The yeast deubiquitinating enzyme Ubp16 is anchored to the outer mitochondrial membrane

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Abstract **We looked for membrane-associated Dubs (deubiquitinating enzymes) among the 16 yeast members of the ubiquitin-specific processing protease (Ubp) family to identify potential regulators of ubiquitin-dependent processes at membranes. For each of the Ubps examined, a certain fraction was found to be membrane associated. This fraction was only small for most Ubps but quite substantial for some Ubps. For Ubp4/Doa4 almost 40% of the protein was found in the membrane fraction suggesting that this protein performs a major function at membranes, probably at endosomes. Among the proteins tested, only one protein (Ubp16) was exclusively membrane associated. By cell fractionation and immunofluorescence experiments, we could show that Ubp16 is localized to mitochondria. Ubp16 contains an N-terminal hydrophobic domain that is similar to N-terminal sequences of other yeast outer mitochondrial membrane proteins. The presence of this putative signal sequence and the result of protease protection experiments suggest that Ubp16 is an integral membrane protein of the outer mitochondrial membrane with an N_{in}–C_{out} orientation. Phenotypic characterization of the Δ*ubp16* mutant and overexpression studies further suggest that Ubp16 is probably not important for the general functioning of mitochondria, but that it rather performs a more specialized function at mitochondria.**

Key words: Ubiquitin; Deubiquitination; Mitochondrion; Membrane association

1. Introduction

A large number of Dubs (deubiquitinating enzymes) has been identified in various organisms. There are two classes of classical Dubs, the ubiquitin C-terminal hydrolases (Uchs) that preferentially cleave ubiquitin from peptides and small adducts (e.g. Yuh1 in yeast) and the extremely divergent family of ubiquitin-specific processing proteases (Ubps) that cleave ubiquitin from protein substrates [1]. The classical Dubs are cysteine proteases. Recently, evidence has been presented for the existence of a new class of Dubs that are metalloproteases [2,3]. Of the 17 classical Dubs in yeast 16 belong to the Ubp class. None of the yeast Ubps proved to be essential under standard growth conditions and despite extensive analysis, no clear phenotypic defects could be detected for most of the single *ubp* mutants [4]. Evidence for a regulatory role in silencing and in the pheromone response pathway has been presented for Ubp3 [5,6].

There are indications that Dubs could also be involved in membrane-associated events. The Dub Ubp4/Doa4 has been implicated in the process of multivesicular bodies (MVB) formation at endosomes [7]. Ubiquitination serves as a signal that targets membrane proteins for degradation via the MVB pathway. Before incorporation into MVB vesicles, ubiquitin appears to be removed from cargo protein. Doa4 is thought to play a major role at this deubiquitination step. However, *doa4* mutations are pleiotropic suggesting that Doa4 could be involved in other cellular functions [8]. This opens up the possibility that other, more specialized Ubps could function at endosomes in addition to Doa4.

To identify potential regulators of ubiquitin-dependent processes at membranes, we looked for membrane-associated Dubs among the 16 yeast members of the Ubp family. Among the proteins tested, only one protein (Ubp16) was exclusively membrane associated. By cell fractionation and immunofluorescence experiments, we could show that Ubp16 is localized to the mitochondrial outer membrane. From the phenotypic characterization of the Δ*ubp16* mutant, we conclude that Ubp16 is probably not important for the general functioning of mitochondria, but that it rather performs a specialized function at mitochondria.

2. Materials and methods

2.1. Strains and plasmids

Yeast strains are derived either from JD52 (*MATa his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52*, J. Dohmen, Köln, Germany) or from BY4741 (*MATa his3-Δ1 leu2Δ met15Δ ura3Δ*, EUROSCARF, Frankfurt, Germany). A polymerase chain reaction (PCR)-based method was used to generate the C-terminal c-myc-tagged Ubp strains and the *UBP16* deletion [9]. The disruption was verified by specific PCRs. Details of plasmid constructions are given upon request.

2.2. Cell fractionation

Floatation gradients were essentially performed as described in [10]. Sucrose gradients were performed as described previously [11]. Protease protection experiments were performed as described in [12].

2.3. Mitochondrial staining and immunofluorescence

For mitochondrial staining, cells were grown in YPGE (1% yeast extract, 2% bacto peptone, 2% glycerol, 2% ethanol) to logarithmic phase. Temperature-sensitive mutants were preshifted to 37°C for 1 h. All further incubation steps were carried out at 37°C as well. Cells from 1 ml culture were harvested by centrifugation for 1 min at 5000 rpm and resuspended in 1 ml SD medium containing 1% casamino acids, 2% glycerol and 2% ethanol as carbon source. After addition of 400 nM MitoTracker Red™ (Molecular Probes, Eugene, OR, USA) the cells were incubated for 45 min in the dark. Then the cells were

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collected by centrifugation, resuspended in phosphate-buffered saline (PBS) pH 7.2 and incubated further for 30 min in the dark. Mitochondrial fluorescence was observed with a Zeiss Axioskop (Zeiss, Göttingen, Germany) equipped with a Zeiss AxioCam digital camera (Zeiss, Göttingen, Germany) using a rhodamine filter set. Immunofluorescence was performed as described elsewhere [13].

3. Results

3.1. Membrane association of Ubps

Membrane association of Ubps was examined by floatation on Optiprep gradients. For detection, the Ubps were marked with a 13myc-tag at their C-terminus. Tagging was successful for 15 of the Ubps, but we repeatedly failed to detect a signal for the Ubp13-myc fusion. Therefore, Ubp13 was excluded from further analysis. Cell extracts at the bottom of the centrifuge tube were overlayed with solutions of lower density. During centrifugation membranes float up to the top of the gradient due to their low density while proteins not attached to membranes remain behind in the lower, denser fractions of the gradient. Six fractions were collected from the gradients. Based on the fractionation pattern of marker proteins, the two top fractions (1 and 2) were considered the 'float fractions' containing the membranes and the remaining four fractions (3–6) were considered the 'non-float fractions' containing mostly soluble proteins. As shown in Fig. 1, the membrane proteins Pep12 (an endosomal SNARE protein) and Pma1 (the plasma membrane ATPase) were mostly found in fractions 1 and 2 of the gradient while the soluble phosphofructokinase (Pfk) was mainly found in fractions 4–6. As further proof of membrane association, cell extracts were treated with 1% Triton X-100 before centrifugation, which should solubilize most of the membranes. As expected, the fractionation profile for Pep12 was shifted towards the non-float fractions after Triton extraction (Fig. 1D). Pma1 is partly associated with detergent resistant membrane domains [10]. Because of this raft association a substantial portion of Pma1 was still found in the float fractions after Triton extraction (Fig. 1F).

For each of the Ubps a certain portion was recovered in the float fractions (Table 1). For some Ubps, however, this fraction was close to the fraction of contaminating soluble proteins carried along with the membranes, which was around 10% for Pfk. These proteins (<25% in float fractions) can therefore be regarded as largely soluble proteins. The smallest portion in the float fractions (16%, Fig. 1C) was observed for Ubp14 which is unique among the Ubps in that it is required for the disassembly of unanchored ubiquitin chains [14]. For some Ubps, on the other hand, the portion in the float fractions was quite high and appears to be significant. One of these proteins is Ubp4/Doa4 (37% in float fractions, Fig. 1B) that has been suggested to function at the endosome in the deubiquitination of cargo for the MVB pathway [4]. Among the Ubps examined only one protein, Ubp16, was exclusively membrane associated. Virtually all of the protein was recovered in the float fractions (Fig. 1A). The hydropathy

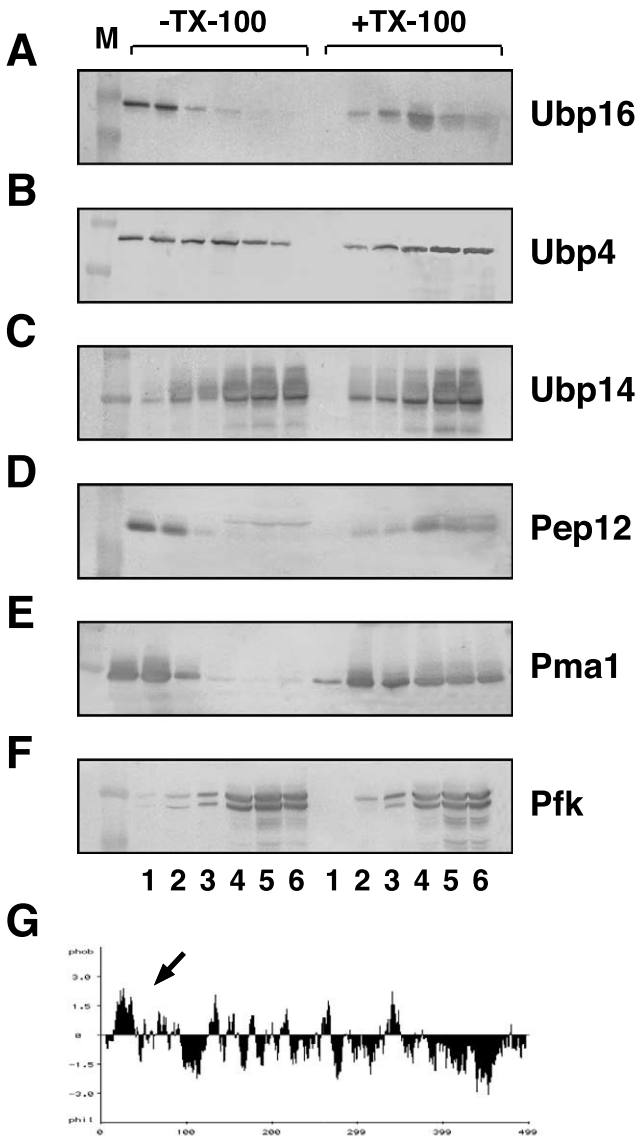

Fig. 1. Floatation analysis of Ubp proteins. Cell extracts of yeast strains encoding C-terminally tagged Ubp variants were fractioned on Optiprep™ floatation gradients. Half of the cell extracts were treated with 1% Triton X-100 before centrifugation (right half of the panels). Six fractions (lanes 1–6) were collected from the gradients (lanes 1, 2, float fraction; lanes 3–6, non-float fraction) and analyzed for the presence of proteins by Western blotting. A: Ubp16-13myc, B: Ubp4/Doa4-13myc, C: Ubp14-13myc, D: Pep12, E: Pma1, F: Pfk, G: hydropathy profile of Ubp16.

Table 1
Membrane association of Ubps

Protein	Float fractions 1, 2 (%)	Non-float fractions 3–6 (%)
Ubp1	32	68
Ubp2	21	79
Ubp3	21	79
Ubp4	37	63
Ubp5	27	73
Ubp6	23	77
Ubp7	27	73
Ubp8	31	69
Ubp9	41	59
Ubp10	19	81
Ubp11	36	64
Ubp12	22	78
Ubp14	16	84
Ubp15	27	73
Ubp16	88	12
Pep12	94	6
Pfk-*c-myc*	12	88
Pfk	7	93

profile of the protein reveals a hydrophobic stretch of amino acids at its N-terminus that could function as a membrane anchor (Fig. 1G). Thus the floatation experiment together with the hydropathy profile suggests that Ubp16 is an integral membrane protein.

3.2. Ubp16 is localized to mitochondria

To obtain information about the intracellular localization of Ubp16, cell extracts were fractionated on sucrose density gradients (Fig. 2). Ubp16 did not co-fractionate with most of the marker proteins tested, specifically it did not co-fractionate with the vacuolar marker alkaline phosphatase (ALP), the ER protein Dpm1, the endosomal protein Pep12 and the plasma membrane ATPase Pma1 (Fig. 3A). Instead, the Ubp16 fractionation pattern closely matched the distribution of porin, a protein of the outer mitochondrial membrane (Fig. 3B). The match, however, was not perfect. While the right halves of the peaks (towards the dense fractions of the gradient) were almost superimposable, the Ubp16 distribution extended slightly more towards the lower density fractions. A possible explanation for this finding is that two populations of mitochondria with different densities exist and that Ubp16 preferentially associates with the lower density population. Evidence for the existence of two different populations of mitochondria has indeed been presented [15].

Localization of Ubp16 was further examined by immunofluorescence experiments with yeast cells overexpressing Ubp16-13myc from a 2μ-plasmid. Overexpression of Ubp16 does not affect the fractionation pattern on sucrose gradients (not shown). As can be seen in Fig. 4, a typical mitochondrial staining pattern with 'worm or sausage-like structures' often extending around the periphery of the cell could be observed with anti-myc antibodies. Although the observed pattern is very suggestive of a mitochondrial localization, a possible ER localization had to be excluded, since somewhat similar

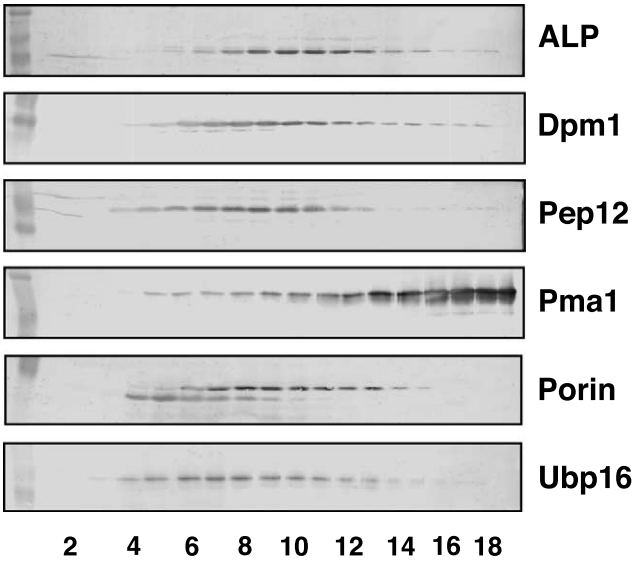

Fig. 2. Fractionation of Ubp16 on sucrose density gradients. Cell extracts of strain RKY1649 (*UBP16-13myc*) were fractionated on sucrose density gradients (20–53% w/w sucrose, fraction 1: low sucrose density). The gradient fractions were analyzed for the presence of marker proteins by Western blotting with specific antibodies as indicated.

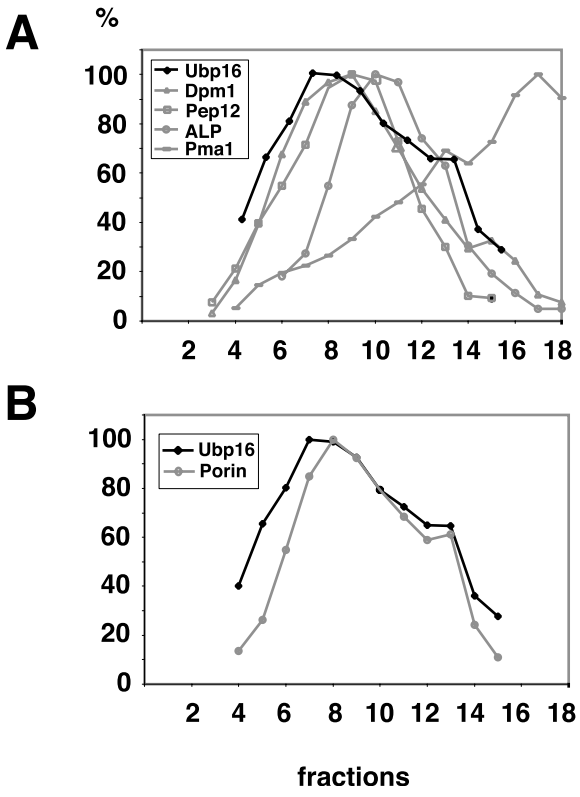

Fig. 3. Fractionation of Ubp16 on sucrose density gradients – densitometric quantification. Western blot signals of the sucrose gradients shown in Fig. 2 were quantified by densitometry. Western blots were scanned and signal intensities were quantified with the program NIH Image 1.62. The strongest signals were set to 100%.

structures can sometimes be also observed for the ER. We therefore performed a double-labeling experiment with anti-myc antibodies and antibodies against the ER protein Kar2. With the Kar2 antibodies, a typical ER pattern was observed with perinuclear and some peripheral staining (Fig. 4A). The Kar2 staining pattern, however, was clearly distinct from the Ubp16 staining. Thus an ER localization can be excluded for Ubp16. In some cells, extranuclear 4,6-diamino-2-phenylindole (DAPI) staining could be observed that closely matched the Ubp16 staining (Fig. 4B). This provides additional evidence for a mitochondrial localization of Ubp16. Further proof of mitochondrial localization was obtained with a Ubp16-GFP (green fluorescent protein) fusion protein expressed from a 2μ-plasmid. The GFP fluorescence perfectly coincided with the signal of a dye (MitoTracker™) that specifically stains mitochondria in living cells (Fig. 4C).

If Ubp16 is localized to mitochondria, it should be highly enriched in purified mitochondrial fractions. To test this prediction, mitochondria were purified from the Ubp16-13myc strain RKY1649 by differential centrifugation and sucrose density gradient fractionation as described in [16]. Indeed, we could show that Ubp16 was highly enriched in the purified mitochondrial fraction along with the mitochondrial markers aconitase (Aco1) and porin while other markers, like the vacuolar ALP, the endosomal t-SNARE Pep12 and the cytoplasmic phosphoglycerate kinase (PGK) were depleted from the mitochondrial fractions (Fig. 5). Thus from our localization experiments, we conclude that Ubp16 is a mitochondrial protein.

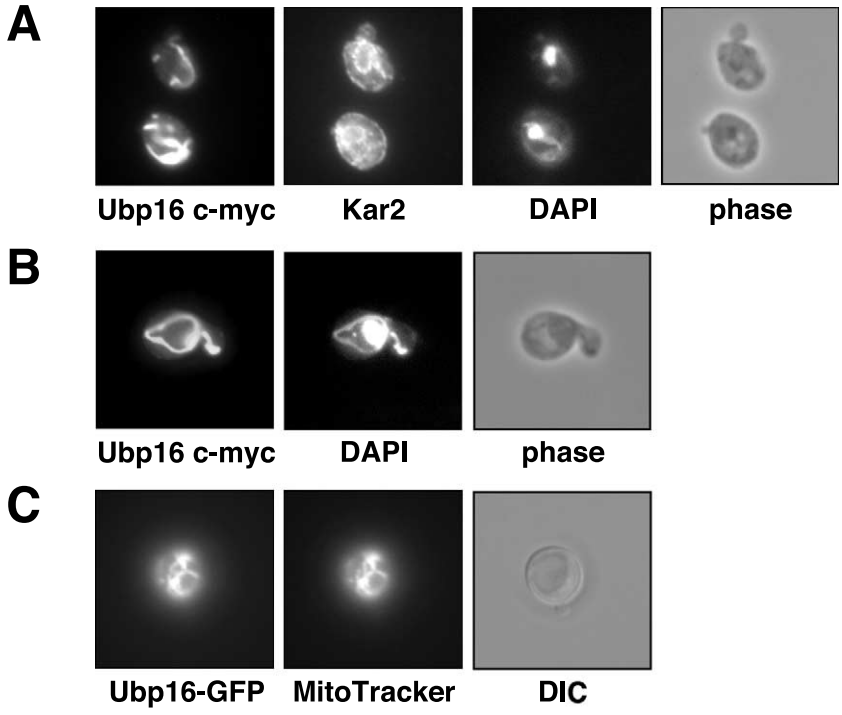

Fig. 4. Ubp16 is localized to mitochondria. A: Double-labeling experiment with strain JD52/pRK802 (*2μ-UBP16-13myc*). Ubp16-13myc was detected by immunofluorescence staining with anti-myc (9E10) primary antibodies and anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. Kar2 was detected with anti-Kar2 primary antibodies and anti-rabbit rhodamine-conjugated secondary antibodies. Nuclei were stained with DAPI. B: Co-localization between extranuclear DAPI staining material and Ubp16-13myc in JD52/pRK802. C: Co-localization between Ubp16-GFP (visualized with FITC filter set) and MitoTracker™ (visualized with rhodamine filter set) in living cells of strain JD52/pRK804 (*2μ-UBP16-GFP*).

3.3. *Ubp16 is localized to the outer mitochondrial membrane*

The submitochondrial localization of Ubp16 was examined by protease protection experiments (Fig. 6). In cell extracts, proteins on the outside of mitochondria should be sensitive towards added protease while proteins inside mitochondria should be protected against degradation. When protease K was added to cell extracts obtained by gentle lysis of yeast spheroplasts, Ubp16-13myc was readily degraded along with the cytoplasmic protein Pfk and the endosomal membrane protein Pep12 which is exposed to the cytoplasm. The mitochondrial matrix protein Mge1, however, was resistant against degradation showing that the mitochondria in the cell extract were intact. When the mitochondrial membranes were dissolved by the addition of detergent, Mge1 was degraded as well. This experiment, therefore, shows that Ubp16 is localized in the outer mitochondrial membrane with its main part oriented towards the cytoplasm.

3.4. *Function of Ubp16*

Actively respiring mitochondria are required for growth on media containing non-fermentable carbon sources. If Ubp16 is important for the general functioning of mitochondria, either deletion of *UBP16* or overproduction should lead to a growth defect on these media. But in neither case, an effect on the growth rate on non-fermentable carbon sources could be observed (not shown). Also, the overall morphology of mitochondria stained with MitoTracker™ appeared to be unaffected by deletion of *UBP16* or overproduction (not shown). Ubiquitination has been implicated in mitochondrial inheritance [17]. However, neither *UBP16* deletion nor overproduction had an effect on mitochondrial inheritance (not shown). This indicates that Ubp16 is not important for the general functioning of mitochondria.

4. Discussion

The Ubp family of Dubs was screened for membrane-associated Ubps to identify potential regulators of ubiquitin-dependent processes at membranes. A varying degree of membrane association was observed for individual Ubps. For most Ubps only a small portion was detected in the membrane fraction. For some Ubps, however, the portion in the membrane fraction was quite high. One of these proteins is Ubp4/Doa4 where 37% of the protein was found in the membrane fraction. It has been proposed that Doa4 functions at the late endosome by deubiquitinating proteins that are destined for degradation via the MVB pathway [4]. An endosomal localization for Doa4, however, could only be demonstrated so far in a Δ*vps4* mutant that accumulates aberrant endosomal structures, the so-called 'class E compartment' [18]. Our data suggest that a large amount of Doa4 is bound to endosomes also under normal conditions. A considerable amount in the membrane fraction was also observed for Ubp9 and Ubp11. Nothing is known so far about the function of these proteins. It will be interesting to see to which membrane compartments these proteins are localized.

Among the collection of yeast Ubps, only one protein, Ubp16, was exclusively membrane associated. By cell fractionation, immunofluorescence and GFP-tagging, we demonstrate that Ubp16 is localized to the outer mitochondrial membrane. Ubp16 contains a hydrophobic stretch of amino acids at its N-terminus that could function as a membrane anchor. It is

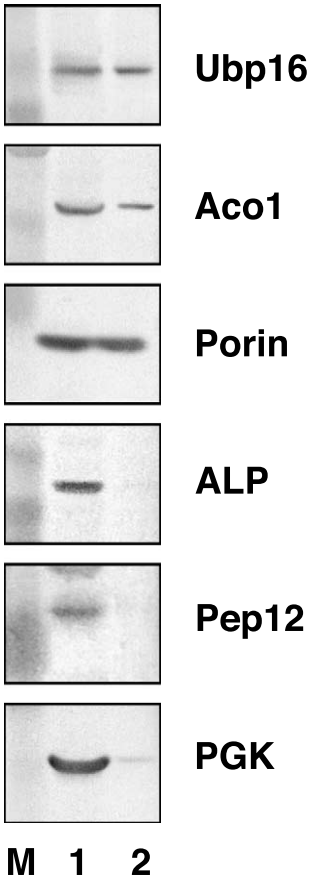

Fig. 5. Purification of mitochondria. Mitochondria were purified from cell extracts prepared from strain RKY1649 (*UBP16-13myc*) as described in [16]. The original cell extract (lane 1) and the mitochondrial fraction (lane 2) were examined for the presence of various proteins by Western blotting as indicated. A fraction of the mitochondrial preparation giving about the same porin signal as the cell extract was loaded onto the gel.

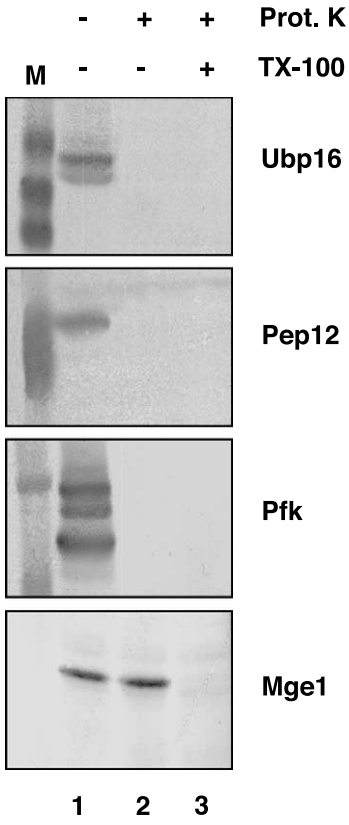

Fig. 6. Protease protection experiment. Cell extracts of strain RKY1649 (*UBP16-13myc*) were treated with proteinase K in the absence or presence of detergent (1% Triton X-100). Proteins were detected by Western blotting with specific antibodies as indicated. Lane 1, no protease; lane 2, protease only; lane 3, protease+detergent.

reasonable to assume that this sequence acts as a targeting signal for the outer mitochondrial membrane. Among the yeast proteins assigned to the outer mitochondrial membrane in the YPD database [19], three proteins (Tom20, Mcr1, OM45) were found with an N-terminal region similar to Ubp16 (Fig. 7). The putative N-terminal signal in these proteins consists of a 18–20 amino acid long alanine-rich central region flanked by sequences with a net positive charge. Not all outer mitochondrial proteins with N-terminal signal sequences conform to our alignment. For instance, no alanine-rich region was discernible in the N-terminal hydrophobic regions of Tom70, Ptc7 and Slc1. This suggests that the proteins listed in our alignment belong to a distinct class of outer mitochondrial proteins that are targeted to the outer mitochondrial membrane by a similar mechanism.

Loss of Ubp16 activity does not have a discernible effect on the pattern of ubiquitinated proteins from whole cell extracts suggesting that Ubp16 does not contribute much to the general deubiquitinating activity of the cell. Thus, it is unlikely that Ubp16 plays a major role in ubiquitin homeostasis under normal conditions, it rather appears to perform a very specialized function. Whatever this function might be, it does not appear to be concerned with the general functioning of mitochondria, since neither deletion nor overexpression of *UBP16* had any effect on the growth rate of yeast cells under conditions requiring actively respiring mitochondria. Also, morphology and inheritance of mitochondria appeared to be unaffected by deletion or overexpression of *UBP16*. But, it is also possible that Ubp16 is not related to mitochondrial function per se. There are indications that mitochondria are tied into cellular signaling networks. It has been shown that a large portion of FKBP12-rapamycin-associated protein (FRAP or mTor), an important sensor of physiologic signals

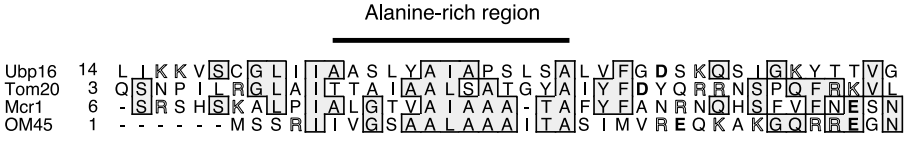

Fig. 7. Alignment of N-terminal signal sequences of outer mitochondrial proteins. Amino acids identical in at least two sequences are boxed. Basic amino acids are highlighted by shading, acidic ones by bold print. The alanine-rich region is marked.

that regulate cell growth, is bound to the outer membrane of mitochondria [20]. Based on these findings, it has been proposed that mitochondria integrate diverse cellular stress signals and generate a response that results in FRAP-mediated regulation of cell growth. If indeed many signaling pathways converge on mitochondria, there is plenty of room for the ubiquitin system (and thus for Ubp16) to contribute to these processes by regulating the turnover or activity of proteins at the outer mitochondrial membrane.

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