Ccz1p/Aut11p/Cvt16p is essential for autophagy and the cvt pathway

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Abstract In a reverse genetics screen, we here identify Ccz1p as an essential component of the cvt pathway and autophagy. Ccz1p is identical with the so far unknown Cvt16p. GFP-Aut7p, a specific cargo of autophagosomes, accumulates in *ccz1*Δ cells at punctate, vesicular structures in the cytosol, suggesting a block in the autophagic pathway prior to vacuolar fusion of autophagosomes. Proteinase protection experiments using hypotonically lysed *ccz1*Δ spheroplasts demonstrate that proaminopeptidase I, another specific cargo of autophagy and the cvt pathway, is trapped inside membrane-enclosed vesicles. Taken together our findings are compatible with a function of Ccz1p in vacuolar fusion of cvt vesicles and autophagosomes.

Key words: Ccz1p; Ypt7p; Vacuolar fusion; Autophagy; Cvt pathway

1. Introduction

To survive non-optimal conditions, cells have developed several mechanisms. One such mechanism is starvation-induced autophagy, which becomes essential for cell survival when an external nitrogen source is limited (reviews: [1,2]). Autophagy is common to all eukaryotic cells, it transports in a self-cannibalistic fashion parts of the cell's own constituents to the lysosome (vacuole) for degradation. Autophagy is an unspecific process also affecting whole organelles such as mitochondria, peroxisomes or parts of the endoplasmic reticulum. It mechanistically starts at the preautophagosomal or perivacuolar compartment [3,4]. From this organelle double membrane-layered cytosol-containing transport vesicles (autophagosomes) are formed [5]. After reaching the vacuole, the outer membrane layer of autophagosomes fuses with the vacuolar membrane and still membrane-enclosed autophagic bodies are released to the vacuolar lumen. Dependent on vacuolar proteinase B and the putative lipase Aut5p (Cvt17p) [6,7], the autophagic bodies are finally broken down together with their contents.

As a model for higher cells *Saccharomyces cerevisiae* was used to genetically dissect autophagy in three independent approaches yielding genes termed *APG* [8], *CVT* [9] and *AUT* [10], respectively. This work showed that under starvation conditions the otherwise unspecific autophagy specifically transports proaminopeptidase I from the cytosol to the vacuole, where it is proteolytically matured [9,11]. In non-starved cells proaminopeptidase I transport is taken over by the cvt pathway. The cvt pathway is morphologically very similar to autophagy and in large part uses the same components. However, its transport intermediates, the cvt vesicles, are smaller in size compared to autophagosomes and exclude cytosol [12].

In previous studies, we identified eight *AUT* genes in a classical screen for mutants using chemical mutagenesis. Since this approach is very time-consuming and we expected the existence of further autophagy genes, we additionally started a reverse genetic screen using the ~5000 yeast deletion strains, which were generated in the 'yeast deletion project'. In this screen we already identified and characterized Aut8p, Aut10p and Mai1p as novel components of the autophagy or the cvt pathway, respectively [13–15].

Here we identify and characterize Ccz1p as a novel component of the cvt pathway and autophagy. Starved and non-starved *ccz1*Δ cells are unable to mature proaminopeptidase I. We confirm the autophagic defect of *ccz1*Δ cells with a newly established assay, based on proteolysis of the specific autophagy cargo green fluorescent protein (GFP)-Aut7p. Fluorescence microscopy using GFP-Aut7p as an autophagosomal marker and proteinase protection experiments are compatible with a function of Ccz1p in vacuolar fusion of autophagosomes and cvt vesicles.

2. Materials and methods

2.1. Chemicals and antibodies

Antibodies: anti-carboxypeptidase Y and anti-3-phosphoglycerate kinase (Molecular Probes, Leiden, The Netherlands). Horseradish peroxidase (HRPO)-conjugated goat anti-rabbit (Medac, Hamburg, Germany) and HRPO-conjugated goat anti-mouse (Dianova, Hamburg, Germany); anti-proaminopeptidase I [15]; anti-GFP (Clontech, Palo Alto, CA, USA). Oligonucleotides (MWG-Biotec, Ebersberg, Germany). For detection of peroxidase-labeled antibodies on immunoblots the ECL detection kit (Amersham, Braunschweig, Germany) was used.

Other chemicals, all of analytical grade, were from Sigma or Roth (Karlsruhe, Germany).

2.2. Media

Standard media [16] were used.

Cells were starved either in 1% potassium acetate or SD-N (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose).

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2.3. Strains

WCG4a	*Mat a* (or *Mat* α, respectively) *his3-11,15 leu2-3,112 ura3*	[10]
YMTA	*Mat a his3-11,15 leu2-3,112 ura3 pep4Δ::HIS3*	[10]
YKMW7	*Mat* α *his3-11,15 leu2-3,112 ura3 ccz1Δ::KAN^R*	(this study)
YKMW10	*Mat a his3-11,15 leu2-3,112 ura3 ypt7Δ::HIS3*	(this study)
YKMW12	*Mat a* Δ *his3-11,15 leu2-3,112 ura3 ccz1Δ::KAN^R*	(this study)
BY4743	*Mat a/*α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 MET15/met15Δ0 lys2Δ0/LYS2 ura3Δ0/ura3Δ0*	(Euroscarf Collection, Frankfurt, Germany)
Y32098	*Mat a/*α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 MET15/met15Δ0 lys2Δ0/LYS2 ura3Δ0/ura3Δ0 pep4Δ::KAN^R/pep4Δ::KAN^R*	(Euroscarf Collection, Frankfurt, Germany)
Y33357	*Mat a/*α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 MET15/met15Δ0 lys2Δ0/LYS2 ura3Δ0/ura3Δ0 apg12Δ::KAN^R/apg12Δ::KAN/^{R}*	(Euroscarf Collection, Frankfurt, Germany)
Y34953	*Mat a/*α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 MET15/met15Δ0 lys2Δ0/LYS2 ura3Δ0/ura3Δ0 ape1Δ::KAN^R/ape1Δ::KAN^R*	(Euroscarf Collection, Frankfurt, Germany)
Y37164	*Mat a/*α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 MET15/met15Δ0 lys2Δ0/LYS2 ura3Δ0/ura3Δ0 ccz1Δ::KAN^R/ccz1Δ::KAN^R*	(Euroscarf Collection, Frankfurt, Germany)
YMS30K1	*Mat a his3-11,15 leu2-3,112 ura3 aut3Δ::KAN^R*	[17]
Cvt3-1	*Mat a SEY6211 leu2-3,112 ura3-52 his3-Δ200 trp1Δ901 ade2-101 suc2Δ9*	[9]
Cvt6-1	*Mat a SEY6211 leu2-3,112 ura3-52 his3-Δ200 trp1Δ901 ade2-101 suc2Δ9*	[9]
Cvt11-1	*Mat* α *SEY6210 leu2-3,112 ura3-52 his3-Δ200 trp1Δ901 lys2-801 suc2Δ9*	[9]
Cvt13-1	*Mat* α *SEY6210 leu2-3,112 ura3-52 his3-Δ200 trp1Δ901 lys2-801 suc2Δ9*	[9]
Cvt14-1	*Mat* α *SEY6210 leu2-3,112 ura3-52 his3-Δ200 trp1Δ901 lys2-801 suc2Δ9*	[9]
Cvt15-1	*Mat* α *SEY6210 leu2-3,112 ura3-52 his3-Δ200 trp1Δ901 lys2-801 suc2Δ9*	[9]
Cvt16-1	*Mat* α *SEY6210 leu2-3,112 ura3-52 his3-Δ200 trp1Δ901 lys2-801 suc2Δ9*	[9]

2.4. Screening on phloxine B plates

According to [15], the strains were grown for 6 h at 30°C in liquid YPD and then dropped on YPD plates and grown overnight. Afterwards the colonies were replica-plated on SD-N plates containing phloxine B (200× stock: 400 mg phloxine B, 850 mg NaCl and 63 mg KH_2PO_4 in 100 ml) and auxotrophic nutrients. Colonies with reduced survival rates turn red after 3–8 days at 30°C.

2.5. Plasmids and DNA manipulation

The deletion plasmid pBSKS+ ypt7::HIS3 (D. Gallwitz, Göttingen) was digested with *Xho*I and *Pac*I and transformed in WCG4a cells. Transformants were selected on plates lacking histidine and chromosomal replacement of the *YPT7* gene confirmed by Southern blotting (not shown). pGFP-AUT7 is described in [3].

2.6. Chromosomal deletion of *CCZ1*

Using oligonucleotides S-ccz1 (CTAAATCGTACAACTATTAAATT GATATATGAAAG ACG GA TCAGCTG AAGCTTCGTACGC) and AS-ccz1 (TATGTCTATCA AATGCTAAAC GTT AC ATTTTT AAATTTCCCGCATAGGCCACTAGTGGATCTG) and plasmid pUG6 [18] we generated by PCR a LoxP-Kan^R-LoxP cassette flanked by sequences of *CCZ1*. This DNA fragment conferring kanamycin resistance was used to chromosomally replace *CCZ1* in WCG4a, generating YKMW7. Gene replacement was confirmed by Southern blotting (not shown).

2.7. Proteinase K digestion

Proteinase protection was done according to [19] with the following modifications. Forty A_{600} unit stationary cells were harvested, washed twice with water and incubated for 15 min in 20 mM dithiothreitol containing 0.1 M Tris–HCl buffer, pH 9.4. The cells were then resuspended in 1 M sorbitol, 50 mM sodium phosphate buffer pH 7.4 containing 50 μg/ml oxalyticase and spheroplasted at 30°C for 30 min. The spheroplasts were hypotonically lysed by resuspending in PS200 (20 mM potassium-PIPES, 200 mM sorbitol, pH 6.8, with 5 mM $MgCl_2$). The lysis solution was repeatedly precleared and the supernatant divided into three 300 μl fractions. 300 μl PS200, PS200 with 100 μg/ml proteinase K, and PS200 with 100 μg/ml proteinase K and 0.4% Triton X-100 were given to each fraction, respectively. After 15 min on ice, digestion was halted through trichloroacetic acid precipitation. The pellets were dissolved in Laemmli buffer.

2.8. GFP-Aut7p degradation

Mid-log cells grown in SMD were starved in SD-N and one A_{600} unit cells were hourly harvested for 4 h. The samples were processed for immunoblotting.

2.9. Direct fluorescence microscopy

pGFP-AUT7 cells were grown to stationary phase in SMD, shifted to SD-N for 4 h and visualized with a Zeiss Axioskop 2 plus with an Axiocam image system.

3. Results

3.1. Identification of *ccz1Δ* cells in a reverse genetics approach due to their defect in maturation of proaminopeptidase I

Identification of genes by complementation of point mutants with a genomic library is very time-consuming. To identify further autophagy genes, we therefore made a reverse genetics approach starting with ∼5000 yeast deletion strains. These strains were generated in the 'yeast deletion project' and cover the non-essential part of the ∼6000 yeast genes. In each of these strains one open reading frame (ORF) was chromosomally deleted. To identify autophagy mutants, we first screened the deletion strains for a reduced survival rate during starvation, a phenotype typical for autophagy mutants [17]. A reduced viability on plates lacking nitrogen can be scored with the dye phloxine B [8] due to the red staining of dead cells. We identified ∼1000 strains, whose colonies compared to wild-type were stained red (not shown). Since a defect in maturation of proaminopeptidase I is typical for almost all autophagy mutants, we further analyzed these strains in immunoblots for proaminopeptidase I maturation and here report identification of *ccz1*Δ cells.

For further analysis we generated a *ccz1* null mutant, by chromosomal integration of a PCR fragment conferring kanamycin resistance into the *CCZ1* locus (see Section 2.6). Gene replacement was confirmed by Southern blotting (not shown).

Under non-starvation conditions the cvt pathway transports proaminopeptidase I to the vacuole. In nitrogen-starved cells, this transport is taken over by autophagy. We checked proaminopeptidase I maturation in growing and in nitrogen-starved cells. The maturation defect in non-starved cells (Fig. 1, lanes 3 and 4) clearly indicated a defective cvt pathway. Even after prolonged starvation proaminopeptidase I maturation was defective (Fig. 1, lanes 5 and 6) suggesting an autophagic defect. Mature proteinase B is essential for intravacuolar breakdown of autophagic bodies [20]. We therefore analyzed the steady-state levels of mature vacuolar carboxypeptidase Y and proteinase B. Previously a defect in carboxypeptidase Y sorting was detected in *ccz1*Δ cells [21],

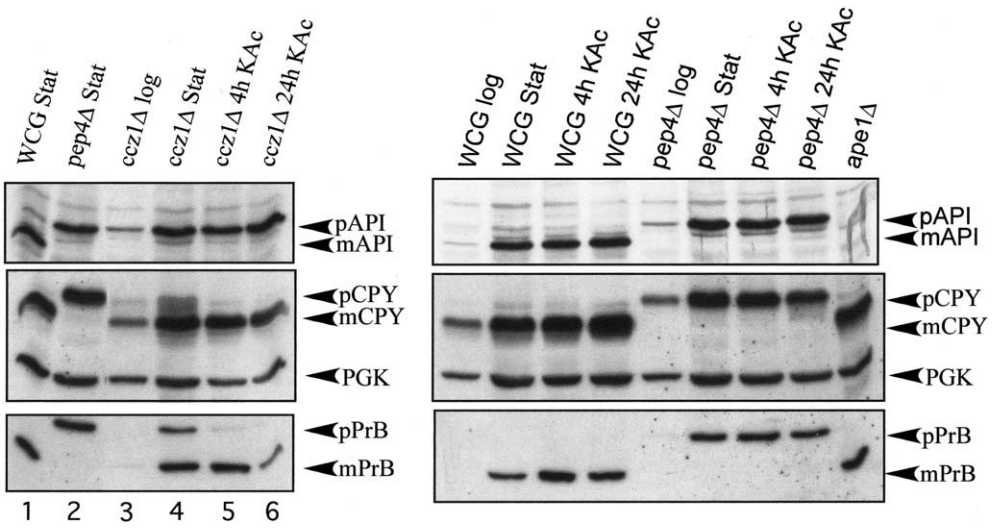

Fig. 1. *ccz1*Δ cells are impaired in proaminopeptidase I maturation. Crude extracts of logarithmic (log) and stationary (stat) cells and extracts from cells starved for the indicated times in 1% K-acetate were immunoblotted and probed with antibodies against (upper panel) proaminopeptidase I (API); (middle panel) carboxypeptidase Y (CPY) and 3-phosphoglycerate kinase (PGK); (lower panel) proteinase B. p: proform; m: mature. Wild-type and proteinase A-deficient (*pep4*Δ) cells are included.

but significant levels of mature proteinase B and Y are detectable in immunoblots (Fig. 1). This argues against a defect in proaminopeptidase I maturation due to impaired vacuolar proteolysis.

3.2. *CCZ1 is identical with previously unknown CVT16*

Cvt mutants were identified for their defect in proaminopeptidase I maturation [9], but so far only some of the mutated genes have been identified. Using the proaminopeptidase I maturation defect we tested if *ccz1*Δ cells are allelic with any of the uncharacterized cvt mutants. Indeed, *cvt16-1* mutant cells are allelic with *ccz1*Δ cells (Fig. 2, lane 7). This demonstrates that *CVT16* is identical with *CCZ1*.

3.3. *The autophagosomal marker GFP-Aut7p accumulates in cytosolic, punctate structures in ccz1Δ cells*

To determine at which step in *ccz1*Δ cells the cvt pathway and autophagy are blocked, we used a biologically active GFP-Aut7p expressed from a centromeric plasmid with its native promoter [3]. Aut7p is coupled via a ubiquitin-like protein conjugation system to phosphatidylethanolamine [22] and plays an important role in the biogenesis of cvt vesicles and autophagosomes. During autophagosome biogenesis Aut7p together with proaminopeptidase I is selectively enclosed in the vesicles and selectively reaches the vacuole, where it is degraded [23]. Direct fluorescence microscopy shows GFP-Aut7p, in stationary wild-type cells, in the cytosol and at the preautophagosomal compartment. During starvation it reaches the vacuole, where GFP is proteolytically released and accumulates due to its proteolysis resistance (Fig. 3A,B) [3,4]. In stationary or starved *ccz1*Δ cells GFP-Aut7p is detected in punctate, vesicular structures in the cytosol (Fig. 3C,D). *ccz1*Δ cells have fragmented vacuoles [21]. After nitrogen starvation no green fluorescence was detectable in the fragmented vacuoles of *ccz1*Δ cells (Fig. 3D). This suggests in *ccz1*Δ cells a defect in the cvt pathway and autophagy before vacuolar uptake of autophagosomes or cvt vesicles.

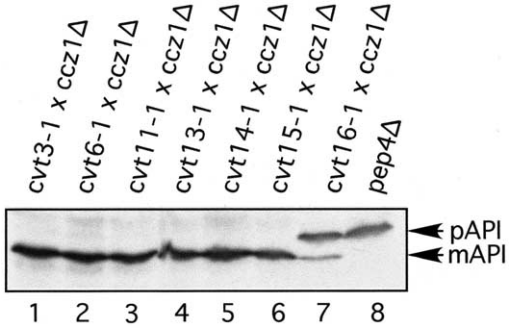

Fig. 2. *cvt16-1* mutants are allelic with *ccz1*Δ cells. The indicated cvt mutants were crossed with *ccz1*Δ cells and crude extracts of stationary cells were immunoblotted with antibodies against proaminopeptidase I.

3.4. *In ccz1Δ cells proaminopeptidase I is membrane-protected*

Ccz1p physically interacts with the rab GTPase Ypt7p [24], and a function in vacuolar delivery of carboxypeptidase Y and in a postinternalization step of the endocytic traffic has been suggested [21,25]. For Ypt7p a function in vacuolar fusion of cvt vesicles and autophagosomes was shown [26]. Accordingly, *ypt7*Δ cells accumulate proaminopeptidase in the cytosol trapped in vesicles [23]. Speculatively, Ccz1p might have a function similar to Ypt7p in proaminopeptidase I transport. To check this, we converted *ccz1*Δ cells to spheroplasts and lysed them hypotonically. Under these conditions proaminopeptidase I-containing vesicles stay intact, as shown by proteinase inaccessibility of proaminopeptidase I in *ypt7*Δ cells (Fig. 4B, lanes 5 and 6). As expected, in lysed *ccz1*Δ spheroplasts proaminopeptidase I was not digested after addition of proteinase K (Fig. 4C, lane 5) but after addition of proteinase K together with the detergent Triton X-100 (Fig. 4C, lane 6). This suggests that proaminopeptidase I-containing vesicles are still formed in *ccz1*Δ cells, but are unable to fuse with the vacuole. We enclosed as a further control *aut3*Δ (*apg1*Δ) cells, which show proteinase-accessible proaminopeptidase I (Fig. 4A).

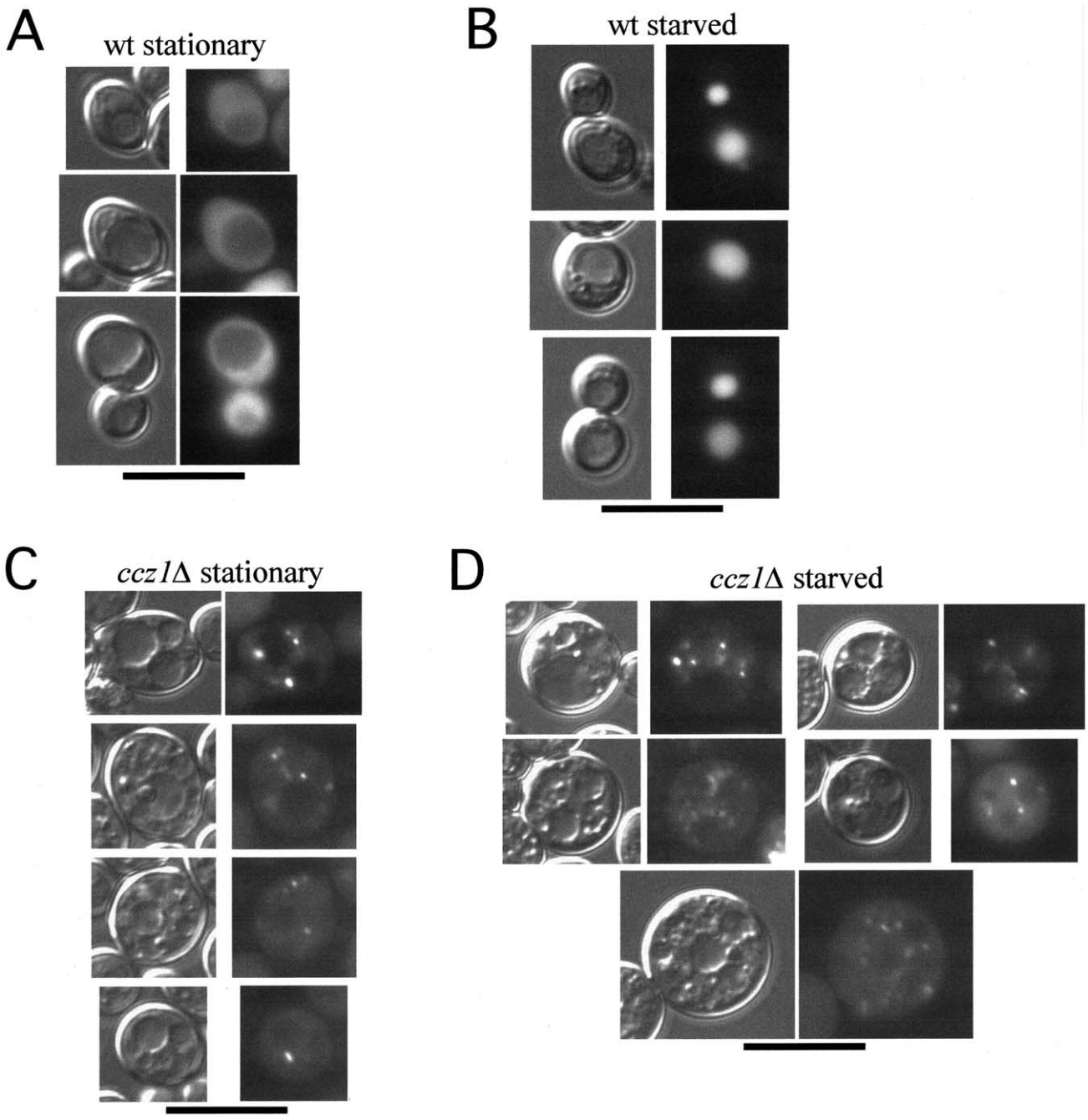

Fig. 3. GFP-Aut7p localizes to punctate, vesicular structures in the cytosol of *ccz1Δ* cells. Wild-type cells expressing GFP-Aut7 from a centromeric plasmid with its native promoter were grown to stationary phase (A) or starved 4 h in SD-N (B). Nomarski optics (left) and direct fluorescence (right) are shown. *ccz1Δ* cells expressing GFP-Aut7p of the stationary phase (C) or starved for 4 h in SD-N (D) were treated analogously. Further details in the text. Bar: 10 μm.

3.5. *ccz1Δ cells are defective in autophagy*

When wild-type cells are starved for nitrogen in the presence of the proteinase B inhibitor phenylmethylsulfonyl fluoride (PMSF), autophagy can be followed by the accumulation of autophagic bodies inside the vacuoles in Nomarski optics [20]. Since *ccz1Δ* cells display fragmented vacuoles this assay was not reliable. A further disadvantage of this 'vesicle accumulation test' is that it gives no quantitative results. We were therefore further interested in establishing a novel quick assay, which allows at least semi-quantitative examination of the autophagic capacity. As outlined GFP-Aut7p selectively reaches the vacuole via autophagy, where proteolytic cleavage of the fusion protein and release of the quite proteolysis-resistant GFP occurs. Wild-type cells expressing GFP-Aut7p therefore should show an increasing level of free GFP during starvation. As shown in Fig. 5A this is indeed detectable in immunoblots of crude cell extracts. As a control, we included *ypt7Δ* cells known to exhibit an autophagic block. As expected in these cells, no free GFP is formed from GFP-Aut7 during starvation. The lack of free GFP generated during starvation of *ccz1Δ* cells expressing GFP-Aut7 further

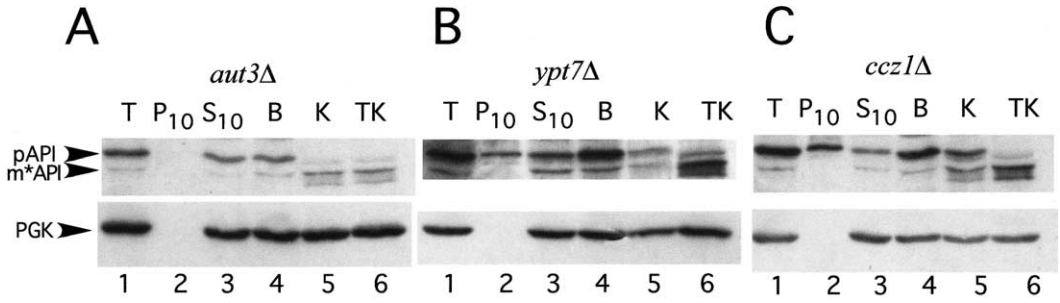

Fig. 4. Proaminopeptidase I in *ccz1*Δ cells is proteinase-protected. Stationary cells were spheroplasted and lysed hypotonically. The lysate was incubated with buffer (B, lanes 4), proteinase K (lanes 5) and proteinase K and Triton X-100 (lanes 6). After immunoblotting the samples were probed with antibodies against proaminopeptidase I. An aliquot of the lysate (T, lanes 1) was further centrifuged at 10 000×*g* yielding a P_{10} pellet fraction (lanes 2) and an S_{10} supernatant (lanes 3). The absence of cytosolic 3-phosphoglycerate kinase in the P_{10} fraction excludes that proteinase protection is mimicked by contaminating unlysed cells. *ccz1*Δ cells are used in C. As controls *ypt7*Δ (B) and *aut3*Δ (A) cells are included. Note that proaminopeptidase I is not degraded by proteinases but converted to a mature-like form (m*API).

confirms their autophagic defect. This finding is consistent with the absence of proaminopeptidase I maturation even after prolonged starvation (Fig. 1). To indicate the essential function of *CCZ1* in autophagy, we termed the ORF *AUT11*, but use *CCZ1* as the official name.

4. Discussion

In a reverse genetics screen we identified *ccz1*Δ cells for their defect in proaminopeptidase I maturation under non-starvation and starvation conditions (Fig. 1). Together with the allelism of *ccz1*Δ with *cvt16-1* cells (Fig. 2), this suggests a defect in the cvt pathway and autophagy. *ccz1*Δ cells are defective in vacuolar protein sorting of carboxypeptidase Y [21], therefore a lack of mature vacuolar proteinases might be responsible for the defect in proaminopeptidase I maturation. The detection of significant levels of mature proteinase B in *ccz1*Δ cells (Fig. 1), however, argues against this possibility. To determine at which stage the autophagic pathway is blocked in *ccz1*Δ cells, we used GFP-Aut7p as a specific marker protein, which together with proaminopeptidase I is selectively enclosed in autophagosomes [3,4,23]. Most interestingly, in contrast to wild-type cells (Fig. 3B), GFP-Aut7p was unable to reach the vacuole in starved *ccz1*Δ cells (Fig. 3D), as indicated by the absence of GFP fluorescence inside their fragmented vacuoles. This confirms the autophagic defect of these cells and suggests a defect prior to vacuolar uptake of autophagosomes. As shown in Fig. 3C,D, GFP-Aut7p accumulates in the cytosol of starved and non-starved *ccz1*Δ cells at punctate, vesicular structures similar to *ypt7*Δ cells (not shown) [3]. These structures might be identical with the pre-autophagosomal compartment or with vesicular intermediates, which bind their specific cargos, but are unable to form membrane-enclosed vesicles. In both cases the cargo proteins would be accessible to proteinases. Alternatively, the structures might be autophagosomes (starved cells) or cvt vesicles (non-starved cells), which specifically enclose GFP-Aut7p and proaminopeptidase I, but fail to fuse with the vacuole. In this scenario the cargo proteins would accumulate membrane-protected and proteinase-inaccessible. Our proteinase protection experiment using proaminopeptidase I as a marker (Fig. 4C) clearly shows accumulation of membrane-protected proaminopeptidase I. Our findings are compatible with a function of Ccz1p in vacuolar uptake (fusion) of these vesicles. This fits with the function of the rab GTPase Ypt7p [24] in vacuolar fusion of autophagosomes and cvt vesicles [26], consistent with a previously shown protein–protein interaction between Ypt7p and Ccz1p [25].

The defect in proaminopeptidase I maturation in *ccz1*Δ cells even after prolonged starvation (Fig. 1) and the lack of GFP accumulation in starved *ccz1*Δ cells expressing GFP-Aut7p (Fig. 3D) indicate an autophagy defect. However, monitoring of autophagy by following the vacuolar accumulation of autophagic bodies during starvation with the proteinase B inhibitor PMSF was prevented by the fragmentation of vacuoles. We therefore further followed the vacuolar degradation of

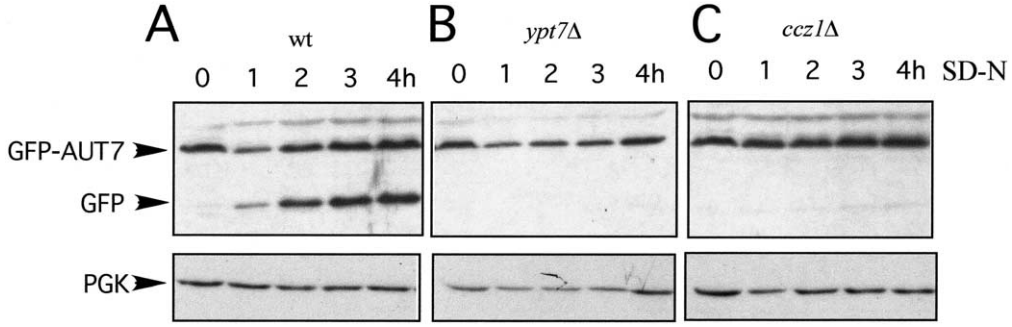

Fig. 5. *ccz1*Δ cells are unable to degrade GFP-Aut7p via autophagy. At the indicated times during starvation in SD-N, aliquots of cells expressing GFP-Aut7p from a centromeric plasmid are withdrawn and after lysis immunoblotted and analyzed with antibodies against GFP. The membranes were reprobed with antibodies against 3-phosphoglycerate kinase (PGK) as a loading control. A: Wild-type; B: *ypt7*Δ; C: *ccz1*Δ cells.

GFP-Aut7p in immunoblots. During starvation in wild-type cells GFP-Aut7p is selectively targeted to the vacuole via autophagy (Fig. 3A). Here GFP-Aut7p is proteolytically attacked and rather proteolysis-resistant GFP is formed in increasing amounts with time (Fig. 5A). The absence of free GFP in both starved *ypt7*Δ and starved *ccz1*Δ cells nicely confirms the autophagic defect of *ccz1*Δ cells. We believe that this novel assay to follow autophagy in immunoblots will be very useful and powerful for many laboratories.

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