Mitochondrial protein turnover: role of the precursor intermediate peptidase Oct1 in protein stabilization

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ABSTRACT Most mitochondrial proteins are encoded in the nucleus as precursor proteins and carry N-terminal presequences for import into the organelle. The vast majority of presequences are proteolytically removed by the mitochondrial processing peptidase (MPP) localized in the matrix. A subset of precursors with a characteristic amino acid motif is additionally processed by the mitochondrial intermediate peptidase (MIP) octapeptidyl aminopeptidase 1 (Oct1), which removes an octapeptide from the N-terminus of the precursor intermediate. However, the function of this second cleavage step is elusive. In this paper, we report the identification of a novel Oct1 substrate protein with an unusual cleavage motif. Inspection of the Oct1 substrates revealed that the N-termini of the intermediates typically carry a destabilizing amino acid residue according to the N-end rule of protein degradation, whereas mature proteins carry stabilizing N-terminal residues. We compared the stability of intermediate and mature forms of Oct1 substrate proteins in organello and in vivo and found that Oct1 cleavage increases the half-life of its substrate proteins, most likely by removing destabilizing amino acids at the intermediate's N-terminus. Thus Oct1 converts unstable precursor intermediates generated by MPP into stable mature proteins.

INTRODUCTION

Virtually all mitochondrial proteins are synthesized on cytosolic ribosomes and are imported into the organelle in a posttranslational manner. Approximately 70% of these mitochondrial preproteins use the presequence pathway for import into mitochondria, involving the translocase of the outer membrane (TOM) and the presequence translocase of the inner membrane (TIM23) complexes (Neupert and Herrmann, 2007; Endo and Yamano, 2009; Vögtle et al., 2009; van der Laan et al., 2010; Schmidt et al., 2010). Those preproteins possess N-terminal positively charged extensions that form amphipathic α-helices required for targeting and import into the organelle. Most presequences are removed upon import by the mitochondrial processing peptidase (MPP) located in the matrix (Witte et al., 1988; Gavel and von Heijne, 1990; Glaser et al., 1998; Gakh et al., 2002; Habib et al., 2007; Huang et al., 2009; Yogev and Pines, 2011).

Some preproteins can undergo a second processing event after MPP cleavage. This can be performed, for example, by the inner membrane peptidase (IMP) or the rhomboid protease processing of cytochrome c peroxidase (Pcp1), which act to regulate submitochondrial sorting of preproteins (Esser et al., 2002; Koppen and Langer, 2007). A further intermediate cleaving peptidase, Icp55, which cleaves preprotein intermediates after initial processing by MPP has recently been identified (Naamati et al., 2009; Vögtle et al., 2009). Icp55 typically removes one amino acid residue from preprotein intermediates that carry a destabilizing amino acid residue at their N-terminus according to the N-end rule for bacterial proteins

(Mogk et al., 2007; Varshavsky, 2008). This leads to the conversion of a destabilizing N-terminus into a stable one, thereby preventing premature degradation of substrate proteins (Vögtle et al., 2009).

Another protease, which cleaves precursor intermediates generated by MPP, is the matrix-located mitochondrial intermediate peptidase (MIP), octapeptidyl aminopeptidase 1 (Oct1). Oct1 cleaves an octapeptide from the N-terminus of the preprotein intermediate, thus generating the mature protein (Gakh et al., 2002). Oct1-dependent substrate proteins were found in the mitochondrial matrix and inner membrane, and their functions are quite diverse. They play a role in the respiratory chain, the citric acid cycle, genome maintenance and translation, the urea cycle, protein folding, and stress response. Based on the presequences of the Oct1 substrates known so far, the cleavage motif RX(↓)(F/L/I)XX(T/S/G)XXXX(↓) has been described (arrows indicate MPP and Oct1 cleavage sites, respectively) (Sztul et al., 1987; Kalousek et al., 1988; Hendrick et al., 1989; Branda and Isaya, 1995; Gakh et al., 2002; Vögtle et al., 2009). However, the functional role of the cleavage of an octapeptide by Oct1 is still not understood. It was speculated that the mature N-termini of dual-processed precursors would be incompatible with MPP processing and that the octapeptide is required to separate the MPP cleavage site from the mature part of the protein (Gavel and von Heijne, 1990; Isaya et al., 1991). In the cases of the Oct1 substrates Cox4 and Rip1, it was proposed that their intermediate forms could be either missorted or involved in regulation of the assembly rate of respiratory complexes (Isaya et al., 1994; Nett et al., 1997). However, it was also shown that the presequence of Rip1 can be cleaved by MPP in one step when the Oct1 cleavage site is replaced by an MPP motif (Nett et al., 1997) and that the Rip1 preprotein can assemble and function also in its intermediate form (Nett and Trumpower, 1999). To date, no explanation for the two-step cleavage by MPP/Oct1 has been found that would be applicable to general features of Oct1 substrate proteins.

We aimed to identify common characteristics of Oct1 substrate proteins that could lead to the uncovering of a functional role of Oct1. A recent proteomic approach led to the identification of five further substrates in Saccharomyces cerevisiae mitochondria (Vögtle et al., 2009) that possess the largest number of Oct1 substrates known. Systematic inspection of their sequences revealed that the most common characteristic is a destabilizing N-terminal amino acid in the intermediate forms. This led to the identification of 1) a novel Oct1 substrate with an unusual sequence motif and 2) a novel function of Oct1 in converting destabilizing N-termini of intermediates into stable N-termini of the mature proteins. Thus Oct1 acts as a quality control system for MPP-processed preproteins.

RESULTS
Ygr031w/Imo32 encodes a novel Oct1 substrate protein with an unusual sequence motif
For the Oct1 substrate proteins identified to date, a motif including three characteristic amino acid residues, RX(↓)(F/L/I)XX(T/S/G) XXXX(↓), has been identified (Hendrick et al., 1989; Gakh et al., 2002). The first arrow represents cleavage by MPP, whereas the second arrow indicates processing by Oct1. The most frequent amino acids within this motif are arginine at position −10 and phenylalanine at position −8 relative to the mature N-termini (Vögtle et al., 2009). While arginine has been considered as strictly required in position −10 leucine and isoleucine were also tolerated in position −8 (Hendrick et al., 1989; Gakh et al., 2002; Vögtle et al., 2009). Screening a large collection of experimentally determined mitochondrial presequences (N-proteome) from yeast for arginine in a −10 position led to the identification of five novel Oct1 substrate

proteins (Vögtle et al., 2009). We now screened the N-proteome for presequences that possess a phenylalanine in position −8. Those candidates were then tested by in organello import of the radiolabeled preproteins into isolated mitochondria from wild-type and oct1Δ yeast cells. For the as yet uncharacterized protein encoded by the open reading frame YGR031W, we found that the membrane potential–dependent processing from an intermediate form into the mature form is completely prevented in oct1Δ mitochondria (Figure 1A), indicating that Ygr031w is a substrate of Oct1. We therefore termed the protein Imo32 for "intermediate cleaved by mitochondrial octapeptidyl aminopeptidase." Imo32 is highly conserved from bacteria to humans, however, the functional role of this protein is still unclear. To test whether the first processing step of the Imo32 preprotein is mediated by MPP, we used a conditional mutant, which expresses one of the two MPP subunits (mas1), from a temperature-sensitive allele (Witte et al., 1988). In this strain, MPP function can be impaired by incubating isolated mitochondria at 37°C during in organello import (Vögtle et al., 2009). When radiolabeled Imo32 was imported into mas1 mitochondria at nonpermissive temperatures, the full-length precursor accumulated without cleavage of the presequence, indicating that MPP processing of Imo32 preprotein precedes processing by Oct1 (Figure 1B). The N-terminus of mature Imo32 has been determined as glutamine 47 (Vögtle et al., 2009). This implies that the predicted MPP cleavage site contains a cysteine at position −2 (position −10 relative to the mature protein) instead of arginine, which would not fit to the characteristic three amino acid motif of Oct1 substrate proteins (Hendrick et al., 1989; Gakh et al., 2002). (To exclude any sequencing error, we also confirmed the DNA sequence coding for the Imo32 presequence including cysteine −10.) In yeast, only one further variation at this position has been found, with Prx1 allowing a lysine instead of arginine at position −10 (Vögtle et al., 2009). To assess the intermediate N-terminus of Imo32, we synthesized radiolabeled truncated versions lacking either the complete presequence (Δ1–46) or the MPP-processed part (Δ1–38), and compared their migration on high-resolution SDS–PAGE with the processed forms after in organello import (Figure 1C). In wild-type mitochondria truncated Imo32$_{\Delta1–46}$ migrated like the imported and fully processed protein (Figure 2C, lanes 1–4; Vögtle et al., 2009), while in oct1Δ mitochondria, the truncated Imo32$_{\Delta1–38}$ migrated like the intermediate form (Figure 2C, lanes 5–8), confirming that the actual cleavage by MPP indeed occurs very close to the predicted cleavage site (the next positively charged residue of Imo32, lysine, is located at position −14 and thus too far away to function as MPP recognition motif). We mutated the cysteine in position −2 (with regard to the predicted MPP cleavage site) to either alanine or aspartate and found that precursors with these residues are also processed by MPP upon in organello import, suggesting the presence of an unusual MPP cleavage site in the presequence of Imo32 (Figure 1D). It must be considered that the peptide removed by Oct1 is shorter or longer than eight residues, and thus a residue different from cysteine −10 would be critical for cleavage by MPP. However, since the next positively charged residue in this case is also too far away, an unusual MPP recognition motif of an Oct1 substrate would be used.

We also noticed that the amino acid leucine at position −5 does not correspond to the published Oct1 motif, which would require threonine, serine, or glycine. This position, however, seems to be generally more variable, as shown by an alignment of the 10 N-terminal amino acids (relative to the mature form) from all experimentally verified Oct1 substrate proteins in S. cerevisiae (Figure 1E). An amino acid frequency profile of this region is shown in Figure 1F.
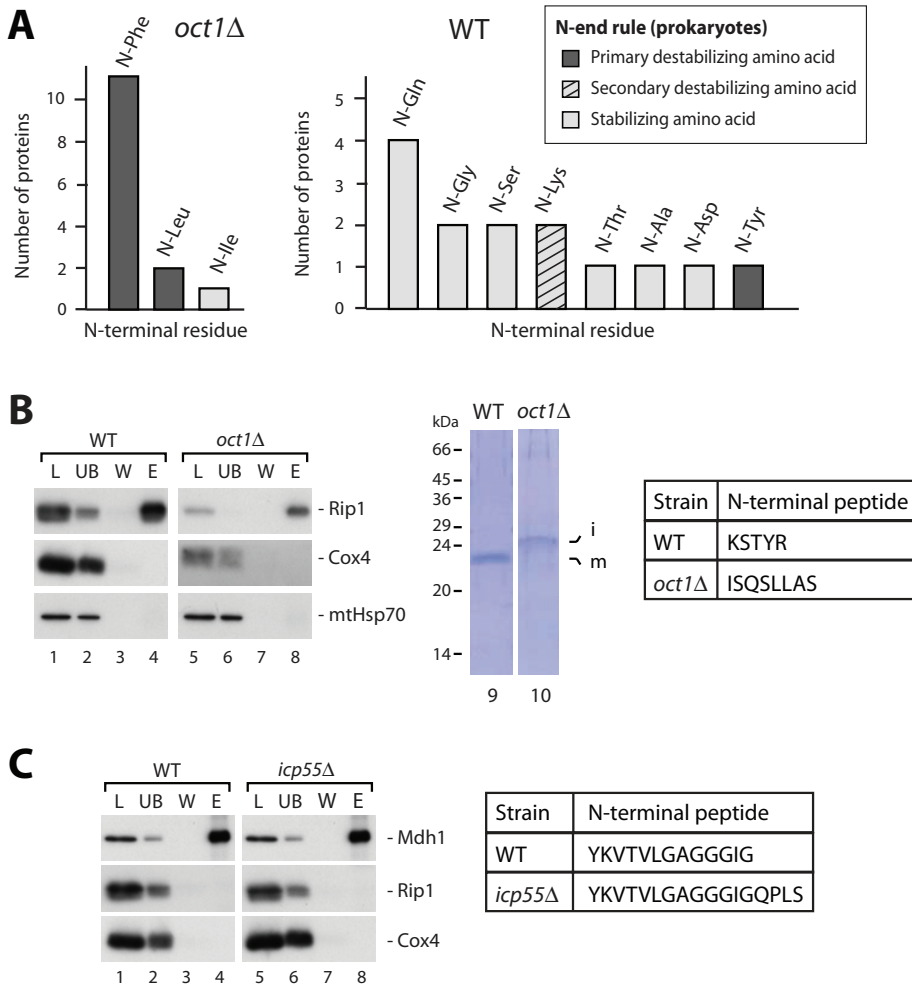
FIGURE 1: Ygr031w/Imo32 is a novel Oct1 substrate with an unusual cleavage site motif. (A) Radiolabeled Imo32 precursor was incubated with mitochondria isolated from wild-type (WT) or oct1Δ yeast cells, treated with proteinase K, and analyzed by SDS–PAGE and digital autoradiography. Where indicated, the membrane potential (Δψ) was dissipated prior to the import reaction. prec. and p, precursor; i, intermediate; m, mature. (B) Import of [35S]Imo32 into wild-type and mas1 mutant mitochondria. Samples were treated as in (A). (C) Radiolabeled Imo32 preprotein was incubated with isolated yeast mitochondria, and then treated with proteinase K. The samples, as well as 35S-labeled truncated forms of Imo32, were analyzed by SDS–PAGE and digital autoradiography. (D) Import of [35S]Imo32 with altered presequences into wild-type mitochondria. Cys-37 (corresponding to position −2 with regard to the proposed MPP cleavage site) was replaced by either alanine/A or aspartate/D. (E) Alignment of the cleavage motif of the 14 Oct1 substrate proteins from yeast. Characteristic three amino acid motif is highlighted in dark gray. Light gray denotes first amino acid of mature protein. Arrows indicate cleavage by MPP and Oct1, respectively. (F) Amino acid blot of relative frequency of the presequences (10 residues of the C-terminal segment) and first mature amino acids of the Oct1 substrate proteins identified in yeast (see Figure 1E). Arrows show cleavage sites of MPP and Oct1.

Thus Imo32 represents a novel Oct1 substrate protein, which matches only in one amino acid (phenylalanine −8) the characteristic three amino acid motif known to date.

Oct1-processing intermediates possess destabilizing N-terminal amino acids

Inspection of the N-terminal amino acid residues from intermediate and mature forms of the Oct1 substrate proteins revealed that most of the intermediates carry a destabilizing amino acid at the N-terminus, while most of the mature forms carry a stabilizing amino acid according to the N-end rule for degradation of bacterial proteins (Figure 2A; Mogk et al., 2007; Varshavsky, 2008). The intermediate forms of 11 substrate proteins start with phenylalanine and 2 with leucine. Both phenylalanine and leucine are primary destabilizing amino acids (Mogk et al., 2007; Varshavsky, 2008). However, in the case of Rip1, the intermediate form carries an isoleucine representing a stabilizing amino acid. Regarding the mature N-termini, we found 10 proteins with a stabilizing amino acid, 2 with secondary destabilizing amino acids, and in the case of Mdh1, the primary destabilizing amino acid was determined to be tyrosine. For Mdh1, it

is important to note that within the group of destabilizing amino acids N-tyrosine was found to be more stable than N-phenylalanine (Bachmair et al., 1986). In the case of Rip1, we observed that the presequence contains a destabilizing leucine in position −9. It has been speculated, that Oct1 could also remove intermediate peptides with 9 amino acids (Gavel and von Heijne, 1990). The N-terminus of the yeast Rip1 intermediate in oct1Δ mitochondria has not yet been determined experimentally. We therefore isolated Rip1 by immunoprecipitation from wild-type and oct1Δ mitochondria, and determined the corresponding N-terminal amino acids by Edman degradation. We confirmed the mature N-terminal amino acid lysine (Beckmann et al., 1987), and found that the intermediate indeed starts with isoleucine (Figure 2B). Regarding Mdh1, we wondered if the N-terminal tyrosine could be additionally cleaved by Icp55, thus generating a stabilizing N-terminus of the mature protein. We isolated Mdh1 from wild-type and icp55Δ mitochondria, and found by Edman sequencing that in both cases the N-terminal residue is a tyrosine (Figure 2C; Thompson et al., 1988).

Our results indicate that, with the exception of Rip1 and Mdh1, Oct1 substrate proteins possess destabilizing N-termini at their

FIGURE 2: Oct1-processing intermediates possess destabilizing N-terminal amino acids. (A) N-terminal amino acids of the 14 Oct1 substrate proteins in *oct1Δ* (left panel) and wild-type (WT; right panel) mitochondria. Amino acids are classified according to the N-end rule for *Escherichia coli*. (B) Immunoprecipitation of Rip1 from wild-type (WT) and *oct1Δ* mitochondria. Samples were analyzed by SDS–PAGE and immunoblotting (left panel) or protein bands of the elution fraction were visualized by Coomassie Blue staining (right panel). L, load (5%); UB, unbound (5%); W, wash (5%); E, elution (100%). The table shows the amino acid sequence of the Rip1 N-terminus from wild-type (WT) and *oct1Δ* mitochondria obtained by Edman degradation. (C) Immunoprecipitation of Mdh1 from wild-type (WT) and *icp55Δ* mitochondria (Vögtle *et al.*, 2009). Analysis was performed as in (B).

preprotein intermediates and stabilizing residues at their mature N-termini.

Oct1 processing of preprotein intermediates in mitochondria increases protein stability

To test whether processing of preprotein intermediates by Oct1 indeed plays a role in regulation of protein turnover, we planned to compare the stability of substrate proteins in wild-type and *oct1Δ* mitochondria. It is known that deletion of *OCT1* results in loss of mitochondrial DNA and, therefore, respiratory deficiency (Isaya *et al.*, 1994). When we compared the import efficiency of the matrix-targeted precursor protein Atp2 into *oct1Δ* and wild-type (Rho$^+$) mitochondria, we found a reduced import rate in *oct1Δ* mitochondria (Figure 3A, Top), which is probably due to a reduced membrane potential in the mutant. However, import capability seemed to be enhanced in *oct1Δ* mitochondria when compared with wild-type mitochondria from a Rho^0 yeast strain (Meisinger *et al.*, 2004) that lacks

mitochondrial DNA (Figure 3A, Bottom). Therefore we decided to reexpress Oct1 under its endogenous promoter and terminator in the *oct1Δ* background and to use it as a control strain. Indeed, the import efficiency for Atp2 was now similar in mitochondria from the *oct1Δ* strain, which reexpressed Oct1 from a plasmid (*oct1Δ* OCT1) and the *oct1Δ* strain transformed with an empty control vector (Figure 3B, Top). Recovery of Oct1 function after reexpression was controlled by full processing of the Cox4 precursor intermediate upon import into isolated mitochondria (Figure 3B, Bottom).

To monitor protein stability, isolated mitochondria from *oct1Δ* and *oct1Δ* OCT1 yeast cells were incubated over an increasing period of time. Samples were taken at regular time points and analyzed by SDS–PAGE and immunoblotting. We chose three different Oct1 substrates for analysis: Cox4 as a typical substrate with respect to the N-terminal amino acids in the intermediate and mature forms, Mdh1 with a destabilizing amino acid at both N-termini, and Rip1 with a stabilizing amino acid at the N-terminus of the intermediate form. For Mdh1 and Cox4, we observed a faster degradation in *oct1Δ* mitochondria compared with mitochondria from the control strain, while Rip1 and the control protein Atp2 were degraded with similar kinetics in both samples (Figure 4A, lanes 1–14). Due to the faster turnover rate of Rip1 and Cox4, a second degradation kinetic with shorter time intervals was performed (Figure 4A, lanes 15–28). Again, Cox4 was found to be less stable in *oct1Δ* mitochondria, while Rip1 stability was unaffected, as were the control proteins Atp2, mtHsp70, Atp3, and Nfs1. Atp3 and Nfs1 were recently identified as substrate proteins of the intermediate cleaving peptidase, Icp55 (Naamati *et al.*, 2009; Vögtle *et al.*, 2009). Quantification revealed a reduction of approximately 50% for Mdh1 (after 166 h) and 70% for Cox4 (after 76 h) in *oct1Δ* mitochondria compared with control samples. Stability of Rip1, however, was unaffected or even slightly higher after 76 h in *oct1Δ* (Figure 4B). We conclude that processing of preprotein intermediates by Oct1 leads to a stabilization of proteins due to the removal of destabilizing N-terminal amino acids that appear after initial cleavage by MPP. Stability of Rip1, which represents the only Oct1 substrate in yeast with a stabilizing N-terminal amino acid in the intermediate form, is not affected, thus fully confirming our finding.

Accelerated degradation of Oct1-dependent precursor intermediates in vivo

We next compared the stability of the intermediate and mature forms of Oct1 substrates in vivo. For this purpose, *oct1Δ* and *oct1Δ* OCT1 cells were treated with cycloheximide to block translation, and samples were taken after different time spans. Whole cell extracts were separated by SDS–PAGE and protein degradation
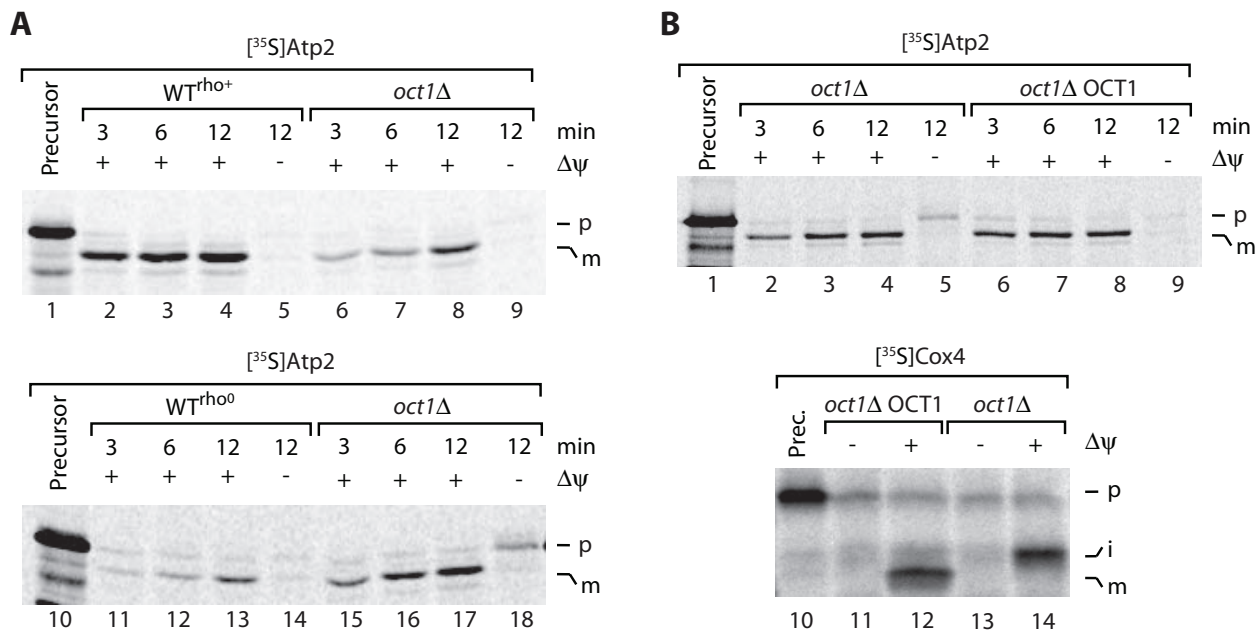
FIGURE 3: Reexpression of Oct1 in *oct1Δ* yeast cells. (A) Radiolabeled Atp2 precursor was incubated with mitochondria from WT^Rho+, *oct1Δ*, or WT ^Rho0 cells over increasing periods of time (3, 6, 12 min). Samples were treated with proteinase K and analyzed by SDS–PAGE and digital autoradiography. For the longest time point, the membrane potential (Δψ) was dissipated prior to the import reaction. p, precursor; m, mature. (B) Mitochondria isolated from *oct1Δ* yeast cells transformed with the empty control plasmid and *oct1Δ* OCT1 yeast reexpressing *Oct1* from its endogenous promoter were incubated with ³⁵S-Atp2 or ³⁵S-Cox4. Samples were analyzed as described in (A) and *Materials and Methods*. i, intermediate.

profiles were analyzed by immunoblotting (Vögtle *et al.*, 2009; Claypool *et al.*, 2011). For Mdh1 we observed a fast degradation of the intermediate form, while the fully processed mature form was stable over the whole time frame tested (Figure 5A). Quantification revealed that the level of the Mdh1 intermediate form decreased to < 40% after 46 h compared with the mature form, while the stability of the control proteins Atp2 and mtHsp70 was not dependent on Oct1 (Figure 5A). The cytosolic Pgk1 was used as a loading control. Due to their faster degradation kinetic, the stability of Cox4 and Rip1 was tested in a shorter time frame (Figure 5B) (the half-lives of different mitochondrial proteins have been found to cover a broad range from hours to several days; Kambacheld *et al.*, 2005; Belle *et al.*, 2006; Major *et al.*, 2006; Yen *et al.*, 2008; Shen *et al.*, 2008; Vögtle *et al.*, 2009; Claypool *et al.*, 2011). Cox4 intermediate form decreased to approx. 50% compared with the mature form after 9 h, while the degradation kinetic of Rip1 was not affected by the absence of Oct1 (Figure 5B). Our results also show that in vivo Oct1-dependent precursor intermediates with destabilizing N-terminal amino acid residues are much more rapidly degraded than their fully processed mature forms.

DISCUSSION

Since its discovery 20 yr ago, several different functions have been assigned to Oct1 and its homologues from other organisms. It was proposed that the removal of an octapeptide after initial processing by MPP separates the MPP cleavage site from mature parts of the N-terminus or regulates submitochondrial protein sorting or is involved in protein complex assembly (Gavel and von Heijne, 1990; Isaya *et al.*, 1991, 1994; Nett *et al.*, 1997). However, so far no satisfying experimental evidence corroborating these different theories has been shown, as all proposed functions were presented only for a single substrate and could never be applied to general features in

the sequence motif of Oct1 substrates. The recent identification of novel substrate proteins by a proteomic approach in yeast enabled a more comprehensive analysis of common characteristics of Oct1 substrates. We found two interesting new features of the cleavage site motif in Oct1-dependent preproteins. First, MPP does not strictly require an arginine in position −10 of Oct1 substrate proteins, but can cleave precursors with lysine (Prx1) or cysteine, alanine, or aspartate (Imo32) residues at this position. This raises the possibility that many more Oct1 substrates with less characteristic sequence motifs await identification. Second, the N-termini of the Oct1 intermediates can be classified as destabilizing amino acids according to the N-end rule, whereas processing by Oct1 converts those N-termini into more stabilizing ones. Mutational analysis of the Oct1 cleavage site motif has shown that the amino acid in position −8 is the crucial one for recognition and cleavage by Oct1 (Isaya *et al.*, 1992). Using in organello and in vivo degradation assays we could show that processing by Oct1 indeed leads to a prolonged half-life of its substrate proteins. A first indication that Oct1-dependent precursor intermediates are degraded faster than the mature proteins was reported by Isaya *et al.* (1994); however, this rapid degradation was explained by a possible missorting of the precursor protein and not connected to a destabilizing N-terminus of the intermediate.

While we cannot exclude further functions of the octapeptide, like allowing a more efficient processing of precursors by MPP, the most common feature of the Oct1 substrates is the different classification of their intermediate and mature N-terminal amino acids according to the N-end rule. Only 2 of the 13 Oct1 substrates analyzed here are showing exceptions in the categorization of intermediate/destabilizing and mature/more stabilizing N-termini. 1) The intermediate form of the Rip1 protein has the stabilizing amino acid isoleucine at its N-terminus and is nevertheless processed by the Oct1
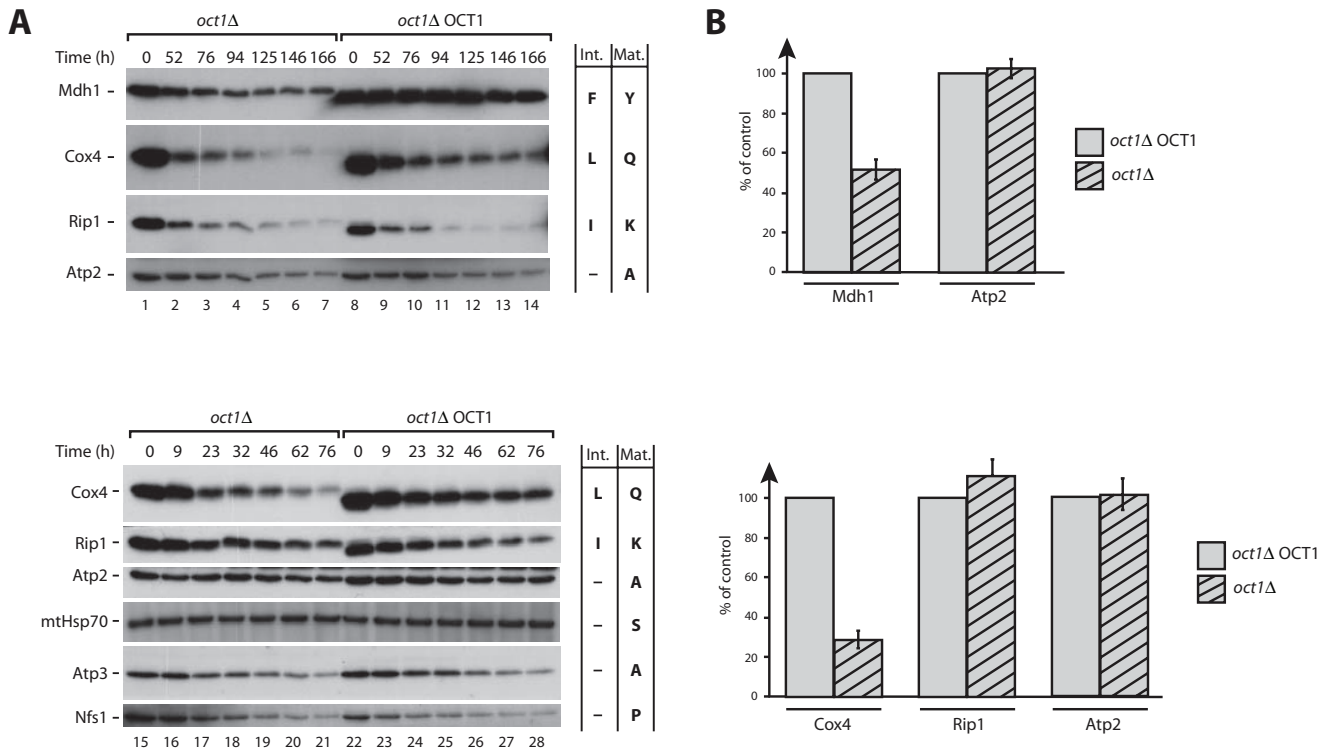
FIGURE 4: The half-life of Oct1 substrate proteins is prolonged upon full processing of the intermediate forms in organello. (A) Mitochondria from *oct1Δ* and *oct1Δ* OCT1 (reexpressing the *OCT1* gene) yeast cells were incubated at 37°C for the indicated time. Mitochondria were reisolated and analyzed by SDS–PAGE and immunoblotting. N-terminal amino acids of Oct1 intermediate (Int.) and mature (Mat.) forms of the proteins tested are indicated. (B) Quantification of protein turnover: graphs indicate averaged protein levels of the last incubation time points (166, 76 h) in comparison to the level at 0 h. Values of *oct1Δ* OCT1 mitochondria were set to 100% (control). Error bars represent SD from three different experiments.

protease. Interestingly, the bovine homologue (Rieske iron–sulfur protein) is processed in a single step resulting in isoleucine at the mature N-terminus. It is likely that the two-step processing has been abolished during evolution, due to the already stabilizing N-terminus after the first cleavage by MPP. In yeast, intermediate Rip1 and mature-sized Rip1 are degraded at a similar rate. Interestingly the N-terminal amino acid of mature Rip1, lysine, represents a secondary destabilizing amino acid according to the N-end rule in prokaryotes (Mogk *et al.*, 2007; Varshavsky, 2008; Dougan *et al.*, 2010). Lysine is also found at the mature N-terminus of another Oct1 substrate protein, Prx1. In the cytosol of eukaryotes and prokaryotes, such residues are typically converted by specific enzymes into primary destabilizing amino acid (Mogk *et al.*, 2007; Varshavsky, 2008; Dougan *et al.*, 2010). However, no homologues of these enzymes have been identified in mitochondria so far. Therefore lysine could be considered as a stabilizing residue in line with our results. 2) Regarding Mdh1, we found that the mature form with an N-terminal tyrosine is more stable than the intermediate form, which carries a phenylalanine. Both residues are classified as primary destabilizing amino acids, but systematic analysis of fusion constructs exposing different residues at the N-termini revealed that proteins with N-terminal tyrosine are three times more stable than those starting with phenylalanine (Bachmair *et al.*, 1986), explaining the more rapid degradation of the Mdh1 intermediate form found in this study. The Mdh1 homologues in mouse and rat have maintained the destabilizing phenylalanine in position −8, but their mature N-termini possess the stabilizing amino acid alanine (Hendrick *et al.*, 1989).

When we compared verified Oct1 substrate proteins from other organisms, we found that they also contain primary destabilizing N-terminal amino acids at the intermediate form and stabilizing ones at the mature form in full agreement with our findings (Hartl *et al.*, 1986; Sztul *et al.*, 1987; Grant *et al.*, 1986; Tropschug *et al.*, 1988; Hendrick *et al.*, 1989). It is not determined which proteolytic systems mediate degradation of mitochondrial proteins according to the N-end rule pathway (Vögtle *et al.*, 2009; Dougan *et al.*, 2010). Due to the absence of a ubiquitin–proteasome system in mitochondria, putative candidates comprise eukaryotic homologues of the ClpP family, which are involved in the bacterial N-end rule pathway, as well as Pim1 (Lon; Meinnel *et al.*, 2006; Mogk *et al.*, 2007; Dougan *et al.*, 2010). (ClpP is present in mitochondria of higher eukaryotes but not in yeast.) Mitochondrial presequences have become essential upon transfer of mitochondrial genes to the nucleus during evolution in order to enable import of nuclear encoded mitochondrial proteins. Due to the presence of the highly conserved Oct1 and Icp55 peptidases in the mitochondrial matrix, an optimization of the presequence cleavage motif by MPP according to the N-end rule has not been necessary. Icp55 and Oct1 apparently recognize different subsets of precursor intermediates. Whereas N-terminal tyrosine is not found at intermediates recognized and cleaved by Oct1, this residue represents the main substrate of Icp55 (Vögtle *et al.*, 2009).

In summary, our results indicate that Oct1 and Icp55 together constitute a quality control system in the mitochondrial matrix, ensuring stabilization of precursor protein intermediates upon import into the organelle.
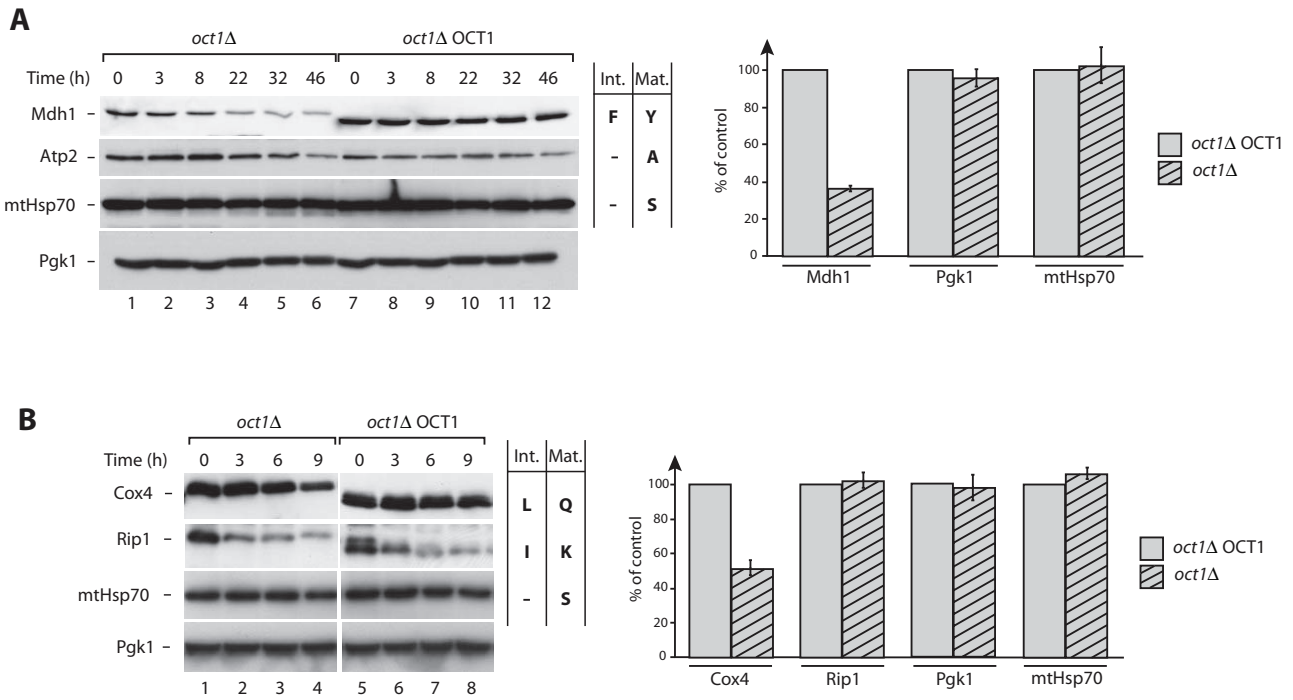
FIGURE 5: Oct1 processing leads to stabilization of its substrate proteins in vivo. (A) and (B) In vivo degradation of proteins analyzed in *oct1Δ* and *oct1Δ* OCT1 yeast cells. Protein translation was blocked by addition of cycloheximide. Samples were obtained at various time points, subjected to postalkaline protein extraction, and analyzed by SDS–PAGE and immunoblotting. N-terminal amino acids of Oct1 intermediate (Int.) and mature (Mat.) forms of the proteins tested are indicated. Quantification was performed as in Figure 4.

MATERIALS AND METHODS

Yeast strains

The *S. cerevisiae* strains used are derived from BY4741: wild-type strain (*Mata*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*) and deletion strain of Oct1 (*Mata*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, YKL134c::kanMX4). For reexpression of OCT1 in the *oct1Δ* strain, the open reading frame, which included the endogenous promoter and terminator region, was cloned into the pRS413 expression vector. Yeast cells were grown on minimal medium (6.7% [wt/vol] yeast nitrogen base without amino acids, 2% [wt/vol] glucose, 0.77% [wt/vol] Complete Supplement Mixture [BD Biosciences] minus histidine).

Isolation of mitochondria

Yeast cells were grown at 30°C on nonfermentable (1% [wt/vol] yeast extract, 2% [wt/vol] Bacto Peptone, 3% [wt/vol] glycerol, pH 5.0) or fermentable (2% [wt/vol] sucrose instead of glycerol) medium. For yeast strains transformed with expression plasmids, cells were grown on minimal medium minus histidine, as described above. Cells were harvested at an OD_{600} of 0.7–1.5 and disrupted by homogenization, and mitochondria were isolated by differential centrifugation, as previously described (Meisinger *et al.*, 2006). Aliquots were stored in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2) at −80°C.

Immunoprecipitation

Mitochondria (3 mg protein) were solubilized in lysis buffer (1% [wt/vol] SDS, 0.5% (vol/vol) Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.2) and incubated for 5 min at 95°C. Protein A Sepharose (GE Healthcare) loaded with antibodies against Mdh1 or Rip1 was equilibrated with lysis buffer without SDS and incubated with mitochondrial extract for 2 h at 4°C. After washing, proteins were eluted with 100 mM glycine, pH 2.5, precipitated with trichlo-

roacetic acid (TCA), and analyzed by SDS–PAGE and Western blotting. Rip1 and Mdh1 protein bands were visualized by staining with Coomassie Brilliant Blue after transfer on PVDF membranes. Amino acid sequences were determined by Edman degradation (pulse liquid-phase sequencer, Procise 492 cLC; Applied Biosystems, Foster City, CA).

In vitro import of preproteins into mitochondria

Radiolabeled precursor proteins were generated by in vitro transcription/translation in the presence of [35S]methionine using a rabbit reticulocyte lysate system (Promega). Mitochondria (40 µg) and 3 µl radiolabeled precursor proteins were incubated in import buffer (10 mM MOPS-KOH, pH 7.2, 3% [wt/vol] bovine serum albumin [BSA], 250 mM sucrose, 5 mM $MgCl_2$, 80 mM KCl, 5 mM KPi) supplemented with 2 mM ATP and 2 mM NADH. To abolish the membrane potential across the inner mitochondrial membrane, 1 µM valinomycin, 20 µM oligomycin, and 8 µM antimycin A (AVO mix) were added. The import reaction was stopped after the indicated time period by adding 0.01 volumes of AVO mix and putting the samples on ice. Samples were treated with 50 µg/ml proteinase K (PK) to remove nonimported precursor proteins. After PK digestion for 10 min on ice, the protease was inhibited by the addition of 2 mM phenylmethylsulfonyl fluoride (PMSF). Mitochondria were reisolated and washed with SEM buffer. For testing full Cox4 processing in *oct1Δ* OCT1 mitochondria, radiolabeled Cox4 was imported for 30 min into isolated mitochondria. After addition of AVO mix, mitochondria were reisolated and incubated for 60 min at 25°C. After SDS gel-electrophoresis, radiolabeled proteins were detected by digital autoradiography (PhosphorImager; GE Healthcare). For analysis of the truncated versions of Imo32, 40 µg mitochondrial extract per lane was loaded together with 0.2 µl of the radiolabeled protein onto SDS–PAGE.

For analysis of Imo32 presequence mutations, Imo32 was cloned into pRS415 and point mutations were introduced with the Quick-Change II Site-Directed Mutagenesis Kit (Stratagene). All constructs were confirmed by sequencing.

Analysis of protein turnover

Mitochondrial protein turnover was analyzed as described (Vögtle *et al.*, 2009; Claypool *et al.*, 2011). Isolated mitochondria were incubated in SEM buffer at 37°C. Samples were taken at various time points and analyzed by SDS–PAGE and immunoblotting. For analysis of protein turnover in vivo, yeast precultures were diluted in minimal medium (minus histidine) to an OD_{600} of 0.6 and grown at 37°C for 2 h. Cycloheximide (50 mg/ml stock in dimethyl sulfoxide [DMSO]) was added to a final concentration of 50 μg/ml to terminate protein translation. Cells were harvested at various time points and whole-cell extracts were obtained by postalkaline extraction. Samples were analyzed by SDS–PAGE and immunoblotting.

Miscellaneous

Western blots were scanned using Multi Gauge (http://multi-gauge .winsite.com) or ImageJ (http://rsbweb.nih.gov/ij/download.html) software. For quantification of protein turnover, the protein amount of the last time point was set in relation to the level of the first time point (0 h). Protein levels in *oct1Δ* were compared with levels of *oct1Δ* OCT1 as control (set to 100%). Standard deviation was calculated from at least three different experiments.

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