Membrane protein turnover by the *m*-AAA protease in mitochondria depends on the transmembrane domains of its subunits

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AAA proteases are membrane-bound ATP-dependent proteases that are present in eubacteria, mitochondria and chloroplasts and that can degrade membrane proteins. Recent evidence suggests dislocation of membrane-embedded substrates for proteolysis to occur in a hydrophilic environment; however, next to nothing is known about the mechanism of this process. Here, we have analysed the role of the membrane-spanning domains of Yta10 and Yta12, which are conserved subunits of the hetero-oligomeric *m*-AAA protease in the mitochondria of *Saccharomyces cerevisiae*. We demonstrate that the *m*-AAA protease retains proteolytic activity after deletion of the transmembrane segments of either Yta10 or Yta12. Although the mutant *m*-AAA protease is still capable of processing cytochrome *c* peroxidase and degrading a peripheral membrane protein, proteolysis of integral membrane proteins is impaired. We therefore propose that transmembrane segments of *m*-AAA protease subunits have a direct role in the dislocation of membrane-embedded substrates.

Keywords: mitochondria; proteolysis; AAA proteases; membrane protein turnover

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INTRODUCTION

The turnover of membrane proteins has been puzzling for a long time as the hydrophobic environment of the lipid bilayer was thought to preclude the cleavage of membrane-embedded protein segments. Since then, studies on ATP-dependent proteases revealed that membrane proteins are often extracted from the membrane bilayer to allow proteolysis to occur in a hydrophilic environment. This is exemplified by 26S proteasomes, which degrade membrane proteins of the endoplasmic reticulum (ER) in the cytosol of eukaryotic cells (Jarosch *et al*, 2003). Membrane dislocation is mediated by the Sec61 translocon and driven by the AAA protein p97/Cdc48 (Bays & Hampton, 2002). AAA proteases comprise another family of ATP-dependent proteases, which mediate the proteolytic breakdown of

membrane proteins after their dislocation from the membrane bilayer (Kihara *et al*, 1999; Leonhard *et al*, 2000). These peptidases by themselves are integral membrane proteins.

Mitochondria harbour two AAA proteases with catalytic sites exposed to opposite membrane surfaces: the *m*-AAA protease is active in the matrix and is composed of the homologous subunits Yta10 (Afg3) and Yta12 (Rca1) in yeast (Arlt *et al*, 1996) and of paraplegin and AFG3L2 in humans (Atorino *et al*, 2003); the *i*-AAA protease is composed of Yme1 subunits and exposes its catalytic sites to the intermembrane space (Leonhard *et al*, 1996; Weber *et al*, 1996). All known subunits of AAA proteases share a conserved domain structure. An amino-terminal transmembrane domain with one or two membrane-spanning segments is followed by an ATPase domain, characteristic of the AAA⁺ superfamily of ATPases (Ogura & Wilkinson, 2001), and a metallopeptidase domain.

AAA proteases control crucial steps during the biogenesis of mitochondria in yeast and mammals (Arlt *et al*, 1998; Atorino *et al*, 2003). Both mitochondrial AAA proteases are part of a quality control system in the inner membrane and ensure the removal of non-native membrane proteins. The membrane topology of substrate proteins seems to determine the involvement of either one or both AAA proteases, which extract membrane proteins to the respective side of the membrane for proteolysis (Leonhard *et al*, 2000). The mechanism of this dislocation process is at present not understood but it is conceivable that it occurs by means of a pore-like structure in the membrane and is driven by ATP-dependent conformational changes of AAA protease subunits.

Here, we have analysed the role of the transmembrane domains of the *m*-AAA protease subunits Yta10 and Yta12 in proteolysis. We demonstrate that the transmembrane domain of either *m*-AAA protease subunit is dispensable for assembly and proteolytic activity, but essential for the degradation of integral membrane proteins. These results point to a crucial function of membrane-embedded parts of the *m*-AAA protease in the proteolysis of integral membrane proteins.

RESULTS

Deletion of membrane domains of *m*-AAA protease subunits

Yeast cells lacking either Yta10 or Yta12 are respiratory deficient and do not grow on nonfermentable carbon sources (Arlt *et al*,

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1996). To examine the role of transmembrane segments in proteolysis, the N-terminal domain of mature Yta10 harbouring both membrane-spanning segments was deleted (Yta10 Δ TM). The mutant variant was expressed from a centromer-based plasmid under the control of the endogenous promoter in Δ yta10 cells (yta10 Δ TM^C; supplementary Fig 6 online), and the respiratory competence of the cells was examined (Fig 1A). yta10 Δ TM^C cells were able to grow on nonfermentable carbon sources, demonstrating that the expression of the catalytic domains of Yta10 is sufficient to restore the respiratory activity of Δ yta10 cells. The growth of these cells was retarded when compared with wild-type cells, but improved significantly on overexpression of the catalytic domain of Yta10 (Fig 1A). In contrast, Δ yta12 mutant cells remained respiratory deficient even in the presence of high levels of Yta10 Δ TM (Fig 1A). Similar experiments were performed on deletion of the N-terminal domain of mature Yta12 harbouring both membrane-spanning segments. Overexpression of the catalytic domains of Yta12 restored respiratory growth of Δ yta12 but not of Δ yta10 mutant cells (Fig 1B). We conclude from these experiments that the transmembrane domains of either Yta10 or Yta12 are dispensable for the maintenance of the respiratory competence of the cells.

The respiratory competence of Δ yta10 Δ yta12 cells was not restored either on expression of Yta10 Δ TM or Yta12 Δ TM, or in the presence of both mutant proteins (Fig 1C). Thus, the suppressive effect of Yta10 Δ TM in Δ yta10 cells depends on the presence of Yta12. Conversely, Yta10 is required for respiratory growth of yta12 Δ TM cells. This functional interdependence pointed to an assembly of both *m*-AAA protease subunits, which was assessed by co-immunoprecipitation. Extracts of wild-type, yta10 Δ TM and, for control, Δ yta10 mitochondria were incubated with Yta10-specific polyclonal antibodies. Yta12 was immunoprecipitated by Yta10-specific antibodies from yta10 Δ TM mitochondria, demonstrating an assembly of truncated Yta10 with Yta12 (Fig 1D). The interaction occurred with lower efficiency when compared with wild-type mitochondria, but was specific as Yta12 was not detected in the precipitate using Δ yta10 mitochondria (Fig 1D). Thus, the transmembrane regions of Yta10 are not essential for the assembly of both proteins. The reduced affinity of Yta10 Δ TM for Yta12 may explain why efficient respiratory growth of Δ yta10 cells was observed only on overexpression of Yta10 Δ TM (Fig 1A).

Respiratory growth requires both catalytic activities

Respiratory growth depends on proteolysis by the *m*-AAA protease (Arlt et al, 1998), suggesting that a mutant protease lacking the transmembrane segment of one subunit is still proteolytically active. To substantiate this conclusion, catalytically active glutamate residues in the consensus metal-binding motif HEAGH of both Yta10 and Yta12 were replaced by glutamine (Yta10 Δ TM^{E559Q}, Yta12 Δ TM^{E614Q}). The corresponding mutations in Yta10 and Yta12 impair proteolytic activity of the respective subunit but lead only to a loss of the respiratory competence of the cell if present in both subunits (Arlt et al, 1998). Expression of Yta10 Δ TM^{E559Q} in Δ yta10 cells restored the respiratory competence of the cells, indicating that complementation of the growth defect does not depend on the proteolytic activity of truncated Yta10 (Fig 2A). If, however, a mutation was introduced in the proteolytic centre of Yta12 in these cells, respiratory growth ceased (Fig 2A). These results provide genetic evidence that the

Fig 1 | Restoration of respiratory growth of Δ yta10 and Δ yta12 cells on expression of Yta10 Δ TM or Yta12 Δ TM, respectively. (A–C) Cells were grown at 30 °C on selective media containing the required auxotrophs and glucose (2%) as the sole carbon source. Fivefold serial dilutions of logarithmically growing cultures were spotted onto yeast peptone medium containing glycerol (3%) and incubated for 2 days at 30 °C. (D) Co-immunoprecipitation of Yta12 with Yta10 Δ TM. Mitochondria isolated from wild-type (WT), Δ yta10 and yta10 Δ TM cells were solubilized in digitonin and incubated with Yta10-specific antibodies. The immunoprecipitate was analysed by SDS-PAGE and immunoblotting using Yta12-specific antiserum.

m-AAA protease retains proteolytic activity after deletion of the transmembrane domain of Yta10. Notably, *yta12^{E614Q}* cells were incapable of growing on nonfermentable carbon sources irrespective of the presence of Yta10 Δ TM or Yta10 Δ TM^{E559Q} (Fig 2A). Thus, respiratory competence is maintained by the proteolytic activity of wild-type Yta12.

How does the truncated, proteolytically inactive matrix domain of Yta10 affect proteolysis by Yta12? AAA proteins are known to form ring complexes that exert ATPase activity only in the assembled state (Ogura & Wilkinson, 2001). Available crystal structures of various AAA domains revealed that the so-called arginine fingers protrude into the catalytic site of adjacent subunits and thereby stimulate ATP hydrolysis. Consistently, mutation of the putative arginine-finger residue 315 in the bacterial AAA protease FtsH was found to impair ATPase activity (Karata *et al*, 1999). We therefore replaced the arginine residues 447 and 450 in the AAA domains of Yta10 Δ TM by alanine and expressed the mutant proteins in Δ yta10 cells. Replacement of either the putative arginine-finger residue 447 or of arginine 450 by alanine abolished the complementing activity of Yta10 Δ TM (Fig 2B). Thus, the ATPase activity of Yta10 Δ TM but not its proteolytic activity is required for respiratory growth.

Proteolysis of integral membrane proteins

We next analysed the stability of various misfolded polypeptides newly imported into isolated *yta10 Δ TM* and *yta12 Δ TM* mitochondria. A derivative of the inner membrane protein Yme2 lacking its intermembrane space domain has been demonstrated to be degraded by the *m*-AAA protease (Leonhard *et al*, 2000). Yme2 Δ C (exposing only 16 amino-acid residues to the intermembrane space) was synthesized in a cell-free system in the presence of [³⁵S]methionine and imported post-translationally into mitochondria isolated from wild-type, Δ yta10 and *yta10 Δ TM* cells. To assess the stability of the newly imported protein, mitochondria were further incubated at 37 °C (Fig 3A). Whereas Yme2 Δ C was degraded in wild-type mitochondria with *t*_{1/2} ~10 min, the proteolytic breakdown was strongly impaired in the absence of the *m*-AAA protease subunit Yta10 (Fig 3A). Overexpression of Yta10 Δ TM in these mitochondria did not result in accelerated degradation (Fig 3A). Similarly, proteolysis of Yme2 Δ C was impaired in mitochondria lacking the *m*-AAA protease subunit Yta12 and was not restored in the presence of Yta12 Δ TM (Fig 3A). These results indicate that the mutant *m*-AAA protease is not capable of degrading Yme2 Δ C.

Oxa1 represents another mitochondrial inner membrane protein that has recently been demonstrated to be degraded by the *m*-AAA protease when misfolded (Käser *et al*, 2003). A radiolabelled temperature-sensitive mutant form of Oxa1 (Oxa1^{ts}) (Bauer *et al*, 1994) was imported into mitochondria lacking *m*-AAA protease subunits or expressing truncated variants of both proteins. The mutant protein was degraded at nonpermissive temperature in wild-type mitochondria, but significantly stabilized in Δ yta10 or Δ yta12 mitochondria (Fig 3B). Proteolysis of Oxa1^{ts} was not accelerated in the presence of Yta10 Δ TM or Yta12 Δ TM (Fig 3B). Thus, neither Yme2 Δ C nor Oxa1^{ts}, both integral inner membrane proteins, can be degraded by a mutant *m*-AAA protease lacking the transmembrane domain of Yta10 or Yta12. We conclude from these experiments that deletion of the transmembrane domain of one *m*-AAA protease

Fig 2 | Respiratory competence of *yta10 Δ TM* cells depends on the proteolytic activity of Yta12 and the ATPase but not the proteolytic activity of Yta10 Δ TM. Cells were grown at 30 °C on YP medium containing glycerol. (A) Expression of a proteolytic site mutant variant of Yta10 Δ TM in Δ yta10 cells (*yta10^{E559Q} Δ TM*) and in Δ yta10 Δ yta12 cells expressing proteolytically inactive Yta12^{E614Q} (*yta10^{E559Q} Δ TM yta12^{E614Q}*) (B) Expression of Yta10 Δ TM variants harbouring mutations in conserved arginine residues of the SRH region in Δ yta10 cells.

subunit impairs the ability of the protease to degrade integral membrane proteins.

Processing of cytochrome c peroxidase

Processing of cytochrome c peroxidase (Ccp1) has recently been demonstrated to depend on the *m*-AAA protease (Esser *et al*,

Fig 3 | Impaired proteolysis of misfolded integral inner membrane proteins in *yta10ΔTM* and *yta12ΔTM* mitochondria. The stability of newly imported, ³⁵S-labelled Yme2ΔC (A) and Oxa1^{ts} (B) was analysed at 37 °C in mitochondria isolated from wild-type (WT), *Δyta10*, *yta10ΔTM*, *Δyta12* and *yta12ΔTM* cells. The average of at least three independent experiments ($\pm$ s.e.m.) is shown.

2002). To monitor the effect of deletion of the transmembrane segments of Yta10 or Yta12 on Ccp1 processing, mitochondria were isolated from *Δyta10*, *yta10ΔTM*, *Δyta12* and *yta12ΔTM* cells and analysed by immunoblotting using Ccp1-specific antibodies (Fig 4). Whereas the precursor form of Ccp1 accumulated in *Δyta10* and *Δyta12* mitochondria, the mature form was detected in *yta10ΔTM* and *yta12ΔTM* mitochondria (Fig 4). Notably, cleavage of Ccp1 occurred with similar efficiencies in *yta12ΔTM* and wild-type mitochondria, whereas only partial maturation was observed in *yta10ΔTM* mitochondria. We conclude that neither the transmembrane domain of Yta10 nor Yta12 is essential for processing of Ccp1.

Degradation of the peripheral membrane protein Atp7

It is conceivable that membrane insertion of *m*-AAA protease subunits is crucial for the turnover of integral membrane proteins but not for the processing of a soluble protein. Alternatively, the mutant *m*-AAA protease may be able to perform single cleavage events but not the complete turnover of a polypeptide. To distinguish between these possibilities, we analysed the stability of Atp7, which is a stalk subunit of the F₁F₀-ATP synthase and peripherally associated with the inner surface of the mitochondrial inner membrane. Radiolabelled Atp7 was rapidly degraded after

import into wild-type mitochondria (Fig 5; supplementary Fig 7 online). Deletion of either Yta10 or Yta12 resulted in a partial stabilization of Atp7, identifying it as a novel substrate of the *m*-AAA protease. Strikingly, although still slightly retarded when compared with wild-type mitochondria, proteolysis was significantly accelerated on expression of Yta10ΔTM or Yta12ΔTM in the respective null mutant mitochondria (Fig 5). Thus, the transmembrane domains of either Yta10 or Yta12 are dispensable for the degradation of the peripheral membrane protein Atp7.

DISCUSSION

AAA proteases are distinct from most other ATP-dependent proteases because they recognize membrane proteins as substrates and are themselves membrane-embedded. We have therefore analysed the role of the transmembrane domains of mitochondrial *m*-AAA protease subunits in proteolysis. Our results demonstrate that the *m*-AAA protease retains proteolytic activity after deletion of the membrane-spanning domains of one subunit. However, the degradation of various substrate proteins by the mutant *m*-AAA protease is affected differentially: although proteolysis of peripheral membrane proteins as well as pre-sequence cleavage can occur, the turnover of integral membrane proteins is impaired.

Fig 4 | Processing of Ccp1 by a mutant *m*-AAA protease lacking the transmembrane domain of one subunit. Mitochondria were isolated from wild-type (WT), $\Delta yta10$, $yta10\Delta TM$, $\Delta yta12$ and $yta12\Delta TM$ cells and fractionated by SDS-PAGE (30 μ g). Samples were analysed by immunoblotting using polyclonal antisera directed against Ccp1 (anti-Ccp1) and, as gel loading controls, cytochrome b_2 (anti-Cyt b_2) and Mge1 (anti-Mge1). p, precursor form of Ccp1; m, mature form of Ccp1.

Studies on the bacterial AAA protease FtsH revealed a crucial role of membrane-spanning regions in the oligomerization of the protease (Akiyama & Ito, 2000). In agreement with these findings, deletion of the transmembrane domains of both Yta10 and Yta12 abolished the respiratory competence of yeast cells even when both subunits were overexpressed. Co-immunoprecipitation experiments using mitochondrial extracts or *in vitro* binding studies using purified catalytic domains of Yta10 or Yta12 did not provide any evidence for a physical interaction of both domains (T.L., unpublished observations). In contrast, assembly of the *m*-AAA protease was evident in the absence of the transmembrane domain of only one subunit, demonstrating that catalytic domains are indeed assembly-competent. The hetero-oligomeric nature of the *m*-AAA protease therefore offered a unique possibility to study the role of the transmembrane domains of AAA proteases in proteolysis, independent of effects on the assembly of the proteolytic complex.

Deletion of the membrane-embedded domain of one type of subunit does not result in an overall inactivation of the *m*-AAA protease. However, our mutational analysis indicates that truncated *m*-AAA protease subunits themselves do not participate in proteolysis. Cells harbouring a truncated variant of Yta10 lost respiratory competence in the presence of a proteolytic site variant of Yta12, irrespective of the integrity of the proteolytic centre of Yta10 Δ C. Thus, respiratory growth of cells expressing a mutant *m*-AAA protease lacking the transmembrane domain of Yta10 depends solely on the proteolytic activity of Yta12.

It should be emphasized, however, that the truncated catalytic domain is not completely inactive. Replacement of the conserved putative arginine finger in truncated Yta10 abolished the complementing activity. This suggests that, similar to the oligomerization-dependent stimulation of the ATPase of other AAA proteins, the ATPase of the wild-type subunit of the *m*-AAA protease is activated by assembly with the catalytic domain of the truncated subunit.

Although these results demonstrate proteolytic activity of a mutant *m*-AAA protease harbouring a truncated subunit, proteolysis of integral membrane proteins was found to be impaired. It seems improbable that membrane domains function only to bring the catalytic domains of the protease subunits in close proximity to

Fig 5 | Degradation of Atp7 by a mutant *m*-AAA protease harbouring truncated subunits. Radiolabelled Atp7 was imported into mitochondria isolated from wild-type (WT), $\Delta yta10$, $yta10\Delta TM$, $\Delta yta12$ and $yta12\Delta TM$ cells, and the stability of newly imported Atp7 at 37 °C was determined as in Fig 3. The average of four independent experiments ($\pm$ s.e.m.) is shown.

the membrane surface allowing the proteolytic attack of integral membrane proteins. Catalytic domains of truncated Yta10 or Yta12 assemble with the other respective wild-type subunit of the *m*-AAA protease and are thereby localized close to the inner surface of the inner membrane. That the mutant *m*-AAA protease indeed exerts proteolytic activity at the inner membrane is illustrated by the degradation of Atp7 peripherally associated with the inner membrane. Thus, the impaired turnover of integral membrane proteins cannot be explained by a deficient membrane anchoring of the protease.

How does the deletion of the transmembrane domain of one subunit affect the proteolytic activity of the *m*-AAA protease? In contrast to substrate proteins peripherally associated with membranes, integral membrane proteins are extracted from the lipid bilayer for proteolysis. In analogy to the proteolysis of ER membrane proteins by 26S proteasomes, substrate extraction may involve protein translocases in the inner membrane, which mediate the membrane insertion of newly synthesized mitochondrial proteins. However, proteolysis of inner membrane proteins was not affected in yeast mitochondria on saturation of TIM23-protein import channels with translocation intermediates (M.K. and T.L., unpublished observations). It therefore seems that different mechanisms underlie the membrane dislocation

processes in the ER and the mitochondrial inner membrane. We propose that the transmembrane domains of *m*-AAA protease subunits are required for this dislocation step, which is presumably driven by ATP-dependent conformational changes of the AAA domains. Given the predicted ring-like structure of AAA proteases, the transmembrane domains by themselves might form a pore-like structure. Alternatively, they may reduce hydrophobicity around membrane-embedded parts of substrate proteins and thereby facilitate membrane extraction.

METHODS

Mutagenesis of YTA10 and YTA12. For the deletion of the N-terminal regions of mature Yta10 or Yta12, DNA segments of YTA10 or YTA12 coding for matrix domains of either protein (amino acids 261–761 for Yta10 and 322–825 for Yta12) were amplified by PCR and ligated with another amplified DNA fragment encoding the promoter region of either gene and the mitochondrial targeting sequence of Yta10 (amino acids 1–43) or Yta12 (amino acids 1–39). The DNA fragments obtained were cloned either into the centromer-based yeast expression vector YCplac111 (YCplac111-*yta10ΔTM*) or the multicopy yeast expression vector YEplac112 (YEplac112-*yta12ΔTM*, YEplac112-*yta10ΔTM*) or YEplac181 (YEplac181-*yta10ΔTM*). A truncated variant of the proteolytic site mutant variant of Yta10 (Yta10^{E559QΔTM}) was constructed by replacement of an *AgeI*–*NcoI* DNA fragment of YEplac112-*yta10ΔTM* by the corresponding fragment of YCplac111::ADH1-*yta10*^{E559Q} (Arlt *et al*, 1996). *yta10*^{R450AΔTM} and *yta10*^{R447AΔTM} were generated by PCR-based site-directed mutagenesis.

Yeast strains. Yeast strains used in this study are derivatives of W303. The mutant strains *Δyta10* (YGS101), *Δyta12* (YGS202), *Δyta10Δyta12* (YHA301) and *yta12*^{E614Q} (YHA203) were described previously. YCplac111-*yta10ΔTM* was transformed in *Δyta10* (*yta10ΔTM*^C; YDK1) cells, and YEplac181-*yta10ΔTM* in *Δyta10* (*yta10ΔTM*; YDK2), *Δyta10Δyta12* (YDK11) and *Δyta10 yta12*^{E614Q} cells (YDK14). Yta12ΔTM (YEplac181-*yta12ΔTM*) was expressed in *Δyta12* (*yta12ΔTM*; YDK8), *Δyta10Δyta12* (YDK12) and *yta10*^{E559Q}*Δyta12* (YDK16) cells. The mutant variants Yta10^{E559QΔTM}, Yta10^{R450AΔTM} or Yta10^{R447AΔTM} were expressed in *Δyta10* mutant cells (generating the strains YDK4, YSW3 and YSW5, respectively).

Co-immunoprecipitation of Yta10ΔTM and Yta12. Mitochondria (200 μg) were resuspended at a concentration of 5 mg/ml in buffer A (30 mM Tris–HCl (pH 7.4), 1% (w/v) digitonin, 20% glycerol, 150 mM K-acetate, 4 mM Mg-acetate, 1 mM ATP, 1 mM phenylmethylsulphonyl fluoride) and lysed by vigorous mixing for 15 min at 4 °C. After a clarifying spin for 30 min at 125,000g, the supernatant was incubated with gentle shaking for 60 min at 4 °C with antiserum directed against a carboxy-terminal peptide of Yta10, which had been coupled to protein A–Sepharose. The beads were washed twice with buffer A (0.5 ml) and once with 30 mM Tris–HCl (0.5 ml, pH 7.4). Immunocomplexes were

dissociated with SDS sample buffer by vigorous shaking for 10 min at 4 °C and incubated for 5 min at 95 °C. The immunoprecipitates were then analysed by SDS–PAGE and immunostaining.

Stability of newly imported proteins in mitochondria. Import and proteolysis of radiolabelled mitochondrial preproteins were performed essentially as described (Käser *et al*, 2003).

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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