid interaction of Yol082p with pAPI, as compared with mAPI, suggests that either Yol082p interacts physically with the amino extension in the context of the native protein or, alternatively, that the extension is required for proper folding and exposure of the transport determinants contained in the mature part of API to Yol082p. Clearly, further research is required to determine whether the determinants involved in its interaction with Yol082p are specific of API.

Our studies on the processing of pAPI in wild-type and Δyol082 cells show that Yol082p is required for targeting and conversion of pAPI into mAPI in the vacuole, both under vegetative growth and short periods of nitrogen starvation. The rescue of the API processing defect in Δyol082 cells transformed with Yol082p expressed from an inducible promoter unambiguously shows the involvement of Yol082p in the vacuolar import of pAPI.

The block in API vacuolar processing shown by Δyol082 cells incubated for short periods in SD(−N) medium is in contrast to the ability of mutants with impeded API transport, such as apg13, vac8, cut3, aut2, and aut7, to overcome the API accumulation soon after their shift to medium without nitrogen (2, 24, 25). Furthermore, it is interesting that the inhibition of the API processing in Δyol082 cells is partially reversed by the extension of the nitrogen starvation period. Under these conditions, mAPI is detected after 12 h in SD(−N) medium, and maturation proceeds slowly to reach a plateau at ~30% in 16 h. These observations strongly suggest that Yol082p is involved in the rapid and specific capture of pAPI by the autophagosomes developed after a short starvation period, but not in the slow and unspecific capture that occurs with the engulfment of large portions of the cytoplasm after prolonged starvation.

Apg mutants (26) often show a correlation between the effect of the mutation in autophagosome biogenesis and the loss in viability under nitrogen starvation, so that mutants with defective autophagosome nucleation die earlier upon nitrogen deprivation (25). In this context it is, therefore, interesting that although Yol082p appears to function in an early step in the pathway of API transport, the Δyol082 mutant is completely starvation-resistant. This resistance to starvation suggests again that Yol082p is not essential for autophagosome biogenesis.

Regarding the API transport step in which Yol082p is involved, we have shown that pAPI assembly into dodecamers takes place normally in the mutant cells. We also show that in the absence of Yol082p, the interaction of the oligomerized pAPI with the sequestering double-membrane sacs appears to be affected. The protease K protection assay performed with metabolically labeled protein reveals that, in the mutant strain, the newly synthesized pAPI remains unprotected in the cytoplasm after 2 h of its synthesis, which is in contrast to the wild-type. This difference suggests that Yol082p may work in the recognition of pAPI by the wrapping membranes or, alternatively, by closing these into vesicles. The recovery of the protease-sensitive pAPI extracted from apg5, apg7, apg9, aut7/apg8, and cut3 cells defective in biogenesis of the API transport vesicles (19, 27–29) with the membranes that float on Ficoll, and the exclusion of the bulk of the pAPI extracted from Δyol082 cells from the membrane fraction, rather supports the first of the two above alternatives.

Confocal fluorescence microscopy studies, performed using the fluorescent protein GFP-Yol082p, show the existence of a pool of protein homogeneously distributed throughout the cytoplasm in equilibrium with a second pool organized into one or more round-shaped structures, which we have called YR. Fur-
thermore, the equilibrium is dramatically shifted by changes in the cell growing conditions, the ability of the cells to use the autophagy pathway, and the functional activity of GFP-Yol082p. Although we lack direct evidence on the meaning of the above changes in distribution, some of these observations suggest that the pool organized into YR structures could be actively engaged in the transport of pAPI to the vacuole by the Cvt pathway. With regard to this, the disappearance of the YR structures from cells incubated for 2 h in SD(−N) medium strongly suggests that they are not needed under conditions in which the autophagy pathway is activated. The difference between the rapid processing of pAPI in mutants with a defective Cvt pathway and the enhanced visibility of YR structures observed in wild-type and truncated forms of yol082ΔC32p-GFP. This shift in equilibrium could again be consistent with our view that YR structures are involved in protein transport through the Cvt pathway, since the functionally inactive protein may cause the jam of the transport machinery and/or the accumulation of the transported material. A second alternative is the possibility that the deletion of the last 32 residues may shift the protein equilibrium toward the YR structures, which could also explain the insensitivity of these to the incubation of cells in SD(−N) medium. Obviously, these two possibilities are not mutually exclusive.

Because of its behavior as a Cvt protein we propose to rename Yol082p as Cvt19p.

Further research is required to characterize the transport step mediated by Yol082p, a step that appears to lay after the assembly of pAPI into dodecamers and before the wrapping of Cvt complexes by the saccular structures.

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