High expression of the yeast syntaxin-related Vam3 protein suppresses the protein transport defects of a pep12 null mutant

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Abstract The Pep12 protein of Saccharomyces cerevisiae is a member of the syntaxin family thought to function as target membrane receptor (t-SNARE) for vesicular intermediates travelling between the Golgi apparatus and the vacuole. Exploiting the temperature-sensitive growth phenotype of pep12 deletion strains, we identified VAM3 as a multicopy suppressor. Vam3p is another syntaxin-related protein which on high expression restored vacuole acidification of pep12 null mutants and effectively suppressed their sorting and maturation defects of vacuolar hydrolases. We conclude that Vam3p acts either as a bypass suppressor or by functionally replacing Pep12p at an endosomal, prevacuolar compartment.

Key words: Syntaxin; t-SNARE; Vacuole; Vesicular transport; Yeast

1. Introduction

The vacuole of the yeast Saccharomyces cerevisiae is the functional equivalent of the mammalian lysosome. Apart from functions in the storage of metabolites and the regulation of ion homeostasis in the cytosol, its most prominent role is the degradation of macromolecules [1], for review. A variety of hydrolases reside in the acidic lumen, most of them being synthesized as inactive precursors in the ER. After passing the early stages of the secretory pathway, soluble vacuolar hydrolases are actively sorted away from secretory proteins in a late Golgi compartment and delivered to the vacuole via intermediate endosome-like compartments [2], for review. Upon arrival in the vacuole, the precursor forms are typically activated by proteolytic processing [3,4] for review.

Mutants defective in vacuole function have been isolated through screening for both biochemical and morphological phenotypes: pep mutants [5] were isolated due to their property of missorting the vacuolar hydrolase proteinase A (PrA) which is also required for activation of the proteinases B (PrB) and C (PrC = carboxypeptidase Y, CPY); vps mutants [6,7] are characterized by the secretion of the Golgi-modified precursor form of CPY, and vam mutants [8] exhibit an altered vacuolar morphology, such as fragmentation. Interestingly, in some of these mutants members of gene families are affected which have been identified as general components of vesicular transport [9,10]. For example, the small GTPases Ypt51p and Ypt7p have also been isolated as Vps24p and Vam4p, respectively [11-14]. Recently, the yeast Pep12p protein has been shown to act in a vesicular transport step between the Golgi apparatus and the vacuole [15]. Pep12p is a transmembrane protein of 288 amino acids which exhibits 25% identity and 56% similarity to rat syntaxin 6 [16]. It is thought to act as a so-called t-SNARE (target membrane soluble NSF attachment protein receptor; NSF = N-ethylmaleimide-sensitive fusion protein) in the docking of transport vesicles at a still ill-defined, prevacuolar (endosomal) compartment [15,17]. Cells carrying a disruption of the PEP12 gene are characterized by a single enlarged, acidification-defective vacuole, the accumulation of 50 nm vesicles and maturation and sorting defects of several vacuolar hydrolases [15]. Furthermore, pep12 deletion strains exhibit a growth defect at 38°C. We exploited this phenotype to screen for multicopy suppressors of the deletion of PEP12. Multicopy suppressor screening previously led to the identification of many components of the vesicular transport machineries, such as t-SNAREs, v-SNAREs (vesicle membrane SNARE) and SEC7-homologues [18-21].

We identified the VAM3 gene as a potent multicopy suppressor of the deletion of PEP12. According to its primary sequence, the Vam3 protein is a member of the syntaxin family [22]. Our results suggest that Vam3p acts as a t-SNARE either replacing Pep12p or activating an alternative, Pep12p-independent pathway.

2. Materials and methods

2.1. Strains, growth conditions and genetic methods

The following yeast strains were used in this study: MSLC-3D: *Mata ura3 leu2 his3 trp1 lys2* (this laboratory); YMG7: *MSUC-3D pep12::kanMX4* (this study); SEY6210: *Mata suc2-D9 ura3-52 leu2-3,113 his3-D200 trp1-D901 lys2-801* [6]; YM65: *SEY6210 pep12:: kanMX4* (this study); YM8: *SEY6210 vam3::LEU2* (this study). Manipulations of *E. coli* and DNA were performed according to standard procedures [23]. Yeast strains were grown in 1% yeast extract (Gibco, Eggenstein), 2% peptone 140 (Gibco, Eggenstein), 2% glucose (YEPD), or in synthetic glucose medium (SD) supplemented as necessary [24]. Solid media were prepared by adding 2% agar (Gibco, Eggenstein). Lithium acetate transformation of yeast cells was performed as previously described [25].

2.2. Construction of recombinant plasmids

To clone the PEP12 gene, a fragment encompassing codons 2 to 189 was amplified using standard PCR techniques. The PCR fragment was digoxigenin-labelled using the DIG-labelling kit (Boehringer Mannheim) and used to screen approximately 19000 *E. coli* clones of a YEp13-based genomic yeast library as described previously [18]. Plasmids were isolated from two candidate colonies and the presence of PEP12 was verified by Southern blotting and DNA-sequencing using the Sequenase 2.0 kit (USB, Braunschweig). A 1200 bp *Clal* fragment was subcloned into *Clal*PstI-cut pBluescript II KS+ (Stratagene, Heidelberg) to create pM1G. A 1290 bp *XhoI*/*EagI* fragment of pM1G was subcloned into the respective polylinker sites of the shuttle vectors pRS316 [26] and pRS326 [27] to create pMG13 and pMG6, respectively. The VAM3 gene was cloned as follows. Utilizing the *XhoI*-polylinker site of the library vector pTSS25, a
1462 bp XhoI/EagI fragment of VAM3 representing base pairs 518330 to 520392 of chromosome XV was cloned into XhoI/EagI-cut pRS326 and pRS316 to create pMG49 and pMG56, respectively. The plasmid pMG48 (pBS-VAM3) was constructed by subcloning the 1768 bp AvrII/NsiI VAM3 fragment of the original suppressor plasmid pTSS1 into Pst/Vbal-cut pBluescript II KS+. The SED5 gene, cloned from a genomic library, was inserted into pRS326 as a 4.2 kb Xbal fragment. All plasmid constructions were controlled by sequencing.

2.3. Gene disruptions

The PEP12 gene was deleted using the LHF-PCR disruption technique as described by Wach [28]. The oligonucleotides MG56 5'-AATTTAACCTCATTCAAGG-3' and MG57 5' -CCCCTTAGT-GAGGTTAATTCTACCTCACAACACAAATTAGTG-3' were used to amplify the 5'-megaprimer, whereas MG58 5'-GCCCTTATAGT-GAGTCTATTACCGGTTACAGGAGCTAAGG-3' and MG59 5' -GTAATACGACTCACTATAGGGC-3' were used to amplify the 3'-megaprimer under standard PCR conditions. Single-step gene disruption of the chromosomal VAM3 gene was done with a linear DNA fragment. Part of the protein-coding region of the gene was removed and replaced by the yeast marker gene LEU2 as follows. From pMG48, a blunt-ended 928 bp StuI/BsrEI fragment containing more than 90% of the protein-coding region of VAM3 (−166 to +792) was removed and replaced by LEU2 on a 2.2 kb Hpal fragment. As a vam3 deletion strain YMG8 was constructed by transforming the haploid strain SEY6210 with a 2.7 kb deletion strain YMG8 was constructed by transform-
Fig. 2. Suppression of the vacuolar acidification defect of a pep12 null mutant. The vacuoles of YMG5 (pepl2−) cells transformed with pRS326 (a,b), pRS326-PEP12 (c,d), pRS326-VAM3 (e,f) or pRS326-SED5 (g,h) were stained with quinacrine as described in Section 2 and viewed by Nomarski (a,c,e) or fluorescence (b,d,f) microscopy, respectively.

Importantly, high expression of SED5 could not suppress the vacuole acidification defect of YMG5 (Fig. 2h).

All vps and pep mutants are defective in vacuolar protein maturation and they missort the soluble vacuolar hydrolases proteinase A (PrA) and CPY [5-7]. As shown in steady-state Western blots (Fig. 3), the mature forms of PrA (42 kDa) and CPY (61 kDa) appeared to be absent from pep12 null mutant cells but they were readily detectable in the pep12− strains transformed with either pRS326-PEP12 or pRS326-VAM3. In the case of PrA, the immature ER- and Golgi-modified forms (48 and 44 kDa species) could be seen in pep12− cells. They disappeared completely in PEP12-expressing cells and, to a large extent, on high expression of VAM3. In contrast, high expression of SED5 had no effect on the processing of both CPY and ALP (results not shown).

Next we followed the maturation kinetics and delivery to the vacuole of soluble CPY and the membrane-bound alkaline phosphatase (ALP). Following a 15-min pulse with Tran35S-label and a 30-min chase with unlabelled amino acids, the maturation of newly synthesized CPY and ALP was severely inhibited in spheroplasts derived from pep12− cells, and a significant portion of CPY was missorted and secreted in its Golgi-modified p2 precursor form (Fig. 4). The maturation of ALP appeared to be less affected. Most importantly, the maturation and sorting defects were effectively suppressed on high expression of VAM3.

To further characterize the genetic interaction of VAM3 and PEP12, we investigated the effect of high expression of the PEP12 gene in the vam3 deletion strain YMG8. Deletion of the vam3 gene resulted in phenotypes previously described for the original vam3 mutants [8]: while the steady-state levels of the mature forms of PrA and CPY were comparable to those of wild-type cells (results not shown), the most prominent phenotype was a highly fragmented vacuole (Fig. 5a,b).

High expression of the PEP12 gene in YMG8 cells resulted in a partial suppression of this phenotype. Although still fragmented, the vacuoles of vam3 cells transformed with pRS326-PEP12 (Fig. 5c,f) appeared larger than the vacuoles of control cells (Fig. 5a,b). However, the single large vacuoles of YMG8 transformed with pRS316-VAM3 (Fig. 5c,d) were not seen.

4. Discussion

Pep12p has been suggested by Becherer et al. [15] to act in transport of Golgi-derived vesicles carrying soluble vacuolar enzymes to a late endosomal (‘prevacuolar’) compartment and it was shown that the membrane-bound ALP might be transported via a Pep12p-independent route. There is evidence that the transport pathways of vacuolar hydrolases and of endocytosed α-factor merge [13,38-40] and it is well possible that some or all of the vacuolar proteins that pass the Golgi ap-

Fig. 3. Processing of soluble vacuolar enzymes in YMG5-transformants under steady-state conditions. Cell extracts of YMG5 transformed with the plasmids indicated were subject to SDS-PAGE and Western blotting with anti-CPY (upper panel) and anti-PrA-antibodies (lower panel) as described in Section 2. The migration positions (in kDa) of molecular mass markers are indicated on the left margin. The migration positions of the precursor (p) and mature (m) forms of CPY and PrA are indicated on the right margin. pPrA indicates the position of the largest (52 kDa) PrA precursor.

Fig. 4. Rescue from transport defects of newly synthesized vacuolar hydrolases in pep12 null mutant cells. Spheroplasts of YMG5 cells transformed with either pRS326, pRS326-PEP12 or pPR326-VAM3 were subjected to a pulse-chase with Tran35S-label. Zero or 30 min after initiating the chase, anti-ALP (upper panel) or anti-CPY (lower panel) antiserum was used for immunoprecipitation. i, internal fraction; e, secreted fraction. The migration positions of the precursor (p) and mature (m) forms of ALP and CPY (in kDa) are indicated to the right. p1CPY, ER-modified form of CPY; p2CPY, Golgi-modified form of CPY.
Fig. 5. Partial suppression of vacuolar fragmentation of a vam3 null mutant. The vacuoles of YMG8 (vam3Δ) cells transformed with pRS326 (a,b), pRS326-PEP12 (c,d) or pRS316-VAM3 (e,f) were stained with CDCFDA as described in Section 2 and viewed by Nomarski (a,c,e) or fluorescence (b,d,f) microscopy.

paratus travel via early and late endosome-like organelles [13]. Different endosomal compartments appear to exist in yeast [41,42]. According to the SNARE hypothesis [17], distinct vesicular and target membrane receptors would be required for consecutive steps of the Golgi-to-vacuole transport. As pep12 and vam3 mutants display phenotypic alterations [15,8] that resemble those of ypt51 and ypt7 mutants, respectively [11–13], the presumptive t-SNAREs are likely to function at different steps of the vacuolar protein transport pathway(s).

How can the suppressor activity of VAM3 in pep12 deletion strains be envisaged? One possibility would be the activation by Vam3p of a putative bypass to the vacuole or of a Pep12p-independent transport route. A high Vam3p receptor density at an endosomal organelle distinct from the Pep12p compartment (or even at the vacuole) would thus allow docking of transport vesicles which under wild-type conditions would dock at the Pep12p-bearing compartment. Alternatively, Vam3p at high dosage might directly replace Pep12p at the endosomal/prevacuolar compartment where Pep12p is thought to reside. Carboxyl terminally anchored type II membrane proteins, such as v- and t-SNAREs, appear to reach their final destination via the secretory pathway [43–45]. It is therefore conceivable that if Vam3p would have to pass the Pep12 compartment on its way to a following organelle, perhaps the vacuole, a receptor density high enough for vesicle docking would be attained at this compartment. In either case one would have to assume that a putative v-SNARE normally pairing with Pep12p would also be able to recognize Vam3p, but most likely with lower affinity. We would like to point out that the suppression of Pep12p-lacking cells by high dosage of Vam3p appears to be specific as the structurally related Sed5 protein was unable to rescue pep12Δ cells and high levels of Pep12p did only marginally affect the phenotypic alterations seen in vam3Δ cells.

The phenotypic resemblance of vam3 and ypt7 mutants [8,13] together with the finding that the Ypt7 GTPase is required for vacuole/vacuole fusion [46] lends credence to the assumption that Vam3p acts as a t-SNARE at the vacuole.

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