Aut2p and Aut7p, two novel microtubule-associated proteins are essential for delivery of autophagic vesicles to the vacuole

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**Introduction**

Eukaryotic cells constitutively deliver their intracellular contents to the vacuole (or lysosome) for degradation. For the delivered proteins this is a non-specific process called autophagocytosis and it is stimulated significantly during nutrient deprivation (for reviews see Dunn, 1994; Mortimore et al., 1996; Seglen et al., 1996; Codogno et al., 1997). Autophagocytosis involves the formation of autophagosomes, which are vesicles containing cytoplasm typically surrounded by two membrane layers, that appear in the cytoplasm. In mammalian cells autophagosomes acquire lysosomal hydrolases very rapidly, usually within 15 min (Dunn, 1990), probably by fusing with pre-existing lysosomes and they mature to become degradative autolysosomes (Lawrence and Brown, 1992; Dunn, 1994).

In the yeast *Saccharomyces cerevisiae* the outer membrane layer of autophagosomes fuse with the vacuolar membrane, releasing autophagic vesicles into the vacuole, where they are lysed. This lysis is dependent on the active vacuolar protease yscB (Baba et al., 1994). During starvation in the presence of the protease yscB inhibitor phenylmethylsulfonyl fluoride (PMSF), accumulation of autophagic vesicles inside the vacuole can be visualized by light microscopy (Straub et al., 1997). This has made the monitoring of autophagy very simple. Also a phenotypic and genetic overlap between autophagy and the selective transport of the precursor of aminopeptidase I from the cytoplasm to the vacuole, with a half-life of ~45 min (Klionsky et al., 1992), has been detected (Harding et al., 1996). Thus, blocking aminopeptidase I precursor maturation, which occurs in almost all aut-mutant cells, suggests an additional way of following autophagic defects in cells.

In wild-type yeast cells autophagosomes can only rarely be detected in the cytoplasm (Baba et al., 1994; our unpublished results), a phenomenon probably due to the fact that they are readily taken up into the vacuole. The rapid fusion of autophagosomes with the lysosome (vacuole) suggests a directed movement of the vesicles rather than diffusion. Indeed, Aplin et al. (1992) reported the accumulation of acidic late autophagosomes lacking hydrolytic enzymes in mammalian cells when treated with nocodazole, a microtubule-depolymerizing drug. They speculated that autophagosomes move along the microtubular network to reach the lysosomes. Also treatment with vinblastine, a microtubule inhibitor, leads to the accumulation of autophagosomes (Seglen et al., 1996).

So far, however, no direct evidence for the attachment of autophagosomes to microtubules, or the proteins involved, has been obtained. Microtubules in *S. cerevisiae* consist of two α-tubulins, Tub1p and Tub3p, and the β-tubulin Tub2p (for reviews see: Solomon, 1991; Winsor and Schiebel, 1997).

To achieve a molecular understanding of the autophagic process, we isolated aut-mutant strains defective in autophagy (Thumm et al., 1994). Two genes, *AUT1* (Schlumpberger et al., 1997) and *AUT3* (Straub et al., 1997), have already been characterized.

Here we report on the identification of *AUT2* and its low copy suppressor *AUT7*, two genes essential for autophagocytosis. Aut7p has significant homology to light chain 3 (LC3), a rat microtubule-associated protein. Aut2p exhibits a direct protein–protein interaction with Tub1p, Tub2p and Aut7p. In *aut2*- and *aut7*-deleted cells autophagosome-like vesicles become detectable. Our results suggest Aut2p and Aut7p are involved in the attachment of autophagosomes to microtubules for delivery to the vacuole.

**Results**

Selection of an autophagocytosis-defective mutant

The *aut2*-1 mutant strain, FIM39, has been identified after ethyl methanesulfonate mutagenesis as an autophagocytosis-defective mutant. This is due to its inability to degrade cytosolic fatty acid synthase and to accumulate autophagic vesicles in the presence of PMSF inside the
Isolation of the AUT2 gene

Homozygous aut2-1 diploid cells show a drastically reduced sporulation frequency. Following a previously described procedure (Schlumpberger et al., 1997), we used this phenotype to isolate the AUT2 gene by complementation with a Ycp50-based genomic library (Rose et al., 1987). Screening of ~30,000 transformants yielded plasmid Ycp50/27 (Figure 1A), which in homozygous aut2-1 mutant cells cured not only the sporulation deficiency but also the accumulation defect of autophagic vesicles in the vacuole during starvation in the presence of PMSF. In addition the defect in maturation of the precursor of aminopeptidase I was complemented by this plasmid in aut2-1 mutant cells (Figure 3A, lane 6).

Subcloning of the 6.5 kb genomic insert of Ycp50/27 identified a 1.8 kb XbaI–BclI fragment (Figure 1A) sufficient for complementation. Sequencing localized this fragment to chromosome XIV. YNL223w was the single complete open reading frame (ORF). Chromosomal integration of the XbaI–BclI fragment into aut2-1 mutant cells followed by crossing with a wild-type strain and subsequent tetrad dissection confirmed the identity of YNL223w (DDBJ/EMBL/GenBank accession No. U20390) with AUT2. AUT2 encodes a protein consisting of 506 amino acids (Figure 1B), with an estimated molecular mass of 56.6 kDa and an isoelectric point of ~4.5. Aut2Δ shares some homology with ZK792.1 (Wilson et al., 1994), an ORF of unknown function from Caenorhabditis elegans, and with regions of tubulin α-chains of other organisms, for example α2-tubulin from maize (Figure 1C).

AUT2 is essential for the autophagic process, but not for growth

An aut2 chromosomal null mutant was constructed, by using a PCR-based deletion method and the plasmid pUG6 (Gündener et al., 1996) to replace the complete ORF with a LoxP–KanR–LoxP cassette (Wach et al., 1994) generating canamycin resistance. Correct gene replacement was confirmed by Southern blot analysis (not shown). Aut2Δ cells grow as the wild-type on rich medium at 16, 30 and

![Fig. 1.](image)

(A) AUT2 is identical to YNL223w. Genomic fragments of complementing plasmids and constructed subclones are illustrated. X, XbaI; B, BclI; N, NruI; H, HindIII. (B) Protein sequence of Aut2p. (C) Aut2p shows weak similarities to part of tubulin α2 from maize (Montoliu et al., 1990).
37°C, respectively (not shown). Aut2Δ cells exhibit all phenotypes found for other aut-mutant strains analyzed so far. As expected Aut2Δ cells were unable to accumulate autophagic vesicles in the vacuole (Figure 2A). While in aut2-1 mutant cells some mature aminopeptidase I can be detected (Figure 3A, lane 5), in aut2Δ cells maturation of aminopeptidase I precursor was completely impaired (Figure 3B, lane 3). The most prominent phenotype of aut-mutants is their lack of starvation-induced protein breakdown (Schlumpberger et al., 1997; Straub et al., 1997). Similar to pepΔ (pra1Δ) cells, which are impaired in vacuolar proteolytic breakdown, aut2Δ cells exhibited only about one third of the total protein breakdown rate found in wild-type cells (Figure 2B). Also the survival rate during starvation for nitrogen was significantly reduced in aut2Δ cells as compared with wild-type cells (Figure 2C).

**Isolation of ORF YBL078c (AUT7), an extragenic suppressor of aut2-1 mutant phenotypes: another gene essential for autophagy**

To identify additional components of the autophagic pathway, we screened for high copy suppressors of the sporulation defect of homozygous diploid aut2-1 mutant cells. Out of 50,000 transformants from an overexpressing Yep24-based genomic library, four independent plasmids Yep24/10, Yep24/24, Yep24/29 and Yep24/34 were isolated, which complemented the sporulation defect. Partial sequencing demonstrated the identity of the insert in Yep24/10 with the AUT2 gene. The other three genomic inserts could be localized to chromosome II (Figure 4A). ORF YBL078c (YBH8) was common to all fragments and subcloning of this DNA as a Stu–SnaBI-fragment (Figure 4A) revealed its ability to complement the maturation defect of the aminopeptidase I precursor (Figure 3A, lane 9) and the vesicle accumulation defect seen in aut2-1 mutant cells (not shown).

As complementation of the aut2-1 mutation also occurred with a centromeric version of YBL078c (DDBJ/EMBL/GenBank accession No. Z35839) or even after chromosomal integration (Figure 3A, lanes 1 and 8), we assume a very close interaction of Aut2p and Aut7p. YBL078c (AUT7) could not substitute completely for AUT2; in aut2Δ cells no suppression was observed (Figure 3B, lanes 6 and 7).

Chromosomal deletion of YBL078c (AUT7) by integrating a LoxP–KanR–LoxP cassette (Wach et al., 1994; Guldener et al., 1996), did not influence growth on rich media at 16, 30 and 37°C, respectively (not shown). Deletion was confirmed by Southern analysis (not shown).

**AUT7 (YBL078c) is essential for autophagy**

Aut7Δ-deleted cells show all the phenotypes of autophagy mutants. When aut7Δ (ybl078cΔ) cells were subjected to nitrogen starvation in the presence of PMSF, no

![Fig. 2.](image-url)
Microtubule-associated rat LC3 protein in the selective cytoplasm-to-vacuole targeting (Cvt) of mutants, defective in autophagy, and Cvt5-1 (3C, lane 5).

Aut7p was complemened by a plasmid borne I precursor (Figure 3C, lane 4). This mutant phenotype was observed (Figure 2A). Also maturation of the aminopeptidase I precursor (Figure 3C), starvation-induced protein breakdown (Figure 2B) and the survival rate during starvation (Figure 2C) were significantly affected in these cells. We therefore named this ORF AUT7.

Recently a phenotypic and genetic overlap between aut-mutants, defective in autophagy, and cvt-mutants impaired in the selective cytoplasm-to-vacuole targeting (cvt) of the aminopeptidase I precursor has been described (Harding et al., 1996). We therefore checked for an alleleism of aut7Δ cells with cvt-mutant strains (Harding et al., 1995, 1996). Interestingly, heterozygous defective cvt5-1 aut7Δ diploid cells showed only the aminopeptidase I precursor (Figure 3C, lane 4). This mutant phenotype was complemented by a plasmid borne AUT7 gene (Figure 3C, lane 5). cvt5-1 itself is allelic with apg8-mutant strains, which are defective in autophagy (Scott et al., 1996).

**Aut7p has significant homology to the microtubule-associated rat LC3 protein**

AUT7 encodes a protein of 117 amino acids with an estimated molecular mass of ~14 kDa and a predicted isoelectric point of ~9.3. Aut7p exhibits a striking homology to proteins from a variety of organisms. It shows 77% identity to the ‘symbiosis-related protein’ from Laccaria bicolor, 71% identity to AC002387 from Arabidopsis thaliana, 56% identity to a ganglioside expression factor-2 (GEF-2) protein from Rattus norvegicus, 53% identity to U23511 from C.elegans (Wilson et al., 1994), 50% identity to Z97341 from A.thaliana, and 28% identity to the microtubule-associated protein 1 LC3 from R.norvegicus (Kuznetsov and Gelfand, 1987; Mann and Hammerback, 1994, 1996) (Figure 4B).

**Protein–protein interactions between Aut2p and Aut7p, Aut2p and Tub1p, and Aut2p and Tub2p**

The homology of Aut7p with a microtubule-associated mammalian protein and the strong genetic interaction of aut2-1 and AUT7 prompted us to search for a possible protein–protein interaction between these proteins and the yeast tubulin proteins Tub1p and Tub2p. A yeast two-hybrid system with HIS3 and LacZ as reporter genes was used for this search (Fields and Song, 1989; Fields and Sternglantz, 1994). Sole expression of fusion constructs of the GAL4 activator and binding domain did not result in any staining indicating that no protein–protein interaction occurred (not shown). In contrast, strong interactions were detected between Aut2p and Aut7p, Aut2p and Tub1p, and Aut2p and Tub2p (Figure 5A), as indicated by formation of the blue reaction product of X-Gal after 3 h incubation at 30°C.

Confirmation of the protein–protein interactions detected with the two-hybrid system, was performed by protein affinity chromatography. A glutathione-S-transferase–Aut2p (GST–Aut2p) fusion protein was bound to a glutathione affinity column. We incubated this GST–Aut2p column with a crude extract of aut7Δ cells expressing a biologically active C-terminal fusion protein of Aut7p with the ‘Green Fluorescent Protein’ (GFP) (Aut7p–GFP-C). After washing with 500 mM NaCl, proteins bound were eluted and analyzed using antibodies directed against GFP. As expected, Aut7p–GFP was bound to the GST–Aut2p affinity column (Figure 5B, lane 3). When GST alone was bound to the column, as a control, no Aut7p–GFP became bound (Figure 5B, lane 6).

Bovine tubulin has been found to interact with yeast tubulin-associated proteins similar to yeast tubulin (Barnes et al., 1992). To confirm the binding of tubulin to Aut2p, we therefore incubated a GST–Aut2p column with bovine tubulin. As shown in Figure 5C, lane 12, bovine tubulin was found attached to the GST–Aut2p column even after washing with buffer containing NaCl. If GST alone was bound to the column no bovine tubulin became bound (lane 9). Protein staining with Coomassie indicate the presence of GST–Aut2p and tubulin (lane 6).

Two-hybrid analysis indicated no direct interaction between Aut7p and tubulin. We also analyzed binding of bovine tubulin to a GST–Aut7p column. As with GST alone, no bound tubulin was detected in this experiment (Figure 5D, lanes 9 and 12). Coomassie staining demonstrated the presence of GST–Aut7p in the eluate fraction (Figure 5D, lane 6). In lane 3 GST is visible. When binding very large amounts of GST–Aut7p to the column, a very faint band, becomes visible (not shown). We consider this faint band not to be due to a specific binding of Aut7p to bovine tubulin.

**aut2Δ and aut7Δ cells are not sensitive to benomyl**

Benomyl is a microtubule-depolymerizing drug. Altered sensitivity (i.e. hypersensitivity or resistance) to benomyl is an indication for a change in microtubule stability.
Fig. 4. (A) AUT7 is identical to YBL078c. Genomic fragments isolated from an overexpression library and leading to suppression of the autophagic defects in aut2-1 mutant cells are illustrated. A complementing Stu–SnaBI subclone carrying only AUT7 as a complete ORF was constructed.

(B) Homologues of Aut7p. Sequences were aligned using the Clustal method. Residues identical with Aut7p are shaded in black. Further details are given in the text.
Fig. 5. (A) Detection of protein–protein interactions using the two-hybrid system. Intense staining indicates an interaction between Aut2p and Aut7p, Aut2p and Tub1p, and Aut2p and Tub2p. AUT7, TUB1 and TUB2 are cloned in-frame with the GAL4-activator domain. AUT2 and AUT7 were fused to the GAL4-binding domain (see Materials and methods). (B) Affinity chromatography confirms protein–protein interaction between Aut2p and Aut7p. A fusion protein of Aut2p with the 26 kDa glutathione-S-transferase (GST) domain from Schistosoma japonicum (see Materials and methods) was bound to glutathione–Sepharose 4B. This GST–Aut2p column was incubated with a crude extract from aut7Δ cen Met25::AUT7–GFP cells. The non-bound supernatant (S) fraction (lane 1) was taken away. Gel beads were successively washed with NaCl solutions of increasing concentrations to remove non-specifically bound proteins. After the final 500 mM NaCl wash (W, lane 2), bound proteins were eluted with Laemmli buffer (E, lane 3). Samples were analyzed in immunoblots with monoclonal antibodies directed against GFP. Using Aut2p–GST, a clear band corresponding to Aut7p–GFP is seen in the eluate fraction (lane 3, arrowhead). As a control, GST alone was attached to the column and no band was detectable (lane 6). (C) As described in (B), bovine tubulin binds to GST–Aut2p glutathione–Sepharose 4B (lane 12), but not to GST glutathione–Sepharose 4B (lane 9). Protein staining with Coomassie is shown in lanes 1–6; in lanes 7–12 tubulin was detected using monoclonal antibodies (Boehringer Mannheim). Further details are given in Materials and methods. (D) Similar to (C), bovine tubulin does not bind to GST–Aut7p glutathione–Sepharose 4B (lane 12) and to GST glutathione–Sepharose 4B (lane 9). Lanes 1–6, protein staining with Coomassie; lanes 7–12 probed with antibodies directed against tubulin.

(Interthal et al., 1995). We therefore expected that overexpression of AUT2 or AUT7 in wild-type cells or chromosomal deletion of AUT2 or AUT7 in cells would show benomyl sensitivity or resistance, if Aut2p or Aut7p were involved in modulating microtubule stability. At concentrations of 15 or 30 μg/ml no altered growth rates were detected (Figure 6) indicating a non-structural function of Aut2p and Aut7p.

**Autophagosomes accumulate in starved aut2Δ and aut7Δ cells**

A detailed electron microscopic analysis of aut2Δ and aut7Δ cells led to the detection of double membrane-layered vesicles with an appropriate diameter of ~170–180 nm in the cytoplasm of these cells during starvation (Figure 7A and B). The two membrane layers surrounding these vesicles can easily be seen (Figure 7B, arrowheads). Vesicular content was indistinguishable from cytoplasm (Figure 7A and B). We further confirmed the accumulation of autophagosome-like vesicles. We isolated these vesicles by modifying an established cell fractionation procedure (Harding et al., 1996), which used hypotonically lysed spheroplasted cells (total fraction, T), (Figure 7C, lane 3). Following this procedure with wild-type cells yielded a 6000 g pellet fraction (P6, Figure 7C, lane 4) consisting of plasma membrane fragments, endoplasmic reticulum and intact vacuoles and a 6000 g supernatant fraction (S6, Figure 7C, lane 5) which was almost entirely cytosolic (Harding et al., 1995). We introduced an additional 100 000 g centrifugation step, which when starting with the S6 fraction, yielded a P100 pellet fraction (Figure 7C, lane 6) and a S100 supernatant fraction (Figure 7C, lane 7). We used cells starved for 20 h on nitrogen-free medium and followed the intracellular localization of fatty acid synthase (Fas) as a suitable cytosolic marker protein (Egger et al., 1993). In pep4Δ (pra1Δ) cells, exhibiting a defect in vacuolar proteolysis, due to the action of the autophagic process, part of Fas is detected in the vacuolar P6 fraction (Figure 7C, lane 4) (Thumm et al., 1994; Schlumpberger et al., 1997). No visible P100 pellet was obtained in this experiment and no Fas could be detected in this fraction (Figure 7C, lane 6). In contrast, following this procedure with aut2Δ cells, a clearly visible P100 pellet was obtained, which observed under the light microscope consisted of vesicles. At the same time significant amounts of Fas could be detected in the P100 fraction of aut2Δ cells (Figure 7D, lane 7). Due to the defect in autophagocytosis, in aut2Δ cells, no Fas was found to be
localization in the vacuole (Figure 7D, lane 4). But proteinase K protection experiments with total fraction T in the absence and presence of Triton X-100 (Figure 7D, lane 1 and 2) demonstrate that part of Fas is membrane-protected in aut2Δ cells. This is a further indication for the accumulation of cytosol-containing vesicles in the cytosol of aut2Δ cells.

Localization of Aut2p and Aut7p
N- and C-terminal fusion proteins of Aut2p and Aut7p with the fluorescent GFP protein from Aequorea victoria (Cubitt et al., 1995; Stearns, 1995) were constructed. The constructs were placed under the control of the inducible MET25 promoter using the plasmids pRN295 and pRN963. Expression of the fusion proteins rescued autophagic defects of the respective chromosomally deleted cells, indicating that the Aut2p–GFP and Aut7p–GFP fusion proteins were biologically active (Figure 3A, lanes 10–13; Figure 3B, lanes 8 and 9; Figure 3C, lanes 2 and 3). Fluorescence of fusion proteins appeared unevenly distributed throughout the cytoplasm, Aut2p and Aut7p seemed to focus in some areas (not shown).

Discussion
Insight into the mechanistic principles of autophagocytosis should result from the study of a variety of previously isolated aut-mutants defective in autophagocytosis (Thumm et al., 1994). Complementation of the sporulation defect of homozygous aut2-1 diploid cells with a genomic library was used to identify the AUT2 gene (Figure 1). AUT2 is not essential for growth on rich media at 16, 30 and 37°C. However aut2-deleted cells exhibit the phenotypes characteristic for aut-mutant cells (Schlumpberger et al., 1997; Straub et al., 1997). (i) They are unable to accumulate autophagic vesicles inside the vacuole during starvation for nitrogen in the presence of PMSF (Figure 2A). (ii) The total protein breakdown during starvation is significantly reduced (Figure 2B). (iii) The survival rate of mutant cells during periods of nutrient limitation is reduced (Figure 2C). (iv) The cell differentiation process of sporulation is almost completely blocked in mutant diploids (not shown). (v) The vacuolar uptake and maturation of the aminopeptidase I precursor is blocked in logarithmically growing cells and in cells starved for nitrogen (Figure 3). Aut2p is predicted to be a soluble protein with no significant homology to proteins of known function, and limited homologies to some α-tubulin chains (Figure 1C).

A screen for high copy suppressors of autophagic defects of aut2-1 cells identified—besides AUT2 itself—the ORF YBL078c. Chromosomally deleted YBL078c cells showed all the characteristic features of mutant strains with autophagic defects listed above (Figure 2A–C and Figure 3C), we therefore named this gene AUT7. Aut7Δ cells were found to be allelic with cvt5-1 cells (Figure 3C). cvt5-1 cells have been isolated for their blockage in the selective cytoplasm-to-vacuole targeting of the aminopeptidase I precursor (Harding et al., 1995). Interestingly they are also allelic to apg8 mutant cells which, in an independent approach, have been isolated for their defect in autophagy (Scott et al., 1996). No DNA sequences have been reported for CVT5 and APG8 so far. Aut7p is a small protein of 13.6 kDa, which shows significant homology to some proteins of unclear biological function and to LC3, a component of microtubule-associated protein (MAP) complexes isolated from brain (Kuznetsov and Gelfand, 1987; Mann and Hammarback, 1994, 1996). LC3 has been found in ternary protein complexes associated with either MAP1A (275 kDa) or MAP1B (244 kDa) and LC1 (27 kDa) as a third protein. Another light chain LC2 (24 kDa) specifically associates with MAP1A. Electron microscopic studies suggested that these protein complexes form long filamentous arms linked to the microtubules and connecting microtubules to each other, and so possibly modulating microtubule shape (Shiomura and Hirokawa, 1987; Sato-Yoshitake et al., 1989; Pedrotti et al., 1996).

AUT7 does not only act as a suppressor of autophagic defects in aut2-1 mutant cells in high copy number, but also in low copy number: chromosomal integration of an additional copy of AUT7 is sufficient for suppression (Figure 3A). This indicated a possible interaction of Aut2p and Aut7p on the protein level. We therefore tried to determine a direct protein–protein interaction between Aut2p and Aut7p using the two-hybrid system. Because of the strong homology between Aut7p and the microtubule-associated LC3 protein from rat, we also included Tub1p and Tub2p as components of the yeast tubulin cytoskeleton in this analysis. We were able to detect strong interactions between Aut2p and Aut7p, Aut2p and Tub1p, and Aut2p and Tub2p. These findings suggested the formation of a protein complex consisting of Aut2p and Aut7p, which is attached to microtubules via interactions between Aut2p and Tub1p, and Aut2p and Tub2p, respectively.
We confirmed this interaction by binding a GST–Aut2p fusion protein to an affinity column followed by incubation with a crude extract of aut7Δ cells expressing a biologically active Aut7p–GFP fusion protein. Indeed, after removing non-specifically bound proteins from the affinity column Aut7p could be detected (Figure 5B). Using bovine tubulin, which has been shown to be equivalent to yeast tubulin in binding yeast-associated proteins (Barnes et al., 1992), we demonstrate the interaction of Aut2p with tubulin (Figure 5C). With a predicted isoelectric point of 4.5, Aut2p is negatively charged at physiological pH. Aut7p on the other hand is positively charged (predicted isoelectric point 9.3). This may be relevant for formation of the Aut2p–Aut7p protein complex attached to microtubules. In S.cerevisiae mutations influencing microtubule stability have been shown to cause altered sensitivity to the microtubule-depolymerizing drug, benomyl (Schatz et al., 1988; Interthal et al., 1995). Cells deleted for aut2Δ

**Fig. 7.** (A) Autophagosome-like vesicles (arrowhead) are accumulating in the cytoplasm of aut2Δ cells starved for 4 h on 1% K-acetate in the presence of PMSF. Bar represents 100 nm. (B) Electron micrographs unravels the accumulation of double membrane-layered autophagosome-like vesicles in the cytoplasm of aut7Δ cells starved for 3 h in nitrogen-free medium in the presence of PMSF. Arrowheads indicate the clearly distinguishable two membrane layers. Bar represents 100 nm. (C and D) Cytosol containing autophagosome-like vesicles accumulating in the cytosol of aut2Δ cells (D) can be isolated with an additional 100 000 g centrifugation step yielding a P100 pellet fraction. In wild-type cells (C), no P100 is detectable. Fatty acid synthase (Fas) was used as a cytosolic protein. Further details are given in the text.
of autophagosomes in mammalian cells (Seglen et al., 1996).

Aut7p is a member of a new protein family consisting of proteins with only scarcely understood functions. They are from various organisms and include the ‘symbiosis-related protein’ from L. bicolor (77% identity), AC002387 protein from A. thaliana (71% identity), GEF-2 protein (56% identity) from R. norvegicus, U23511 protein (53% identity) from C. elegans (Wilson et al., 1994), Z97341 protein (50% identity) from A. thaliana, and to microtubule-associated protein 1 LC3 (28% identity) from R. norvegicus (Kuznetsov and Gelfand, 1987; Mann and Hammarback, 1994, 1996), (Figure 4B). LC3 from rat has been shown to bind directly to microtubules (Mann and Hammarback, 1994). Two-hybrid analysis (Figure 5A) and the use of GST–Aut7p affinity columns with bovine tubulin (Figure 5D) does not support a direct interaction of Aut7p with tubulin. We cannot exclude, however, an interaction of Aut7p with yeast tubulin in vivo. Interestingly, two proteins with different homologies to Aut7p has been found so far in R. norvegicus and in A. thaliana, respectively. It is conceivable that, the GEF-2 protein sharing 56% identity with Aut7p rather than LC3 with only 28% identity is the functional homologue.

Nevertheless, it is tempting to speculate that some Aut7p relatives in other organisms, might have the same or similar functions as Aut7p in S. cerevisiae, namely transport of vesicles along microtubules. It will be a challenging task to test this hypothesis.

Materials and methods

Chemicals and media

Yeast cells were starved in 1% K-acetate. Other media were prepared according to standard protocols (Ausubel et al., 1987). Chemicals were of analytical grade.

Strains

WCG4a Mata his3-11,15 leu2-3,112 ura3 (Thumm et al., 1994); YMTA469 Mata his3-11,15 leu2-3,112 ura3 pral Δ.HIS3 prb1 Δ. (Thumm et al., 1994); FIM397b Mata leu2-3,112 ura3 his3-11,15 aut2-1, (backcrossed three times with WCG4) (Thumm et al., 1994); YTL4 Mata his3-11,15 leu2-3,112 ura3 ade2Δ aut1-1 (this study); YTL5 Mata his3-11,15 leu2-3,112 ura3 ade2Δ aut1-1 (this study); YTL6 Mata his3-11,15 leu2-3,112 ura3 ade2Δ aut1-1 (this study); YTL7 Mata his3-11,15 leu2-3,112 ura3 ade2Δ aut1-1 (this study); YTL8 Mata his3-11,15 leu2-3,112 ura3 ade2Δ aut1-1 (this study); YTL9 Mata his3-11,15 leu2-3,112 ura3 ade2Δ aut1-1 (this study); YTL10 Mata his3-11,15 leu2-3,112 ura3 ade2Δ aut1-1 (this study); YTL11 Mata his3-11,15 leu2-3,112 ura3 ade2Δ aut1-1 (this study); DYY101 Mata leu2-3,112 ura3-52, his3-2A200 trpl-1Δ901 lys2-801 suc2-901 vet8-1 Δ. LEU2 (Harding et al., 1995); THY313 Mata leu2-3,112 ura3-52 his3-2A200 trpl-1Δ901 ade2-101 suc2-901 vet5-1 (Harding et al., 1995), Y190a Mata gal4A GAL80 his3 trpl-1Δ901 ade2-101 ura3-52 leu2-3,112 URA3::GAL → lacZ, LYS2:: GAL(UAS) → HIS3 (S.J. Elledge, Houston, USA).

YTL4 was obtained by chromosomal deletion of the ADE2 gene in aut1-1 mutant with a 2.3 kb BamHI fragment from pL131 (Schlumpberger et al., 1997). YTL8 was obtained by chromosomal integration of HisAp linearized pRS306/AUT7 in aut2-1 mutant cells. Using oligonucleotides Δaut2 KAN1, Δaut2 KAN2 or Δaut7 KAN1; Δaut7 KAN2 and plasmid pG6, two DNA fragments for the chromosomal replacement of AUT2 or AUT7 with a LoxP–KAN8–LoxP cassette were created by PCR. The strains YTL9 or YTL11 were made by transforming the single LoxP–KAN8–LoxP cassettes into WCG4a. Y190a was used for the two-hybrid assay, YTL5 for isolation of AUT2 and AUT7 according to Schlumpberger et al. (1997).

GST–Aut2 and GST–Aut7 fusion proteins

A EcoRI–XhoI fragment containing AUT2 was generated by PCR using oligonucleotides GST1 AUT2, GST2 AUT2, and pRS316/ Aut2. It was introduced in the EcoRI–XhoI site of pGEX-4T-3 (Pharmacia, Freiburg,
Germany). After inducing protein expression for 4 h with 0.1 mM isoprrol-β-D-galactopyranoside, the 82 kDa fusion protein was isolated following the manufacturer’s protocol and bound to glutathione–Sepharose 4B (Pharmacia, Freiburg) during a 30 min incubation. Similarly, a BamHI–XhoI fragment containing AUT7 was generated using oligonucleotides (ATT ACT GGA CCC ATG AAG TCA TAT TTA AGT CTG GCC AAA AAG TAT TTG AGG ACT GCC ACT AGT TCC TAT TTC TCC CCC GAG ACT TCC AAT AGG ACT GTG AAT ACC TAC CGT TTC CTT 5′-TTC CTT GTC GAC GCA TTT TTC ATC AAT AGG ACT-3′; AUT2-Hybrid2 5′-ATT ACT GGA CCC ATG AAG TAT TTA AGT CTG GCC AAA AAG TAT TTG AGG ACT GCC ACT AGT TCC TAT TTC TCC CCC GAG ACT TCC AAT AGG ACT GTG AAT ACC TAC CGT TTC CTT 5′-TTC CTT GTC GAC GCA TTT TTC ATC AAT AGG ACT-3′). It was introduced into pGEX-4T-3.

Protein affinity chromatography

Twenty OD midlog cells of strain YTL1 pGFP–AUT7 were lysed using glass beads. Crude extracts in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4) were incubated overnight at 4°C with gentle shaking with GST–Aut2p (or GST) bound to glutathione–Sepharose 4B. Supernatant (S) was removed and the beads were washed successively with 50 μl each of a solution containing 15.6, 31.2, 62.5, 125, 250 and 500 mM NaCl (fraction W, wash) in PBS buffer to remove unspecifically bound proteins. After eluting all proteins (fraction E) with 50 μl Laemmli buffer, 10 μl of all fractions were analyzed in immunoblots with anti-GFP antibodies (Molecular Probes, Leiden, The Netherlands).

Bovine tubulin binding

GST–Aut2p (or GST) bound to glutathione–Sepharose 4B (~10 μg) was incubated with 30 μl of PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4) and bound to glutathione–Sepharose 4B. Supernatant (S) was removed and the beads were washed successively with 50 μl each of a solution containing 15.6, 31.2, 62.5, 125, 250 and 500 mM NaCl (fraction W, wash) in PBS buffer to remove unspecifically bound proteins. After eluting all proteins (fraction E) with 50 μl Laemmli buffer, 10 μl of all fractions were analyzed in immunoblots with anti-GFP antibodies (Molecular Probes, Leiden, The Netherlands).

Cell fractionation

Subcellular fractionation were carried out as described by Harding et al. (1995) with the following modifications. Cells were starved 20 h in 1% glycerol incubated with 30 μM isopropyl-β-D-thiogalactopyranoside for 2 h. Cells were lysed using a cell disruptor (Braunschweig, Germany). Digestion of DNA was carried out using PvuII (Pharmacia, Freiburg) and Mg2+ to prevent formation of spheroplasts. DNA fragments were digested with corresponding enzymes. DNA fragments were generated by PCR using (i) pRS316/AUT2 and oligonucleotides AUT2-2hybrid and AUT2-2hybrid,1; (ii) pGAD-C1/AUT2 and oligonucleotides AUT2-2hybrid and AUT2-2hybrid,2; and (iii) pAS1-CYH2/AUT2 and oligonucleotides AUT2-2hybrid and AUT2-2hybrid,3 yielding pGAD-C1/AUT2, pGFP-C/AUT2, and oligonucleotides AUT2-2hybrid and AUT2-2hybrid,1; (ii) pGFP-C/AUT2 and oligonucleotides TB1 and TB1,1; (iii) pGFP-C/AUT2 and oligonucleotides TB2 and TB2,1. In-frame fusions with the GAL4 DNA-binding domain were similarly constructed by ligating (i) a 1.5 kb BamHI–XhoI fragment carrying AUT2; (ii) a 1.37 kb smal–PstI fragment carrying TB2 in pGAD-C1 (James et al., 1996), digested with BamHI–SalI, yielding pGAD-C1/AUT2, and pGAD-C1/TB1, respectively. Ligation of (i) a 1.37 kb smal–PstI fragment carrying TB2 in the respective sites pGAD-C1 yielded pGAD-C1/TB2. DNA fragments were generated by PCR using (i) pRS316/AUT2 and oligonucleotides AUT2-2hybrid and AUT2-2hybrid,1; (ii) pB306 (D.Botstein, Stanford, CA) and oligonucleotides TB1 and TB1,1; (iii) pB312 (D.Botstein, Stanford, CA) and oligonucleotides TB2 and TB2,1. In-frame fusions with the GAL4 DNA-binding domain were similarly constructed by ligating (i) a 1.5 kb Ncol–SalI fragment containing AUT2; (ii) a 0.35 kb BamHI–XhoI fragment containing AUT7 into the BamHI–XhoI sites of pAS1-CYH2 (S.J.Elledge, Houston, USA), yielding pAS1-CYH2/AUT2 and pAS1-CYH2/AUT7. Also these DNA fragments were generated by PCR with (i) pRS316/AUT2 and oligonucleotides AUT2-2hybrid and AUT2-2hybrid,1; (ii) pRS316/AUT7 and oligonucleotides AUT7-2hybrid,2 and AUT7-2hybrid, (iii) PCR fragments were purified from agarose gels and digested with corresponding enzymes.

Plasmids pGAD-C1/AUT2, pAS1-CYH2/AUT2 and pAS1-CYH2/AUT7 were able to complement the vesicle accumulation defect of aut2Δ and aut7A cells, respectively, indicating biological activity.

GFP-Aut2 and GFP-Aut7 fusion proteins

pBR229 and pBRN96 (J.Hegemann, Giessen, Germany) were digested with XbaI–HindIII and a 1.5 kb XbaI–HindII fragment containing AUT2 was ligated in frame, yielding pGFP-N/AUT2 and pGFP-C/AUT2. The DNA fragment was generated by PCR using pRS316/AUT2 and oligonucleotides GFP1 AUT2, GFP2 AUT2, pGFP-N/AUT7 and pGFP-C/AUT7 were created similarly by ligating a 0.35 kb XbaI–HindIII fragment containing AUT7 in-frame into XbaI–HindIII of pBR229 and pBRN96, respectively. The 0.35 kb DNA-fragment was also generated by PCR using pRS316/AUT7 and oligonucleotides GFP1 AUT7, GFP2 AUT7. Biological activity of all GFP fusion proteins were confirmed by complementation tests.

pGFP-C/AUT2, pGFP-N/AUT2, pGFP-C/AUT7 and pGFP-N/AUT7 were transformed in aux2-1 mutant cells to generate the corresponding conditional null mutant strains YTL7 and YTL11. Expression of the MET25 promoter, strains were incubated overnight in methionine-free CM medium. Localization of GFP was examined by fluorescence microscopy using a Zeiss Axioscope.

Measurement of protein turnover and survival rates during starvation, microscopy using Nomarski optics and preparation for electron microscopy using permanganate fixation and epon embedding was performed as described elsewhere (Straub et al., 1997).

We routinely used starvation on nitrogen-free media to induce the autophagic process.

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References


