Aut7p, a soluble autophagic factor, participates in multiple membrane trafficking processes

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Received for publication, February 4, 2000, and in revised form, May 11, 2000
Published, JBC Papers in Press, June 2, 2000, DOI 10.1074/jbc.M000917200

Aut7p, a protein recently implicated in autophagic events in the yeast Saccharomyces cerevisiae, exhibits significant homology to a mammalian protein, p16, herein termed GATE-16 (Golgi-associated ATPase Enhancer of 16 kDa), a novel intra-Golgi transport factor. Here we provide evidence for the involvement of Aut7p in different membrane trafficking processes. Aut7p largely substitutes for the activity of GATE-16 in mammalian intra-Golgi transport in vitro. In vivo, AUT7 interacts genetically with endoplasmic reticulum to Golgi SNAREs, specifically with BET1 and SEC22. Aut7p interacts physically with the following two v-SNAREs: Bet1p, which is involved in endoplasmic reticulum to Golgi vesicular transport, and Nyv1p, implicated in vacuolar inheritance. We suggest that, in addition to its role in autophagocytosis, Aut7p has pleiotropic effects and participates in at least two membrane traffic events.

Membrane trafficking in eukaryotic cells is a highly regulated process that is essential for secretion of macromolecules, as well as for the maintenance of distinct subcellular compartments (1, 2). This process encompasses a series of highly regulated events, including cargo selection and vesicle budding at the donor membrane, followed by transport, docking, and fusion of the transport vesicle with the target organelle. We previously identified a cytosolic factor, GATE-16, which participates in intra-Golgi transport (3, 4). However, the yeast homologue of GATE-16, Aut7p, was recently shown to participate in autophagy (5, 6). In this study, we have questioned whether Aut7p plays a role in constitutive protein transport, in addition to its involvement in autophagy.

Vesicular transport between the ER and the Golgi apparatus in the yeast Saccharomyces cerevisiae has been extensively studied. The first step in this process, vesicle budding, involves the assembly of the COPII coat, composed of the Sec13p-Sec31p complex (7–9), the Sec23p-Sec24p heterodimer (10), as well as a small GTPase, Sar1p (11), and the multidomain protein Sec16p (12, 13). Docking of an ER-derived COPII vesicle with the cis-Golgi compartment takes place just after, or concurrently with, a tethering event mediated by Uso1p (14), the yeast homologue of p115 (15, 16). It has been further suggested that docking involves the interaction of ER to Golgi v-SNAREs, Bet1p, Bos1p, Sec22p, and Ykt6p (17–20), with Sed5, the cognate t-SNARE on the Golgi (21) to form the v-t-SNARE complex. This complex binds the yeast SNAP (Sec17p) and NSF (Sec18p), which in turn catalyze its disassembly (19) after a round of fusion, thus allowing a new round to take place (22, 20).

Homotypic vacuolar fusion is the last step in yeast vacuole inheritance. Like many membrane trafficking processes, it is mediated by a number of membrane and soluble factors, including Vam3p (a t-SNARE), Nyv1p (a v-SNARE), Ypt7p (a Rab protein), Sec17p, Sec18p, and a low molecular weight factor, LMA1 (24–28). Vacular homotypic fusion has been divided into the following three distinct subset reactions: priming, docking, and fusion. Priming of SNARE molecules for a new fusion event is mediated by Sec17p, Sec18p, and LMA1 (22, 29). Based on a cell-free system reconstituting vacuolar homotypic fusion, it appears that the formation of the SNARE complex is only an intermediate step in the overall fusion reaction (30). Accordingly, SNARE molecules are involved in docking between donor and acceptor membranes, whereas another set of proteins participates in subsequent stages of the fusion process. This model for the course of events is supported by Peters and Mayer (31), who have suggested that calmodulin and other yet-unidentified factors are involved in mediating late stages of vacuolar fusion.

The yeast vacuole takes in membrane-bound traffic through at least the following five different transport pathways: the carboxypeptidase Y (CPY) pathway, the alkaline phosphatase I (Alp1) pathway, the endocytic pathway, autophagy, and the cytoplasm to vacuole targeting (Cvt) pathway (32). Each of these pathways has different cargo, transport intermediates, and genetic requirements. Autophagy is a bulk protein degradation process by which cytoplasmic components, including organelles, become enclosed in double membrane structures (autophagosomes), which are then delivered to the vacuole for degradation (33). Recent studies have revealed that the autophagic process in the budding yeast Saccharomyces cerevisiae is similar to that of higher eukaryotes (34–36). Autophagy in yeast may be dissected into the following series of subreactions: starvation signaling, formation of autophagosomes, targeting of autophagosomes to the vacuole, docking and fusion with the vacuolar membrane, and degradation of the autophagosomal body within the vacuole (37, 38). Transport of autophagosomes to lysosomes or vacuoles should therefore be regarded as a membrane traffic process. The Cvt pathway is a constitutive biosynthetic process that shares many common transport components with autophagy (39, 40). Both Cvt and the autophagy pathways involve at least two membrane fusion events, which are dependent on Sec18p and on the vacuolar t-SNARE Vam3p; the latter acts as...
a multispecific receptor for heterotypic membrane docking and fusion reactions (24). Additionally, Tlg2p, a member of the syntaxin family of t-SNARE proteins, and Vps45p, a Sec1p homologue, are reported to be required for the constitutive Cvt pathway (41).

In this study, we demonstrate that Aut7p can largely replace GATE-16 activity in vitro, indicating that the two proteins share a similar, conserved function. Aut7p is a peripheral membrane protein localized predominantly on the Golgi complex and vacuolar membrane. It interacts genetically and physically with Bet1p, a v-SNARE involved in ER to Golgi protein transport. Aut7p also interacts physically with the vacuolar membrane protein localized predominantly on the Golgi complex and the docking and transport. Aut7p also interacts with the vacular membrane protein localized predominantly on the Golgi complex and the docking and transport.

Involvement of Aut7 in Membrane Trafficking

**TABLE I** Yeast strains used in this study

| Strain       | Genotype                     | Source         |
|--------------|------------------------------|----------------|
| W303-ab      | Mat a ade2 his3-11,15 leu2-3,112 trp1-1 ura3-1 | J. Gerst       |
| W303-1a      | Mat a ade2 his3-11,15 leu2-3,112 trp1-1 ura3-1 | J. Gerst       |
| W303-1b      | Mat a ade2 his3-11,15 leu2-3,112 trp1-1 ura3-1 | J. Gerst       |
| RSY944       | Mat a lys2-801 ura3-52 bet1-1 | R. Schekman    |
| RSY271       | Mat a his4-619 ura3-52 sec18-1 | R. Schekman    |
| RSY976       | Mat a ura3-52 ypl1-3 | R. Schekman    |
| RSY271       | Mat a his4-619 ura3-52 sec18-1 | R. Schekman    |
| RSY979       | Mat a ura3-52 sec7-4 | R. Schekman    |
| RSY941       | Mat a ura3-52 leu2-3,112 sec12-1 | R. Schekman    |
| RSY314       | Mat a ura3-52 sec13-3 | R. Schekman    |
| RSY1010      | Mat a leu2-3,112 ura3-52 sec21-1 | R. Schekman    |
| RSY324       | Mat a ura3-52 sec24-2 | R. Schekman    |
| RSY641       | Mat a leu2-3,112 ura3-52 sec23-2 | R. Schekman    |
| CBY285       | Mat a trp1 ura3 leu2 lys2 sed5-1 | C. Barlowe     |
| RSY955       | Mat a leu2-3,112 sec32-1 | R. Schekman    |
| RSY952       | Mat a leu2-3,112 ura3-52 sec31-1 | R. Schekman    |
| RSY101       | Mat a sec20-1 ura3-52 his4-619 | R. Schekman    |
| MLY101       | Mat a trp1-1 ade2-1 can1-100 leu2-3,112 his3-11,15 ura3-52 ufe1::TRP1 + (CEN LEU ufe1-1) | J. Lewis       |
| RSY955       | Mat a leu2-3,112 sec32-1 | R. Schekman    |
| ZE1          | Mat a ade2 leu2 ura3 trp1 ura3 asn7::KAN | This study     |
| ZE14         | Mat a ade2 leu2 trp1 ura3 his3 asn7::KAN | This study     |
| ZE15         | Mat a ade2 leu2 trp1 ura3 his3 asn7::KAN | This study     |
| ZE15-bet1    | Mat a ade2 leu2 trp1 ura3 asn7::KAN bet1-1 | This study     |
| ZE15–18      | Mat a ade2 trp1 his3 ura3 asn7::KAN sec18-1 | This study     |

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antisera against Bet1p, Sec22p, and Bos1p were a generous gift from R. Schekman and S. Ferro-Novick. Anti-CPY and anti-glycosphosphatidylinositol-anchored surface glycoprotein (Gas1p) were obtained from H. Riezman. Anti-Ufe1p antibodies were obtained from H. Pelham. Affinity-purified anti-Sed5p were obtained from D. Gallwitz. Anti-3-phosphoglycerate kinase (PGK) and anti-dolichol phosphate mannose synthase (Dpm1p) antibodies were purchased from Molecular Probes, Inc. Horseradish peroxidase-conjugated secondary antibodies were obtained from Bio-Rad.

**Strains and Media**—The yeast strains used in this study are listed in Table I. Yeast strains were grown in complete medium (YPD; 1% yeast extract, 2% peptone, and 2% glucose), synthetic complete medium (SC; 2% glucose, 0.67% yeast nitrogen base without amino acids, supplemented with amino acids and nutrients), or in synthetic minimal medium (SD; 2% glucose, 0.67% yeast nitrogen base without amino acids, supplemented with the appropriate auxotrophic nutrients). Transformation of *S. cerevisiae* was done by the lithium acetate method (42). Standard yeast techniques for sporulation, tetrad analysis, and gene disruption were employed as described (43). *Escherichia coli* transformations were done as described previously (44).

**Plasmids**—Constructs containing the AUT7 open reading frame were generated by PCR amplification of yeast genomic DNA using Vent polymerase (Biolabs) and subsequent ligation into the vectors pRS424 or pYPEP50 (2 μm URA3 and CEN-based URA3, respectively), behind the AUT7, ADH, or the GAL1-inducible promoters.

To produce His6-Aut7p fusion protein, an EcoRI-BamHI fragment containing the AUT7 gene generated by PCR was introduced into pQE30 (Qiagen) and expressed in the *E. coli* XL-1-blue strain. After inducing protein expression for 2 h with 1 mM isopropyl-β-D-galactopyranoside, the fusion protein (His6-Aut7p) was affinity-purified on Ni2+–nickel-nitriotriacetic acid (NTA)-agarose beads (Qiagen) and further purified by Mono S (cation exchange) column.

**Antibody Production and Purification**—Rabbit polyclonal antiserum were produced against recombinant His6-Aut7p. Pure His6-Aut7p, emulsified in Freund’s complete adjuvant, was injected subcutaneously into two rabbits (0.6 mg/rabbit). Polyclonal antibodies were affinity-purified on nitrocellulose strips containing pure His6-Aut7p.

**Intra-Golgi Transport Assay**—The standard assay mixture (25 μl) contained 0.4 μCi of UDP-[3H]N-acetylgalactosamine (American Radiolabeled Chemicals), 5 μl of a 1:1 mixture of donor and acceptor Chinese hamster ovary Golgi membrane, and crude bovine brain cytosol. Transport reactions were incubated at 30 °C for 2 h, and the incorporation of [3H]N-acetylgalactosamine into vesicular stomatitis virus (VSV)-G protein was determined as described previously (45). The GATE-16-dependent assay was performed as described previously (3). Briefly, each assay contained 0.4 μCi of UDP-[3H]N-acetylgalactosamine, 5 μl of a 1:1 mixture of donor and acceptor Chinese hamster ovary Golgi membrane, 100 μg of β (a crude cytosolic fraction obtained by anion exchange chromatography), 0.5 μg of p115, 5 ng of His6-NSF, 0.25 ng of SNAP, 10 mM palmitoyl coenzyme A, and ATP and UTP regeneration systems.

**Disruption of AUT7**—A fragment of 1700 base pairs containing full-length genomic AUT7 was PCR amplified, using oligonucleotides containing BamHI and KpnI sites and yeast genomic DNA as a template. The resultant PCR product was subcloned into the BamHI and KpnI sites of the pSK+LoxP cloning vector to yield pSK-AUT7. Next, LoxP-KAN-LoxP module selection marker was inserted into the AccI and HpaI sites of pSK-AUT7, removing ~350 base pairs from the open reading frame of AUT7. AUT7 disruption strain in a wild type background was constructed by transforming the pUWII fragment containing the aut7::KAN disruption into the diploid yeast strain W303. Disruption of one of the AUT7 loci was verified by Southern analysis and PCR analysis. Upon sporulation of this diploid strain (ZE1), the resulting tetrads were dissected to yield haploid ∆aut7 cells (ZF14 and ZE15). ZE15 cells were then crossed to bet1–1 and sec18–1 mutants to give diploid strains that sporulated and dissected to yield haploid yeast bearing both mutations (Table I).

**Secretion of Media Proteins and Pulse-Chase Analysis**—Secretion of proteins into the medium was assessed using the method described by Gaynor and Emr (46). Intracellular protein processing was monitored by pulse-chase analysis with [35S]methionine (Amersham Pharmacia Biotech), using anti-CPY and anti-Gas1p antibodies in immunoprecipitation reactions as described (47, 48). Autoradiography was performed with a fluorescence enhancer.

**Subcellular Fractionation and Gradient Analysis**—Cellular fraction of yeast cells was performed as described (49) with minor modifications. Briefly, cells (25 A260 units) were harvested during log phase and lysed with glass beads in 500 μl of buffer 88 (20 mM Heps, pH 7.0, 10 mM KCl, 1.5 mM MgCl2, 50 mM NaCl, 0.5% Triton X-100, 0.1% sodium dodecyl sulfate, 100 mM glucose), and the lysate was centrifuged at 10,000 × g for 10 min. The supernatant (post-nuclear supernatant fraction) was added to the gradient. The gradient was centrifuged at 100,000 × g for 16 h in a Beckman SW32Ti rotor, and 15 fractions were collected from the bottom of the gradient. The protein concentration of each fraction was determined using the Bio-Rad protein assay.
Aut7p Substitutes for GATE-16 Activity in Intra-Golgi Transport in Vitro—Aut7p is a conserved yeast protein exhibiting high homology to proteins found in eukaryotes, with no other homologues in S. cerevisiae. Caenorhabditis elegans has two AUT7-related genes, Arabidopsis thaliana has at least five different AUT7-related genes, and mammals have three different AUT7 homologues. We have recently isolated one of the mammalian homologues of Aut7p, GATE-16, which exhibits 56% amino acid sequence identity and 75% similarity, and demonstrated that it participates in intra-Golgi protein transport (3, 4).

To determine whether Aut7p plays a role in membrane trafficking, we have performed a series of biochemical and genetic experiments. Aut7p is a 117-amino acid protein, constitutively expressed in S. cerevisiae. Anti-Aut7p polyclonal antibodies specifically recognized on Western blot a 14-kDa poly peptide corresponding to Aut7p in a wild type strain and absent in an aut7 null strain (Fig. 1A). These antibodies also recognized GATE-16 (the mammalian homologue of Aut7p) in bovine brain cytosol (Fig. 1A) and recombinant GATE-16 expressed in E. coli (data not shown). When added to a cell-free intra-Golgi transport assay, affinity purified anti-Aut7p antibodies specifically inhibited transport (Fig. 1B), whereas antibodies preincubated with recombinant Aut7p did not. Clearly, in addition to their sequence homology, Aut7p and GATE-16 share immunogenic determinants that are recognizable by anti-Aut7 antibodies. To test whether recombinant Aut7p could stimulate intra-Golgi transport, similar to GATE-16, we purified recombinant His6-Aut7p from E. coli on an Ni2+-nitrotriatric acid (NTA)-agarose column followed by Mono S chromatography. When added to the GATE-16-dependent transport assay (see “Experimental Procedures”), Aut7p significantly stimulated transport, although to a somewhat lesser extent than its mammalian homologue (Fig. 1C). These experiments suggest that...
Aut7p and GATE-16 share a similar function in mediating membrane trafficking.

**Aut7p Is a Peripheral Membrane Protein**—Many soluble proteins involved in vesicular transport are associated with the membrane. To determine the subcellular distribution of Aut7p, we subjected yeast homogenate to differential centrifugation and obtained a soluble fraction, S200 (representing the cytosol), and two membrane fractions, P13 (pelleted at 13,000 \( \times g \)) and P200 (pelleted at 200,000 \( \times g \)). With this series of differential centrifugation steps, all organelles of the secretory pathway are present in one or more of the pellet fractions; the ER and vacuole are located primarily in the P13 fraction, whereas the Golgi partitions in the P200 fraction. Using anti-Aut7p antibodies, Western blot analysis revealed that Aut7p was distributed between the membrane fraction and the cytosol (Fig. 2A). The membrane-associated Aut7p co-fractionated with the cis-Golgi marker Sed5p, found in the P200 pool, and to a somewhat lesser extent with the ER marker Dpm1p, found in the P13 pool. Phosphoglycerate kinase, serving as a control cytosolic factor, exclusively fractionated to the S200 fraction (Fig. 2A). We then further fractionated the P200 fraction, loading it on top of a 20–60% sucrose gradient that was ultracentrifuged at 100,000 \( \times g \) for 17 h. Fractions collected from this gradient were separated by SDS-PAGE and analyzed by Western blotting using different antibodies. Aut7p was found in the 30–49% sucrose fraction, co-fractionating with Sed5p (Fig. 2B), thus corroborating the differential centrifugation experiments.

To find whether Aut7p is a peripheral membrane protein, we attempted to extract the protein from the membrane using a detergent, high salt, or high pH. Although integral, luminal, and peripheral membrane proteins are extractable by detergents that solubilize membranes, a peripheral membrane protein is characteristically extracted by high salt or high pH, conditions that often disrupt protein-protein interactions. Most of the Aut7p associated with the membrane was extracted with Triton X-100, 1 M NaCl, or with 100 mM sodium carbonate, pH 11.5 (Fig. 2C). A similar extraction pattern was observed for Sec17p, serving as a control for peripheral membrane proteins, but not for Sec22p, an integral membrane protein. Evidently, Aut7p is peripherally associated with membranes probably by interaction with other proteins, whose identities remain to be elucidated.

**AUT7 Interacts Genetically with BET1 and SEC22**—Considering the involvement of Aut7p in autophagocytosis under starvation and in protein transport processes under normal conditions, we analyzed systematically the genetic interaction of AUT7 with genes involved in membrane traffic. The ability of Aut7p to largely replace the activity of its mammalian homologue, GATE-16, in intra-Golgi transport suggested participation in early steps of the secretory pathway. We therefore examined whether the AUT7 could act as a multicyclic suppressor of the temperature-sensitive growth phenotype in mutants defective in these transport steps. Table II summarizes a multicyclic-suppression experiment performed on a number of temperature-sensitive secretion mutants. It appears that overexpression of Aut7p specifically suppresses the temperature sensitivity of bet1–1 and sec22–2 mutants (Fig. 3A), whose gene products are ER to Golgi v-SNAREs. The suppression effect of Aut7p on these mutants was accompanied by a complete recovery of total protein secretion under non-permissive conditions (Fig. 3B). Thus, Aut7p interacts genetically with ER to Golgi v-SNAREs by restoring the transport function of their temperature-sensitive alleles.

**Aut7p and Bet1p Form a Protein Complex**—To determine whether the protein products of AUT7 and BET1 interact physically, total yeast membrane extracts were prepared and subjected to immunoprecipitation with anti-Aut7p antibodies. Protein A-Sepharose beads coupled to anti-Aut7p antibodies were mixed with Triton X-100 membrane extracts and then washed, and the eluted material was subjected to Western blot analysis with various antibodies. Anti-Aut7p antibodies specifically precipitated Aut7p and significant amounts of Bet1p (Fig. 3C). Similarly, when anti-Bet1p antibodies were used to precipitate Bet1p from the membrane extract, Aut7p co-immunoprecipitated. Other proteins, such as Ufe1p, Sec17p, and Bos1p, did not precipitate with the anti-Aut7p antibodies (Fig. 3D). The immunoprecipitation observed in these experiments was specific; no Bet1p was precipitated with the anti-Aut7p antibodies when the aut7 null strain (\( \Delta \text{aut7} \)) was used as a source for the
Aut7p suppresses the temperature-sensitive phenotype of ER to Golgi secretion mutants

| Mutant   | Function of gene product |
|----------|--------------------------|
| bet1-1   | ER-Golgi v-SNARE         |
| sec22-2  | ER-Golgi v-SNARE         |
| sec18-1  | NSF                      |
| usol-1   | p115                     |
| sec21-1  | COPI subunit             |
| sec12-1  | COPII subunit            |
| sec13-1  | COPII subunit            |
| sec31-1  | COPII subunit            |
| ypt1-3   | ER-Golgi docking/fusion regulator |
| sly1-1   | ER-Golgi SNARE-associated protein |
| tip20-1  | ER-Golgi transport factor |
| sec7-1   | ARF exchange factor      |
| bos1-1   | ER-Golgi v-SNARE         |
| sed5-1   | ER-Golgi t-SNARE         |
| ufe1-1   | ER t-SNARE               |
| sec9-1   | FM t-SNARE               |

Table II

Aut7p suppresses the temperature-sensitive phenotype of ER to Golgi secretion mutants

The AUT7 gene was tested for its ability to suppress the temperature-sensitive (ts) growth defect of different mutants. Mutants were transformed with multicopy plasmid containing the AUT7 gene and incubated at 37 °C. ARF and PM stands for ADP-riboseylation factor and plasma membrane, respectively.

We also found that upon starvation, which triggers autophagocytosis, this mutant strain shows lower rates of survival and protein degradation (data not shown), consistent with reduced autophagocytosis. In addition, the level of Aut7p was dramatically elevated, reaching a peak 5 h after cells were transferred into a starvation medium (data not shown). These data strongly suggest that upon starvation, Aut7p is an essential and possibly rate-limiting factor for autophagocytosis.

During autophagocytosis, autophagosomes containing cytosol fuse with the vacuolar membrane. We have tested whether Aut7p is in fact present on the vacuolar membrane. For that purpose, vacuolar membranes were isolated by flotation through a discontinuous Ficoll step gradient as described by Conradt et al. (50). The Ficoll interphase (0–4%) was collected and tested by Western analysis using specific antibodies. Aut7p was found in this fraction together with the vacuolar markers Vam3p and Nyv1p (Fig. 5A); the Golgi marker, Sed5p, and the ER marker, Dpm1, were absent.

As we have shown that Aut7p interacts with Bet1p, an ER to Golgi v-SNARE protein (Fig. 3), we tested by co-immunoprecipitation whether Aut7p also interacts with a vacuolar SNARE. As shown in Fig. 5B, anti-Aut7p antibodies specifically precipitated Aut7p from yeast membrane extracts, together with Nyv1p, a vacuolar v-SNARE. Only a small fraction of the vacuolar t-SNARE Vam3p co-precipitated with Aut7p. When the yeast membrane extract was incubated with anti-Nyv1p antibodies, significant levels of Aut7p were found in the pellet, this time together with higher amounts of Vam3p (Fig. 5B). Aut7p does not interact with v-SNAREs such as Snc1p and Snc2p involved in Golgi to plasma membrane transport (data not shown). No precipitation of Nyv1p with anti-Aut7p antibodies was detected when Δaut7 mutant strain was used (Fig. 5B), confirming the specificity of the interaction.

SNARE molecules can be found either as free monomers or assembled into a v-t-SNARE complex. It has been demonstrated that NSF/Sec18p, together with SNAP/Sec17p, assembles these complexes in the presence of ATP (30, 51). This reaction is accompanied by dissociation of Sec17p from the membrane (22). To test whether Aut7p interacts with the Nyv1p/Vam3 complex or with a free Nyv1p, membranes were isolated and resuspended in the presence or absence of 1 mM MgATP. After incubation for 10 min at 25 °C the membrane proteins were extracted with detergent and immunoprecipitated with the anti-Aut7p antibodies. The resulting immunoblot indicates that whereas Sec17p was removed from the membranes in the presence of ATP (Fig. 5C), no significant change was observed in the overall amount of Nyv1p that co-immunoprecipitated with Aut7p (Fig. 5D). Furthermore, in both cases, only a small level (less than 0.5%) of Vam3p co-immunoprecipitated with Aut7p. In addition, anti-Vam3p antibodies failed to co-precipitate Aut7p from membrane extracts (data not shown). These results indicate that Aut7p primarily interact with Nyv1p and to a lesser extent with the Nyv1p/Vam3p complex. The association of Aut7p/Nyv1p implies a function of Aut7p at the docking stage.

**DISCUSSION**

Aut7p, previously identified as an autophagic factor (5, 6), is shown here to take part in multiple intracellular membrane trafficking processes. We have demonstrated that Aut7p specifically interacts with different v-SNARE molecules involved in ER to Golgi transport and in vacuolar fusion. Our findings imply that in addition to its involvement in transport to the vacuole, Aut7p also participates in membrane fusion events that take place in the early secretory pathway.

That Aut7p is essential for autophagocytosis (5, 6) is consistent with the notion that it participates in membrane traffic. Lang et al. (5) proposed that Aut7p and Aut2p are involved in the delivery of autophagosomes to the vacuole along microtubules. Kirisako et al. (6) suggested that Aut7p plays an important role in autophagosome formation. We propose that the
formation of autophagosomes and/or their fusion with the vacuolar membrane are SNARE-dependent and that Aut7p is essential for the fusion process. The t-SNARE Vam3p, a protein known to participate along with other vacuolar protein sorting gene products in directing endosomes to vacuole transport, has also been shown to be involved in autophagy (24). Vam3p interacts primarily with Nyv1p, the vacuolar v-SNARE involved in homotypic fusion (25). As we show here, Aut7p and Nyv1p form a complex, suggesting that Aut7p may play a role in the fusion between autophagosomes and vacuoles by interacting with the docking/fusion machinery. It was reported that Nyv1p is not involved in transport of AP1 to the vacuole; in
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Overexpression or depletion of Aut7p in yeast results in vacuolar defects (41). To determine the nature of the vacuolar membrane traffic disruption caused by overexpression of Aut7p, we isolated vacuolar membranes from an aut7-1 strain using a modified procedure. The procedure involved the following steps: (i) lysis of spheroplasts in a buffer containing MgCl2, (ii) centrifugation to remove soluble cytosolic materials, (iii) washing of pellets with buffer containing DAE-decstet, and (iv) re-isolation of vacuolar membranes by centrifugation and resuspension in buffer containing SDS-sample buffer. The membrane proteins were analyzed by Western blot using specific antibodies as indicated. The results showed that the membrane proteins were eluted and loaded on 13% SDS-PAGE and analyzed by Western blot. 10% of the input extract was shown (Total (10%)).

**FIG. 5.** Aut7p co-immunoprecipitates with Nyv1p. A, Aut7p is found in isolated vacuolar membranes. Vacuolar membrane was isolated according to Conradt etal. (50). Spheroplasts were prepared from wild type strain and carefully resuspended in cold 10% Ficoll buffer containing DEAE-dextran. The vacuoles were isolated by flotation through a discontinuous Ficoll step gradient. The Ficoll interphase (0–4%) was collected (left lane) and tested by Western blot using specific antibodies as indicated. The left lane contains total lysate. B, Nyv1p interacts with Aut7p. Total membrane extract was prepared from WT and from aut7Δ strain (as a control). Extracts were incubated with anti-Aut7p or anti-Nyv1p antibodies and protein A-agarose beads overnight at 4 °C. Beads were washed five times, and the precipitates were eluted and loaded on 13% SDS-PAGE and analyzed by Western blot. 10% of the input extract was shown (Total (10%)). IB, immunoblot; IP, immunoprecipitation. Densitometric quantification of the Western blots revealed that 0.8% (± 0.6%) of total Vam3p was precipitated with anti-Aut7p antibodies, whereas 10% (± 1.4%) of total Nyv1p was precipitated with anti-Aut7p antibodies. In reciprocal experiments 7% (± 0.5%) of total Aut7p was precipitated with anti-Nyv1p antibodies. C, total membranes were re-isolated and incubated for 10 min at 25 °C with buffer 88 containing 3 mM MgCl2 in the presence or absence of 1 mM ATP. Membranes were re-isolated by centrifugation, resuspended with SDS-sample buffer, and analyzed by Western blots with anti-Sec17p antibodies and anti-Nyv1p antibodies. D, after ATP treatment the membrane proteins were extracted with detergent buffer, immunoprecipitated with anti-Aut7p antibodies, and analyzed by Western blot.

In contrast, Vti1p, a v-SNARE found in Golgi-derived vesicles and known to be involved in many transport events, is required for this transport step (52). Additionally, Tig2p, a member of the syntaxin family of t-SNARE proteins, and Vps45p, a Sec1p homologue, are required in the constitutive Cvt pathway but not in inducible macroautophagy (41). It is therefore likely that Aut7p may interact with an as yet unidentified related v-SNARE molecule that mediates fusion between autophagosomes and the vacuole. Defining the origin of the donor membrane required for the formation of autophagic vesicle, and isolation of these vesicles using Aut7p as a marker, will allow a better understanding of the roles of the various proteins involved in this pathway.

It appears that Aut7p interacts with v-SNAREs and thereby affects their activity. Several regulatory proteins that interact with SNAREs have been reported. Sec1p in yeast and its homologues nSec1p, Munc18, and unc18 in higher eukaryotes have been shown to regulate exocytosis (53–58). This protein family acts directly on the syntaxin t-SNAREs (54, 59–61). Furthermore, experiments in vivo in Dro sophila melanogaster and Caenorhabditis elegans showed that the Sec1p homologues inhibit neurotransmitter release (62, 63). It has therefore been suggested that these proteins act as negative regulators of fusion. Vam1p, another factor that interacts with v-SNARE (Snc2p), was recently suggested to regulate Snc2p entry to the SNARE complex (64). Finally, LMA1, a low molecular weight heterodimer composed of thioredoxin and the protease B inhibitor IB2, originally identified as a protein required for in vitro vacuolar homotypic fusion (28, 65), as well as for ER to Golgi transport (14, 66), was shown to interact with the vacuolar t-SNARE Vam3p in a Sec18p-dependent manner (67). Possibly, Aut7p acts on v-SNAREs in a similar manner to that by which LMA1 acts on the t-SNARE Vam3p.

Bet1p, Bos1p, and Sec22p, three v-SNAREs, have been implicated in transport between the ER and the Golgi. It has been recently proposed that Bos1p participates exclusively in anterograde transport from the ER to the Golgi, Sec22p is involved in retrograde transport from the Golgi to the ER, whereas Bet1p acts in both directions (68). We show here that Aut7p specifically interacts with Bet1p but not with Bos1p, suggesting that it is not involved in anterograde transport but rather in retrograde transport from the Golgi to the ER. This is supported by the suppression effect of AUT7 on the retrograde mutant sec22Δ. The viability of the aut7 null strain and the lack of a detectable transport defect in this strain suggest that Aut7p plays a regulatory role in this process or that other factors may substitute its function. Because under starvation conditions Aut7p is essential for autophagy, we speculate that Bet1p or Sec22p may participate in the formation of the autophagic membrane or that under starvation Aut7p interacts with other SNARE molecules mediating autophagy.

We have recently found that GATE-16, the mammalian homologue of Aut7p, interacts specifically with a Golgi v-SNARE in an NSF- and SNAP-dependent manner (4). Although the precise mechanism for the function of Aut7p in membrane traffic is uncertain, we propose that it may act as a positive regulator of v-SNAREs. The data presented in the present study support the notion that the function of Aut7p is closely related to the activity of SNAREs.

Our findings are summarized by the model described in Fig. 6. Accordingly, under normal growth conditions Aut7p functions in early steps of the secretory pathway by interacting with v-SNARE molecules such as Bet1p. This Aut7p activity can probably be replaced by other unidentified factor(s). Under constitutive steady-state conditions, Aut7p is essential for the Cvt pathway. Upon nitrogen starvation, Aut7p expression levels are significantly increased, and it becomes involved in autophagy. Based on the fact that Aut7p interacts with the vacuolar v-SNARE, Nyv1p, we propose that Aut7p may be involved in vacuolar fusion or that Nyv1p is involved in autophagy triggered upon nitrogen starvation. Further experiments are required to resolve this issue.

**FIG. 6.** A model for trafficking pathways involving Aut7p. We propose that under normal growth conditions, Aut7p interacts with Bet1p and Sec22p and participates in ER to Golgi transport. Aut7p activity in this process, however, can be bypassed in vivo. Aut7p is required for the constitutive Cvt pathway to the vacuole, and under nitrogen starvation conditions, Aut7p is essential for transport of autophagosomes to the vacuole, as described previously (5, 6). Aut7p interacts with Nyv1p, which is required for vacuolar fusion, but the functional relevance of this finding is not yet known. PVC stands for pre-vacuolar compartment.
Acknowledgments—We are grateful to J. E. Gerst, O. Giladi, and S. Fishburn for helpful advice and for providing the reagents. We thank R. Schekman, H. Riezman, D. Gallwitz, A. Mayer, S. Ferro-Novick, H. Pelham, J. Lewis, and C. Barlowe for providing strains and antibodies. Special thanks go to Hedva Later and Frida Shimron for technical help. We are grateful to the members of Dr. Elazar’s group for stimulating discussion.

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