Genetic and Phenotypic Overlap between Autophagy and the Cytosol to Vacuole Protein Targeting Pathway*

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We have explored the phenotypic and genetic overlap between autophagocytosis and cytoplasm to vacuole targeting in the yeast Saccharomyces cerevisiae. Complementation analysis was performed with mutants in each of these groups (aut and cvt, respectively), and three complementation groups were found to overlap. Also, most of the unique aut mutants accumulated precursor aminopeptidase I in the cytoplasm, while maintaining wild type kinetics and maturation of proteins targeting in the cytoplasm, but is made specific, saturable, and constitutive by API has been partially characterized. API is synthesized as a soluble cytosolic protein containing an amino-terminal propeptide. Upon delivery to the vacuole, this propeptide is proteolytically removed in a proteinase B (PrB)-dependent manner, to generate the mature form of the enzyme. The signal used by API that allows it to be directed to the vacuole has been localized to a putative α-helix at the very amino terminus of the propeptide (3). Overproduction of API results in accumulation of the precursor form of this hydrolase, suggesting that one or more of the component(s) required for its import may be saturable (1). Precursor accumulation might result from the titration of either cytosolic or membrane proteins required for API import. Recently, we have isolated a group of unique mutants that block the targeting of API to the yeast vacuole (4).

The actual mechanism of protein import employed in the targeting of API is still open to question, but two distinct mechanisms are possible: entry through a proteinaceous pore or via a vesicle-mediated process. The observation that API import is inhibited at 14 °C (5) argues against direct translocation through a protein channel. Also, precursor API in the cytosol appears to be folded, as indicated by protease resistance (3), rather than in an extended form. There are two known vesicle-mediated pathways, endocytosis and autophagy, used for degradative delivery of proteins to the vacuole/lysosome from the plasma membrane/cell surface or cytoplasm, respectively. In mammalian cells, autophagy is known to be used for bulk, nonselective transport of cytosol and organelle fragments to the lysosome (6). There is also a more selective pathway, which requires the presence of a consensus KFERQ motif in the protein (7).

Although there is currently no evidence of a selective yeast counterpart to the mammalian KFERQ pathway, bulk autophagy is known to occur in yeast. During starvation, there is a significant turnover of cellular proteins and dramatic changes in cell physiology, including the induction of autophagy. The vacuole, with its numerous hydrolases, is essential in this turnover process. As in mammalian cells, autophagy in yeast transports cytosolic components destined for degradation into the vacuolar lumen. This process, however, is fundamentally different under nutrient starvation and is slow compared to the processing half-time observed for API. Mutants in the yeast autophagic pathway (aut and apg) have been isolated (8, 9). In an effort to begin to define the potential overlap between API targeting and autophagy, we compared the phenotype of aut and cvt mutants and checked for complementation between these two groups. We found a significant amount of phenotypic and genetic overlap. However, a few specific mutants in each group showed little phenotypic overlap, implying that the pathway is shared only partially, or that it branches. Our observations are consistent with the idea of a selective process for the import of API from the cytoplasm to the vacuole.

EXPERIMENTAL PROCEDURES

Strains and Media—Wild type yeast haploid strains SEY6210 (MATa leu2-3,112 ura3-52 his3-120 trp1-190 lys2-801 suc2-30), and SEY6231 (MATa leu2-3,112 ura3-52 his3-120 trp1-190 ade2-101 suc2-30) were derived by EMS mutagenesis of SEY6210 and SEY6231, respectively. In part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: ER, endoplasmic reticulum; API, aminopeptidase I; aut, autophagy; CPY, carboxypeptidase Y; cvt, cytoplasm to vacuole targeting; EMS, ethyl methanesulfonate; PGK, phosphoglycerate kinase; PrA, protease A; PrB, protease B; PVDF, polyvinylidene difluoride; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis.

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SEY6210 and back-crossing to SEY6211. For mutants in autophagy, the wild-type strain WCG (MATa his3-11,15 leu2-3,112) was also identified. All mutants in autophagy, the wild-type strain WCG (MATa his3-11,15 leu2-3,112 ura3) (12), proteinase A-deficient strain YMT A (MATa pep4::HIS3 his3-11,15 leu2-3,112 ura3), and FIM35 (aut1) were isolated. YBK18 (aut4), YBK303 (aut5), YBK355 (aut6), and YBK367 (aut9) were derived by EMS mutagenesis of strain WCG and susceptible backcrossing. All yeast strains were grown in YPD (1% yeast extract, 2% Bacto-peptone, and 2% dextrose) for Western blot analysis, or in SMD (synthetic minimal medium supplemented with the appropriate amino acids (13)) for radiolabeling and immunoprecipitations. Yeast were typically grown at 30°C and harvested at an A600 of 0.8 to 1.2, unless otherwise noted. Lithium acetate yeast transformations, genetic crosses, diploid selection, tetrad dissection and analysis, and complementation testing were carried out essentially as described (13).

Reagents—Zymolyase 20T was obtained from ICN Biomedicals (Irvine, CA). [35S] Met Protein Labeling Mix and autoradiographic film were from DuPont NEN. Glass beads (0.45–0.52 mm) were from Thomas Scientific (Swedesboro, NJ), and acrylamide and proteinase K were from Boehringer Mannheim. Immobilon-P (polyvinylidene fluoride, PVDF) was from Millipore. Antiseras to CPY and PrA (14), PGK (15), and antisera to peptides within the mature region of API (1) were described previously. Autofluor was from National Diagnostics, Inc. (Manville, NJ). All other chemicals were from Sigma.

Isolation of Mutants Defective in Autophagy and in API Maturation—Mutants defective in autophagy were isolated as described previously (8). In addition, a modification of the initial screen was used that omitted PMSF prior to starvation and microscopy. The aut1 and aut5 mutants were identified microscopically as strains that accumulated autophagic vesicles inside the vacuole under these conditions.

Yeast mutants, aut, that accumulate precursor API were isolated as described previously (4). Additional mutants were identified as follows. Yeast cells (SEY6210 transformed with pRN1(1)) were mutagenized using EMS similarly to our previous study (4), resulting in ~50% decrease in viability. Batches of 5 colonies were screened by immunoblot analysis for accumulation of precursor API as described previously (4). Individual isolates which showed defects in API maturation were characterized further.

Indirect Measurement of Autophagy—To induce formation of autophagic vesicles, cells were grown overnight at 30°C in YPD or SMD to A600 = 3–5, diluted to A600 = 0.5, and allowed to double once at 30°C. 1 ml of culture was then harvested, washed twice, and resuspended in 1% potassium acetate with 1 mM fresh PMSF in isopropyl alcohol. Cells were incubated 2–4 h at 30°C with continuous shaking, then visualized using a Nikon Labophot-2 microscope equipped with differential interference contrast optics for Nomarski photomicrographs.

Other Procedures—Cell fractionation, immunoprecipitations of radiolabeled proteins, and Western blot analyses were carried out as described previously (4).

RESULTS

Autophagy Mutants Share the Phenotypic Traits of cvt Mutants—Recently, mutants for phenotypic characteristics of cvt mutants (4) yielded cvt9 to cvt17 (see “Experimental Procedures”). Autophagy is characterized as an exclusively degradative pathway, responsible for recycling of cytoplasmic components under conditions of nutrient stress. In contrast, the Cvt pathway is a biosynthetic pathway, responsible for transport of at least one newly synthesized protein (API) to its site of action, the vacuole. However, since both pathways transport fully synthesized proteins from the cytoplasm into the vacuole lumen, we were interested in discovering whether mutants from one group would affect the other process. Therefore, we first examined the aut mutants for phenotypic characteristics of cvt mutants. The cvt mutants were identified as those that accumulate high steady-state levels of precursor API outside of the vacuole without severely affecting vacuolar protein targeting via the secretory pathway (4). We determined the maturation state of API in the aut mutants by immunoblot and compared it to that of proteinase A (PrA), a typical secretory pathway-targeted vacuolar protein. Although there were a few exceptions (see Fig. 4), the majority of the aut mutants (aut1, 3, 5, and 9) showed no significant difference in CPY kinetics (Fig. 2 and data not shown). We also observed the subcellular localization of proAPI in those aut mutants that accumulate proAPI. Yeast cells were converted to spheroplasts and subjected to differential osmotic lysis (4). Using this lysis procedure, we are able to separate a vacuole-enriched, pelletable fraction from the remainder of the cell lysate. Once again, the majority of the aut mutants behaved like cvt mutants, in that the accumulated precursor API was soluble and did not co-localize with the vacuole marker PrA (Fig. 3A). Treatment of an aliquot of the lysed spheroplasts with proteinase K showed that accumulated proAPI was protease-accessible even in the absence of detergent. This shows that proAPI in these mutants is neither in the vacuole nor trapped in a vesicle outside of the vacuole.

Genetic Overlap between aut and cvt Complementation Groups—Having found five aut mutants which shared significant phenotypic characteristics with previously identified cvt mutants, we crossed these aut mutants with the unique cvt mutants (cvt1 to cvt7, excluding cvt4 and cvt8) to check for complementation of the cvt defects; diploids were selected and examined by immunoblot. Using precursor API accumulation as an indication of noncomplementarity (4), we identified the following overlaps: aut9 does not complement cvt7, aut3 does not complement cvt10, and aut5 does not complement cvt17 (data not shown). This partial genetic overlap...
Partial Overlap between cvt and aut Mutants

FIG. 2. Autophagy mutants do not show a kinetic defect in targeting via the secretory pathway. Cells of WCG (WT), YMT A (Δ), and the aut strains indicated were labeled 5 min using [35S]methionine, followed by a nonradioactive chase for the times indicated. Extracts were immunoprecipitated with antisera to CPY as described under “Experimental Procedures.” Positions of precursor (p1 and p2) and mature (m) species are shown.

Induced (starvation) conditions, occurs with a delay—

components for API maturation may be a secondary effect. Alternatively, the apparent need for autophagocytosis indicates that these two pathways, cytoplasm to vacuole targeting and autophagy, may share some essential components. The cvt Mutants Show Some Defects in Autophagic Process—Autophagy is an inherently slow process and, even under induced conditions, occurs with a delay of ∼28 h. A recently developed diagnostic tool for monitoring autophagy involves starvation of yeast cells in the presence of PMSF, a potent inhibitor of PrB, which is responsible for degradation of autophagic vesicles inside the vacuole (16). Wild type cells under these conditions will accumulate large numbers of small vesicles inside of the vacuole, whereas mutants in the autophagic pathway are unable to accumulate these vesicles, and the vacuoles remain empty. After a 3-h starvation in acetate with PMSF, most of the cvt complementation groups showed a complete block or severe reduction in the accumulation of autophagic vesicles when compared to an isogenic wild type strain (data not shown). Three groups, cvt3, 6, and 9, appeared less defective in autophagy than the aut mutants using this test. These results are summarized in Table I.

Some cvt and aut Mutants Do Not Share Common Phenotypic Characteristics—Although the majority of the mutants isolated in both the autophagic and Cvt pathways share significant phenotypic characteristics, there are a few complementation groups that can be distinguished as truly unique. The first such class of mutants, which includes aut4 and aut6, are those that mature API fully (Table I). As shown in Fig. 4, neither of these mutants affects maturation of API. The subcellular fractionation profile of API is also not disturbed in these strains; i.e. API is located in the vacuole lumen (Fig. 3B and data not shown). Interestingly, aut4 and aut6 have different autophagy defects: aut4, like aut5, is defective in lysis of autophagic vesicles after delivery to the vacuole; vesicles accumulate in the vacuole in the absence of PMSF. aut6, similar to aut1, 2, 3, and 9, is defective in accumulation of vesicles (Table I).

Alleles of aut5/cvt17, which are defective in degradation of autophagic vesicels in the vacuole (Table I), comprise a second class of mutant which doesn’t exhibit the classic cvt phenotypes. Specifically, as seen in Fig. 3B, after osmotic lysis the proAPI accumulated in these mutants is found in the vacuole-enriched fraction and is protease-protected in the absence of detergent. This implies that API is targeted correctly to a membrane-bound compartment but is not matured upon its delivery. This phenotype is reminiscent of that seen with PrB mutants. However, the defect in aut5/cvt17 is not complemented by transformation with a plasmid carrying PRB1, nor is protease B activity itself affected in these mutants (data not shown). This indicates that there is another component(s) either required for protease B function in this pathway or directly needed for the degradation of autophagic vesicles.

**DISCUSSION**

Although isolation of mutants in the Cvt pathway promises to shed light on the components necessary for targeting of API, the actual mechanism of movement across the vacuole membrane currently remains open to speculation. API may bind directly to the surface of the vacuole membrane and in some manner trigger a process involving invagination of the membrane followed by the pinching off of a vesicle, similar to the process of endocytosis. This process of “endocytosis” would result in the formation of single membrane vesicles inside the vacuole lumen, containing API until they are disassembled. For such a process, the targeted protein need not be unfolded prior to targeting and in fact could be in a multimeric complex during transport. This process would perhaps be similar in mechanism to mammalian microautophagy, except that the protein being targeted is not destined for degradation.

Table I

| Strain | Matures API | ProAPI | Accumulates vesicles |
|--------|------------|--------|---------------------|
| WT     | Yes        | ND     | –                   |
| Δpop4  | No         | Vacuolar | +                   |
| cvt1   | No         | Cytosolic | –                   |
| aut5/cvt17 | No     | Cytosolic | –                   |
| cvt2, 5, 11, 12, 13 aut1, 2, 3, 9 aut9/cvt7, aut3/cvt10 | No | Cytosolic | –                   |
| cvt3, 6, 9 | No      | Cytosolic | –                   |
| aut4   | Yes        | ND     | +                   |
| aut6   | Yes        | ND     | –                   |

* ND, not detectable at steady state.

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A possible alternative mechanism of targeting is formation of vesicles in the cytosol which engulf API and are then targeted to the vacuole. In yeast, recent characterization of nonspecific, bulk-phase autophagy has demonstrated that this process occurs through the formation of double membrane vesicles in the cytoplasm, followed by fusion of the outer vesicular membrane with the vacuole membrane (17). This also results in the release of a single-membrane vesicle into the vacuole lumen. Since autophagy in yeast has been characterized as a slow process, targeting of API via this pathway would probably require that a receptor in some manner concentrate the protein prior to targeting. Essentially, this would be receptor-mediated macroautophagy.

Both of these models involve the formation of vesicles that contain proAPI that in some way end up in the vacuole lumen. However, the origin of these membranes would differ. In the first case, the vesicles would consist of vacuole membrane. In the second case, the autophagic vesicle would be derived through assembly using cytoplasmic membrane sources. Work is currently under way to isolate vesicles found inside of the yeast vacuole to analyze both the protein content and lipid constituents of these structures.

The genetic and phenotypic overlaps explored in this paper could result from one of two situations. First, the API maturation defect in aut mutants is a secondary effect. One could envision, for instance, that autophagy defects alter the characteristics of the vacuole membrane such that it is incompetent for delivery of API. Second, it is possible that both API and nascent autophagic vesicles are taken into the vacuole via an invagination (endocytosis) process, whereby the components of the pathway are conserved but the specifics of uptake from the cytosol are different for API traffic and autophagic transport.

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FIG. 4. API maturation is unaffected in aut4 and aut6 mutants. Samples were prepared as in Fig. 1; WT, WCG. Numbers at the right indicate the molecular mass in kDa.