Autophagy and the cytoplasm to vacuole targeting pathway both require Aut10p

Henning Barth, Khuyen Meiling-Wesse, Ulrike D. Epple, Michael Thumm*

University of Stuttgart, Institute of Biochemistry, Pfaffenwaldring 55, 70569 Stuttgart, Germany

Received 20 September 2001; accepted 9 October 2001

First published online 19 October 2001

Edited by Horst Feldmann

Abstract We here report the identification of *AUT10* as a novel gene required for both the cytoplasm to vacuole targeting of proaminopeptidase I and starvation-induced autophagy. *aut10*Δ cells are impaired in maturation of proaminopeptidase I under starvation and non-starvation conditions. A lack of Aut10p causes a defect in autophagy prior to vacuolar uptake of autophagosomes. Homozygous *aut10*Δ diploids do not sporulate. Vacuolar acidification indicated by accumulation of quinacrine is normal in *aut10*Δ cells and mature vacuolar proteinases are present. A biologically active Ha-tagged Aut10p, chromosomally expressed from its endogenous promoter, localizes in indirect immunofluorescence microscopy in the cytosol and on granulated structures, which appear clustered around the vacuolar membrane. This localization differs from known autophagy proteins.

Key words: Autophagy; Cytoplasm to vacuole targeting pathway; Aminopeptidase I

1. Introduction

To survive nutrient limitation eukaryotic cells have evolved a sophisticated protein transport pathway called autophagy. Autophagy delivers parts of the cells to the lysosome (vacuole) for degradation [1,2]. This breakdown affects nearly half of all cellular proteins in a day and recycles building blocks and energy.

Autophagy starts with formation of autophagosomes. These double-membrane vesicles unspecifically enclose parts of the cytoplasm and even whole organelles. After fusion of the outer membrane layer of autophagosomes with the vacuole a still membrane-enclosed autophagic body is released to the vacuolar lumen and broken down together with its cytosolic content [3,4].

Our knowledge of autophagy increased significantly due to three independent screens for autophagy-defective mutants in *Saccharomyces cerevisiae*, and the identification of autophagy genes termed *APG* [5], *AUT* [6] and *CVT* [7], respectively. Landmarks in the analysis of autophagy have been the identification of two ubiquitin-like conjugation systems required for formation of autophagosomes. One system covalently couples Apg12p to Apg5p, the other covalently links Aut7p to the membrane lipid phosphatidylethanolamine [8].

Interestingly proaminopeptidase I (proAPI) is selectively targeted to the vacuole via autophagy under starvation conditions [9]. In growing cells, however, proAPI reaches the vacuole via the cytoplasm to vacuole targeting (cvt) pathway. The cvt pathway significantly resembles autophagy in using double-membrane cvt vesicles and requiring almost all autophagy proteins. But compared to autophagosomes, cvt vesicles are smaller and exclude cytosol [9]. Furthermore, the cvt pathway depends on some specific gene products not essential for autophagy [10,11].

Our initial screen for autophagy mutants identified eight *AUT* genes. Since we expected the existence of further autophagy genes, we started a novel screen using the ~5000 yeast deletion strains which cover the non-essential part of the ~6000 yeast genes. In each strain an individual gene was deleted in the 'Yeast Deletion Project'. We screened these 5000 deletion strains for autophagy mutants and have already reported the identification of *AUT8* [12]. Here we report the identification and characterization of *AUT10*, which we demonstrate to be essential for both autophagy and the cvt pathway. *aut10*Δ cells are unable to mature proAPI and are defective in autophagy prior to vacuolar fusion of autophagosomes. Furthermore, homozygous diploid *aut10*Δ cells do not sporulate. In *aut10*Δ cells mature vacuolar proteinase B and carboxypeptidase Y are detectable and vacuolar acidification appears normal. Indirect immunofluorescence microscopy localizes a chromosomally expressed, biologically active Ha-tagged Aut10p (Aut10-Hap) in the cytosol and on granulated structures, which appear clustered around the vacuole.

2. Materials and methods

2.1. Strains, media, antibodies and reagents

Media were prepared according to [13]. Starvation was done in 1% potassium acetate. Antibodies used: anti-Ha: clone 16B12 (BabCo, USA); monoclonal anti-green fluorescent protein (GFP): (Clontech, USA); anti-3-phosphoglycerate kinase (PGK) and anti-carboxypeptidase Y (CPY) (Molecular Probes, Leiden, The Netherlands); horseradish peroxidase (HRPO)-conjugated goat anti-rabbit (Medac, Hamburg) and HRPO-conjugated goat anti-mouse (Dianova, Hamburg). Antibodies to proAPI are described in [12].

Chemicals: Zymolyase-100T (Seikagaku, Tokyo, Japan); phenylmethylsulfonyl fluoride (PMSF) and phloxin B (Sigma, Deisenhofen, Germany); oligonucleotides (MWG-Biotec, Ebersberg, Germany), other chemicals were of analytical grade and from Sigma or Merck (Darmstadt, Germany). For detection of peroxidase-labeled antibodies on immunoblots the ECL detection kit (Amersham, Braunschweig, Germany) was used.

YHB1, YHB2 and YHB3 are isogenic to wild-type strain WCG4a *Matα ura3 his3-11,15 leu2-3,112* [14] and were generated as described

*Corresponding author. Fax: (49)-711-6854392.
E-mail address: thumm@po.uni-stuttgart.de (M. Thumm).

0014-5793/01/$20.00
PII: S0014-5793(01)03016-2

below. Homozygous diploid strains used were from Euroscarf, Frankfurt, Germany and isogenic to BY4743 (*MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0*).

2.2. Chromosomal deletion of AUT10

A PCR fragment consisting of the kanamycin resistance gene flanked by *AUT10* sequences was generated using fr021w-1 (tgttccagt taactctgtatcctt ttctcttc ggcctg acacagctgaa gcttcgtacgc), and fr021w-2 (tgcgttgtga cgtacggaaggca gcgcgagacacttcc gtgagcataggcc actagtggatctg) and plasmid pUG6 [15]. Chromosomal replacement of *AUT10* with this fragment in WCG4a yielded YHB1. Gene replacement was confirmed by Southern blotting (not shown).

2.3. Generation of tagged AUT10 species

YHB2 was generated by chromosomal integration of a fragment consisting of GFP and a *Schizosaccharomyces pombe HIS5* marker in WCG4a. The fragment was created by PCR using plasmid pFA6a-GFP(S65T)-HIS3MX6 [16], YFR-GFP-1 (gcgattgcttaa tattgtcacagtattccatctt gatggatcggatccc cgggttaattaa), and YFR-GFP-2 (tgcgttgtgacgta cggaagg cagcgcg agacacttccgtga gaattcgagctcgtttaaac).

Chromosomal integration of a PCR fragment consisting of a triple Ha tag and an *S. pombe HIS5* marker in WCG4a yielded YHB3. The fragment was created using plasmid p3xHA-HIS5 (S. Munroe, Cambridge, UK), YFR-HA-1 (gcgattgcttaatatt gtcacagtattccatctt gatggatcatcatcatca tcatcatggagcaggggcgggtgc), and YFR-HA-2 (tgcgttgt gacgta cggaaggcagc gcgagacacttccgtg agaggtcgacggta tcgataag).

Positive transformants were selected on plates lacking histidine. Southern blotting confirmed correct gene replacement (not shown).

2.4. Quinacrine staining

Cells were harvested and washed in 10 mM HEPES, 2% glucose pH 7.4, then incubated in the same buffer for 3–5 min with 200 μM quinacrine [17] and washed with buffer.

2.5. Indirect immunofluorescence

Immunofluorescence was done according to [18] with the following modifications. Cells were fixed by adding formaldehyde to a concentration of 3.5% and 1 M potassium phosphate pH 6.5 to a concentration of 100 mM. After 2 h at room temperature the cells were washed three times with SP buffer (1.2 M sorbitol, 0.1 M KH_2PO_4 pH 6.5), resuspended in SP buffer containing 20 mM β-mercaptoethanol and 45 μg/ml Zymolyase T100 and spheroplasted at 30°C for 30 min. After labelling with mouse anti-Ha antibody, Cy™3-conjugated goat anti-mouse IgG (Dianova, Hamburg, Germany) was used as secondary antibody. The cells were then covered with mounting solution containing DAPI (0.4 μg/ml) and visualized with a Zeiss Axioskop 2 plus and an Axiocam image system. For overlays pictures were pseudo-colorized with Photoshop.

3. Results

3.1. Identification of YFR021w/AUT10

We screened the ~5000 yeast deletion strains for a reduced ability to survive nitrogen starvation [12]. This phenotype is typical for autophagy mutants [5,6] and can be monitored on starvation plates containing the red dye phloxin B [5]. Due to the increased number of dead cells, autophagy mutants turn red on these plates, while wild-type colonies stay almost white. Starvation sensitivity turned out to be quite unspecific, however, more than 1300 colonies were positive. We further checked these deletion strains in immunoblots for a defect in maturation of proAPI and here report identification of *yfr021wΔ* cells in this screen. As shown below, *YFR021w* is essential for autophagy, we therefore term this open reading frame *AUT10*.

To analyze *YFR021w/AUT10* we chromosomally deleted it in our wild-type strain WCG4a by replacement with a PCR-generated cassette conferring resistance to kanamycin. Correct gene replacement was confirmed by Southern blotting (not shown).

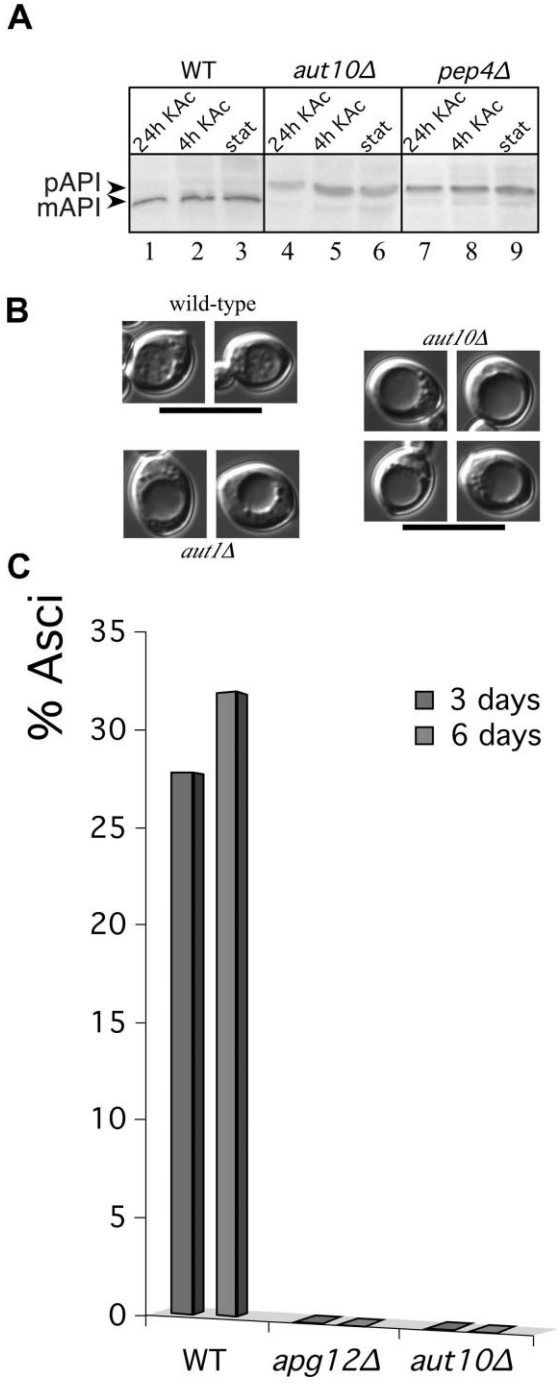


Fig. 1. *AUT10* is essential for the cvt pathway and autophagy. A: *aut10Δ* cells of the stationary growth phase (lane 6), starved for 4 h (lane 5) and 24 h (lane 4) in 1% K-acetate are impaired in maturation of proaminopeptidase I. Wild-type (lanes 1–3) and cells defective in vacuolar proteinase A (*pep4Δ*) (lanes 7–9) are included. Crude extracts of cells were subjected to SDS–PAGE, blotted on polyvinylidene fluoride (PVDF) membranes and probed with antibodies against aminopeptidase I. B: *aut10Δ* cells do not accumulate autophagic bodies in their vacuoles during starvation with PMSF. Wild-type cells and *aut1Δ* cells [23] defective in autophagy are included. Cells were starved 4 h in 1% K-acetate and visualized with Nomarski optics. Bar = 10 μm. C: Homozygous diploid *aut10Δ* cells do not sporulate. Diploid wild-type cells and homozygous diploid *apg12Δ* cells [24] defective in autophagy are included. Cells were sporulated in 1% K-acetate and the number of asci was counted after 3 days (left bar) and 6 days (right bar) and expressed as a percentage of total cells.

3.2. *aut10Δ (yfr021wΔ) cells are defective in the cvt pathway and autophagy*

In growing cells proAPI reaches the vacuole, where it is proteolytically matured, via the cvt pathway. During nitrogen starvation autophagy takes over the transport of proAPI to the vacuole. Therefore, cells with a defect in the cvt pathway but not autophagy show proAPI under non-starvation conditions and mature aminopeptidase I (mAPI), when autophagy is induced by starvation. *aut10*Δ cells grown to early stationary phase as well as starved cells show an almost complete block in maturation of proAPI (Fig. 1A, lanes 4–6). This suggests a function of Aut10p in both the cvt pathway and autophagy. Next we directly checked the autophagic capacity of *aut10*Δ cells.

3.3. *aut10Δ cells are defective in autophagy prior to vacuolar uptake of autophagosomes*

In *S. cerevisiae* autophagic bodies are broken down in the vacuole dependent on vacuolar proteinase B. Addition of the proteinase B inhibitor PMSF to starving wild-type cells therefore leads to the accumulation of autophagic bodies in the vacuole (Fig. 1B) [4,6]. *aut10*Δ cells do not accumulate autophagic bodies in their vacuoles under these conditions (Fig. 1B), indicating a defect in the autophagic pathway prior to the fusion of autophagosomes with the vacuole.

Autophagy mutants are unable to undergo the cell differentiation process of sporulation [1,2]. Accordingly, homozygous diploid *aut10*Δ cells do not sporulate (Fig. 1C).

3.4. *Features and homologues of Aut10p*

Aut10p is a protein of yet unknown function with a predicted molecular mass of 55 kDa. Aut10p is not classified as a membrane protein by all algorithms, but a rather hydrophobic region at amino acids 48–64 is present (Fig. 2A). Consistent with the sporulation defect of *aut10*Δ cells (Fig. 1C), Aut10p was termed Nmr1p for 'needed for premeiotic replication' (http://genome-www.stanford.edu/Saccharomyces/), but no data or publications are known.

Aut10p exhibits two WD40-like repeats at amino acids 234–274 and 277–318. WD repeat proteins, whose best studied member is the Gβ subunit of heterotrimeric G-proteins, contain at least four repeats [19]. There is no common function attributed to WD repeat proteins, but they are suggested to share a common β-propeller fold, which is presumed to form a scaffold for interaction with other proteins. With only two repeats Aut10p is not a typical WD repeat protein. Aut10p shares homologies with ∼40 proteins from *Homo sapiens* to *S. pombe*. Phylogenetic analysis groups these proteins into four subfamilies (Fig. 2B). From the proteins of the Aut10p subfamily (Fig. 2C) no function can be deduced.

3.5. *In aut10Δ cells mature vacuolar proteinases are present and vacuolar acidification appears normal*

Immunoblot analyses of *aut10*Δ cells of the early stationary

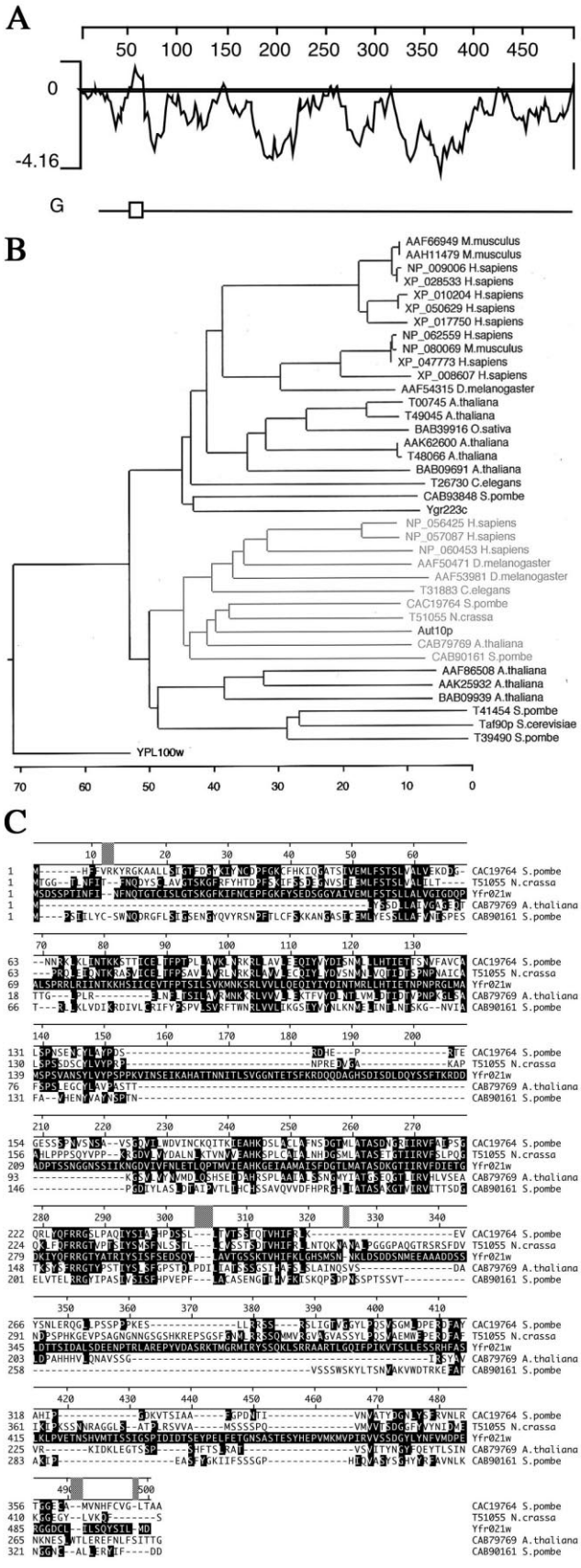


Fig. 2. A: Aut10p shows a hydrophobic region between amino acids 48 and 64. Hydrophobicity analysis was done according to [25]. B: Phylogenetic analysis of proteins with homologies to Aut10p unravels four different families. Phylogenetic analysis was done using Clustal [26]. C: The homology of Aut10p with other proteins is not restricted to the WD40 repeats.

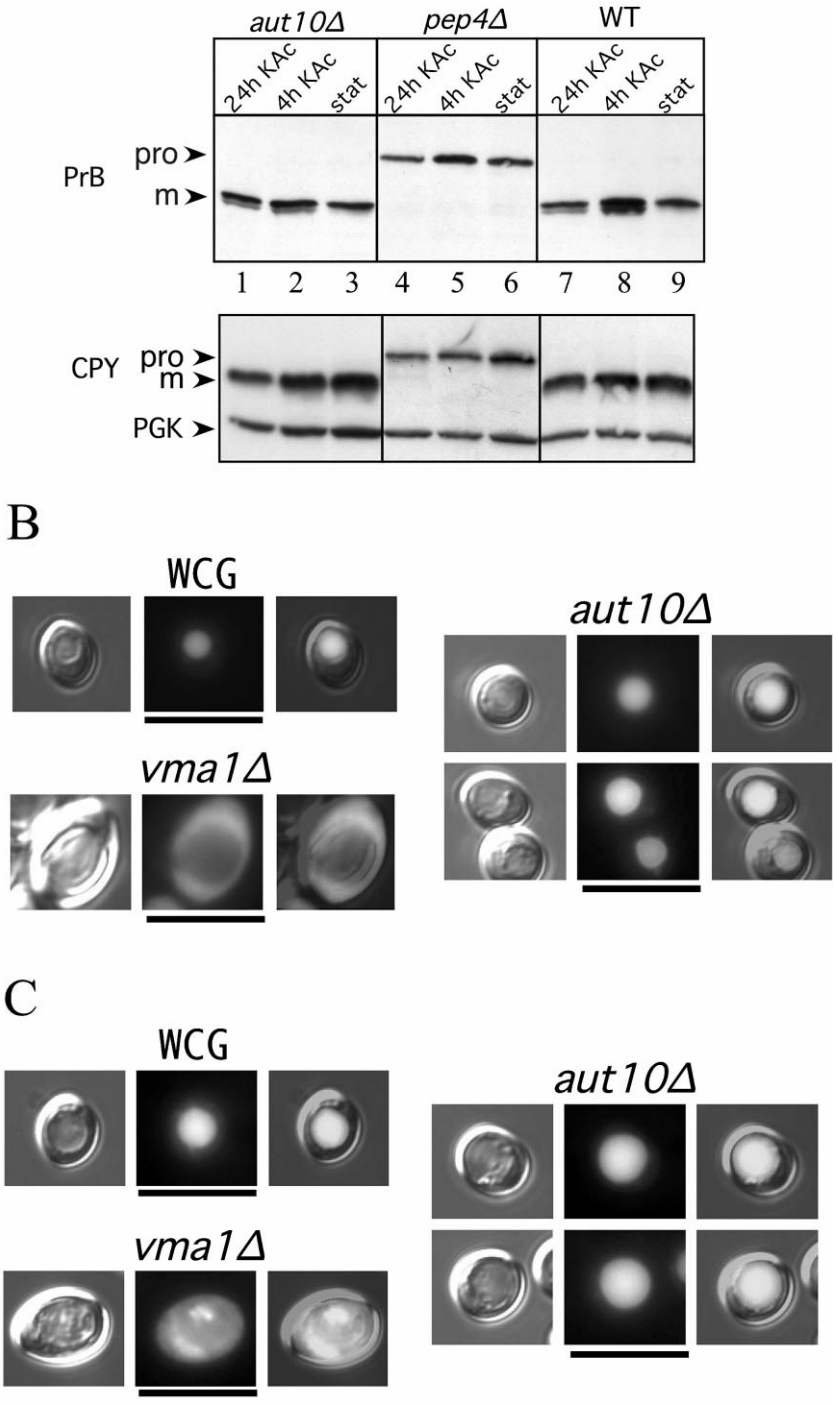


Fig. 3. A: In *aut10*Δ cells grown to stationary phase (lane 3), starved for 4 h (lane 2) and 24 h (lane 1) in 1% K-acetate mature proteinase B (PrB) and mature carboxypeptidase Y (CPY) are detectable. Wild-type (lanes 7–9) and cells lacking vacuolar proteinase A (*pep4*Δ) (lanes 4–6) are included. Crude extracts were separated by SDS–PAGE, blotted on PVDF membranes and probed with antibodies against proteinase B (upper panel). The membrane was then reprobed with antibodies against carboxypeptidase Y (lower panel) and antibodies against 3-phosphoglycerate kinase (PGK). B,C: Accumulation of the fluorescent dye quinacrine in the vacuoles of *aut10*Δ cells indicates normal vacuolar acidification. Wild-type cells (WCG) and *vma1*Δ cells defective in vacuolar acidification are included. B: stationary cells; C: cells starved for 4 h in 1% K-acetate. From left to right: Nomarski, fluorescence and an overlay of Nomarski and fluorescence. Bar = 10 μm.

growth phase and those starved for nitrogen, respectively, showed mature vacuolar proteinase B and mature carboxypeptidase Y, but no significant amounts of proproteinase B or procarboxypeptidase Y (Fig. 3A). Quinacrine is a fluorescent dye, which accumulates in acidic vacuoles [17]. Wild-type-like accumulation of quinacrine in the vacuoles of *aut10*Δ cells grown to stationary phase and those starved for nitrogen indicates normal vacuolar acidification (Fig. 3B,C).

3.6. Localization of biologically active Ha-tagged Aut10p

To localize Aut10p, we chromosomally integrated a PCR-generated cassette consisting of GFP [20] and an auxotrophic

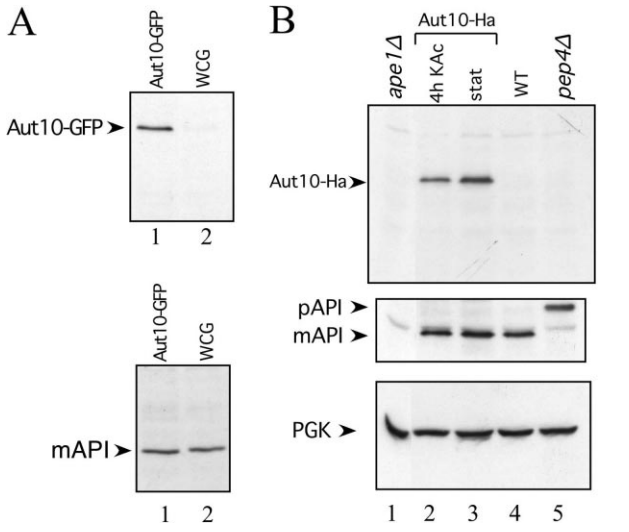
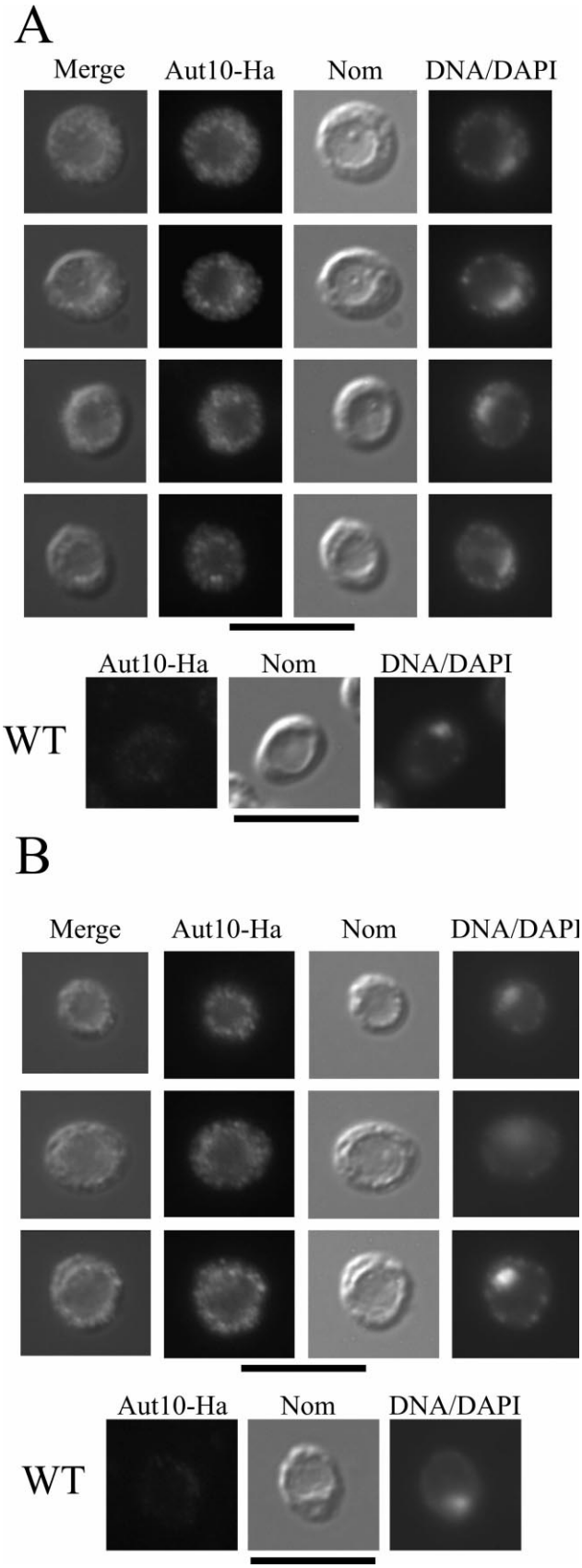


Fig. 4. A: An Aut10 fusion protein with GFP, chromosomally expressed with its native promoter, is detectable in immunoblots using anti-GFP antibodies (upper panel). The fusion protein is biologically active, as indicated by maturation of aminopeptidase I (lower panel). WCG: wild-type. Crude extracts were separated by SDS–PAGE, blotted on PVDF membranes and probed with antibodies against GFP and aminopeptidase I, respectively. B: An Aut10p carrying a triple Ha tag at its carboxy-terminus is detectable in immunoblots. Aut10-Ha is biologically active, indicated by maturation of aminopeptidase I (middle panel). Crude extracts were separated by SDS–PAGE, blotted on PVDF membranes and probed with anti-Ha antibodies. The membrane was then reprobed with antibodies against aminopeptidase I and 3-phosphoglycerate kinase (PGK). Cells chromosomally expressing Aut10-Ha were grown to stationary phase (lane 3) or starved for 4 hours (lane 2). Lane 4: wild-type cells; lane 5: cells lacking vacuolar proteinase A; lane 1: cells lacking aminopeptidase I.

marker at the 3'terminus of *AUT10* (see Section 2.3). Correct integration was confirmed by Southern analysis (not shown). Immunoblot analysis clearly confirmed the presence of Aut10-GFPp, which as indicated by maturation of aminopeptidase I was biologically active (Fig. 4A). However, direct and indirect fluorescence microscopy failed to detect the fusion protein within the cells. Therefore, we further generated an Aut10-Hap, by chromosomal integration of a PCR fragment consisting of a triple Ha tag and an auxotrophic marker at the 3′-terminus of the *AUT10* gene (see Section 2.3). The resulting Aut10-Hap, expressed from the chromosome under control of the endogenous promoter, was detectable in immunoblots as expected at ~60 kDa using monoclonal antibodies to Ha (Fig. 4B). Aut10-Ha was biologically active as demonstrated by maturation of proaminopeptidase I (Fig. 4B) and by accumulation of autophagic bodies in the vacuole, when starved in the presence of PMSF (not shown). In indirect immunofluorescence microscopy using an anti-Ha antibody Aut10-Ha was



→

Fig. 5. Indirect immunofluorescence localizes Aut10-Ha, chromosomally expressed from its native promoter, in the cytosol and on granulated structures, which appear clustered around the vacuolar membrane. A: stationary cells; B: cells starved for 4 h in 1% K-acetate. Fixed cells were spheroplasted and processed with antibodies to Ha. Wild-type cells lacking a Ha tag are included. From left to right: overlay of immunofluorescence and Nomarski (Merge), immunofluorescence (Aut10-Ha), Nomarski (Nom) and nuclear staining with DAPI (DNA/DAPI). Bar = 10 μm.

visible in both stationary grown and starved cells in the cytosol (Fig. 5A,B) and on granulated punctate structures, which appear to cluster at the vacuolar membrane.

4. Discussion

Autophagy involves membrane flux and formation of unconventional double-membrane transport vesicles. Despite the progress in autophagic research numerous questions remain open. What is the membrane source of autophagosomes? Where and how are autophagosomes formed? What is the nature and function of the organelle defined by the integral membrane protein Aut9/Apg9 [21,22]? An important step in answering these questions is identification of the components of the autophagic machinery. After completion of the 'Yeast Deletion Project' we used the yeast deletion strain collection to screen for novel autophagy genes. Typical for a reverse genetics approach, identification of a mutant implies knowledge of the respective gene. We here report the identification of *YFR021w*/*AUT10* in this screen. Our study clearly indicates the requirement of Aut10p for both the cvt pathway and autophagy (Fig. 1A–C). In autophagy, a lack of Aut10p leads to a defect prior to vacuolar fusion of autophagosomes (Fig. 1B). We further checked for effects on vacuolar biogenesis. Deletion of *AUT10* has no obvious influence on vacuolar acidification (Fig. 3B,C), no significant amounts of the proforms of vacuolar proteinase B and carboxypeptidase Y are detectable in immunoblots (Fig. 3A) and the vacuole is not fragmented (Figs. 1B and 3B,C). The localization of Aut10-Hap at granulated, punctate structures, which appear clustered around the vacuole (Fig. 5), is highly interesting, since it is different from known autophagy proteins. Clarification of the nature of these structures will therefore surely give new insights. Recruitment of Aut10-Hap at structures together with the existence of two WD40 repeats in Aut10p might be a hint for interactions with other proteins. Speculatively, Aut10p might dimerize, thus bringing together four WD40 repeats necessary to form a platform for protein interactions. Phylogenetic analysis puts Aut10p in a family consisting of 11 proteins of so far unknown function from species such as *H. sapiens*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *S. pombe*, *Arabidopsis thaliana* and *Neurospora crassa*. Most likely all these proteins share a similar function. With Ypl100wp (19% identity) and Ygr223c (19% identity) Aut10p has two homologues with unknown function in yeast. Studies are under way to check if these relatives have a function similar to Aut10p.

Acknowledgements: We thank the labs of the 'Yeast Deletion Project' and D.H. Wolf for discussions and support. This work was supported by DFG Grant Wo210/12-4 and the 'Fonds der Chemischen Industrie'.

References

[1] Klionsky, D.J. and Ohsumi, Y. (1999) Annu. Rev. Cell Dev. Biol. 15, 1–32.
[2] Thumm, M. (2000) Microsc. Res. Tech. 51, 563–572.
[3] Baba, M., Takeshige, K., Baba, N. and Ohsumi, Y. (1994) J. Cell Biol. 124, 903–913.
[4] Takeshige, K., Baba, M., Tsuboi, S., Noda, T. and Ohsumi, Y. (1992) J. Cell Biol. 119, 301–311.
[5] Tsukada, M. and Ohsumi, Y. (1993) FEBS Lett. 333, 169–174.
[6] Thumm, M., Egner, R., Koch, B., Schlumpberger, M., Straub, M., Veenhuis, M. and Wolf, D.H. (1994) Febs Lett. 349, 275–280.
[7] Harding, T.M., Hefner-Gravink, A., Thumm, M. and Klionsky, D.J. (1996) J. Biol. Chem. 271, 17621–17624.
[8] Ohsumi, Y. (2001) Nat. Rev. Mol. Cell Biol. 2, 211–216.
[9] Baba, M., Osumi, M., Scott, S.V., Klionsky, D.J. and Ohsumi, Y. (1997) J. Cell Biol. 139, 1687–1695.
[10] Abeliovich, H., Darsow, T. and Emr, S.D. (1999) EMBO J. 18, 6005–6016.
[11] Scott, S.V., Nice, D.C., Nau, J.J., Weisman, L.S., Kamada, Y., Keizer-Gunnink, I., Funakoshi, T., Veenhuis, M., Ohsumi, Y. and Klionsky, D.J. (2000) J. Biol. Chem. 275, 25840–25849.
[12] Barth, H. and Thumm, M. (2001) Gene 274, 151–1156.
[13] Ausubel, F.M., Brent, R., Kingston, R.E. and Moore, D.D. (1987) Current Protocols in Molecular Biology, Greene, New York.
[14] Suriapranata, I., Epple, U.D., Bernreuther, D., Bredschneider, M., Sovarasteanu, K. and Thumm, M. (2000) J. Cell Sci. 113, 4025–4033.
[15] Güldener, U., Heck, S., Fielder, T., Beinhauer, J. and Hegemann, J.H. (1996) Nucleic Acids Res. 24, 2519–2524.
[16] Longtine, M.S., McKenzie 3rd, A., Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P. and Pringle, J.R. (1998) Yeast 14, 953–961.
[17] Roberts, C.J., Raymond, C.K., Yamahiro, C.T. and Stevens, T.H. (1991) Methods Enzymol. 194, 644–661.
[18] Egner, R., Mahe, Y., Pandjaitan, R. and Kuchler, K. (1995) Mol. Cell. Biol. 15, 5879–5887.
[19] Smith, T.F., Gaitatzes, C., Saxena, K. and Neer, E.J. (1999) Trends Biochem. Sci. 24, 181–185.
[20] Cubitt, A.B., Heim, R., Adams, S.R., Boyd, A.E., Gross, L.A. and Tsien, R.Y. (1995) Trends Biochem. Sci. 20, 448–455.
[21] Lang, T., Reiche, S., Straub, M., Bredschneider, M. and Thumm, M. (2000) J. Bacteriol. 182, 2125–2133.
[22] Noda, T., Kim, J., Huang, W.P., Baba, M., Tokunaga, C., Ohsumi, Y. and Klionsky, D.J. (2000) J. Cell Biol. 148, 465–480.
[23] Schlumpberger, M., Schaeffeler, E., Straub, M., Bredschneider, M., Wolf, D.H. and Thumm, M. (1997) J. Bacteriol. 179, 1068–1076.
[24] Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M.D., Klionsky, D.J., Ohsumi, M. and Ohsumi, Y. (1998) Nature 395, 395–398.
[25] Engelman, D.M., Steitz, T.A. and Goldman, A. (1986) Annu. Rev. Biophys. Biophys. Chem. 15, 321–353.
[26] Higgins, D.G. and Sharp, P.M. (1989) Comput. Appl. Biosci. 5, 151–153.