Autocatalytic processing of the ATP-dependent PIM1 protease: crucial function of a pro-region for sorting to mitochondria

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The biogenesis of the ATP-dependent PIM1 protease of mitochondria was studied by mutational analysis. The ATPase and proteolytic activities of PIM1 were shown to be essential for mitochondrial function. A proteolytically inactive mutant form of PIM1 protease accumulated as a pro-form in mitochondria, revealing a two-step processing of PIM1: the matrix targeting signal is removed by the mitochondrial processing peptidase and then a pro-region of 61 amino acids is cleaved off in an autocatalytic reaction. This latter process depended on the ATP-dependent assembly of PIM1 protease subunits and can occur by an intermolecular and, most probably, also an intramolecular pathway. The respiratory competence of cells harboring mutant PIM1 protease lacking the pro-region was strongly impaired. Subcellular fractionation revealed a cytosolic localization of mutant PIM1 protease. This demonstrates the requirement for the propeptide for efficient sorting of PIM1 protease to mitochondria.

Keywords: autocatalytic processing/mitochondria/PIM1 protease/pro-region/protein sorting

Introduction

The maintenance of mitochondria in a functional state depends on the regulated degradation of mitochondrial proteins by ATP-dependent proteases present in various subcompartments of the organelle (Langer and Neupert, 1996; Rep and Grivell, 1996; Suzuki *et al.*, 1997). These proteases are known to degrade excess or misfolded polypeptides, but in addition fulfill essential regulatory functions during the biogenesis of the organelle.

Fractionation of isolated mammalian mitochondria provided initial evidence for an ATP-dependent proteolytic activity in the matrix space (Desautels and Goldberg, 1982; Watabe and Kimura, 1985). The corresponding genes were identified in human and yeast and termed *PIM1* (for proteolysis in mitochondria) or *LON* (Wang *et al.*, 1993; Suzuki *et al.*, 1994; Van Dyck *et al.*, 1994). Yeast cells lacking *PIM1* (*LON*) are respiratory deficient, indicating essential functions of PIM1 protease during

mitochondrial biogenesis: PIM1 is required for the maintenance of the integrity of the mitochondrial genome (Suzuki *et al.*, 1994; Van Dyck *et al.*, 1994) and for the synthesis of mitochondrially encoded cytochrome oxidase I and cytochrome *b* (L.van Dyck and T.Langer, unpublished observations). Non-assembled or misfolded polypeptides, accumulating in the mitochondrial matrix space, are degraded by PIM1 protease in cooperation with the mitochondrial Hsp70 system, which stabilizes misfolded polypeptides and prevents aggregation (Wagner *et al.*, 1994).

PIM1 protease is a member of the highly conserved family of Lon-like proteases, ATP-dependent serine proteases present in eubacteria, archaeobacteria and mitochondria of eukaryotic cells (Goldberg *et al.*, 1994; Gottesman, 1996). Functional conservation recently has been demonstrated for prokaryotic and eukaryotic Lon-like proteases. Expression of *Escherichia coli* Lon protease fused to a mitochondrial targeting sequence in yeast restored the respiratory competence of $\Delta pim1$ mutant cells (Teichmann *et al.*, 1996). The *E.coli* Lon protease did not, however, substitute for PIM1 at high growth temperature, suggesting functional differences between both proteases.

Overexpression of PIM1 protease suppresses the respiratory deficiency of yeast cells lacking *m-AAA* protease, pointing to a functional overlap between both proteases (Rep *et al.*, 1996a). The *m-AAA* protease is composed of multiple copies of the homologous subunits, Yta10p (Afg3p) and Yta12p (Rca1p), integral inner membrane proteins with catalytic sites facing the matrix space (Arlt *et al.*, 1996). Cells lacking the gene for either of the subunits are respiratory deficient and exhibit defects in the degradation of non-assembled inner membrane proteins (Pajic *et al.*, 1994; Tzagoloff *et al.*, 1994; Guélin *et al.*, 1996). As overexpression of proteolytically inactive PIM1 protease has been observed to restore the respiratory competence of $\Delta yta10\Delta yta12$ mutant cells, a chaperone function which is independent of its proteolytic activity has been proposed for PIM1 protease in the assembly of mitochondrial protein complexes (Rep *et al.*, 1996b).

Here we describe site-directed mutagenesis studies which demonstrate the requirement for the ATPase and proteolytic activities of PIM1 protease for the biogenesis of the respiratory chain. The analysis of proteolytically inactive PIM1 led to the identification of a pro-region which is cleaved off autocatalytically upon ATP-dependent assembly of newly imported PIM1 molecules with pre-existing subunits. The propeptide plays a crucial role in the biogenesis of PIM1 protease. A mutant variant lacking the propeptide accumulates in the cytosol, demonstrating the requirement for the pro-region for sorting of PIM1 protease to mitochondria.

Results

Respiratory competence depends on the proteolytic function of PIM1 protease

To examine the role of PIM1-mediated proteolysis for the maintenance of the respiratory competence of the cells, a proteolytically inactive mutant form of PIM1 was generated by replacement of the catalytic site Ser1015 by alanine (PIM1^{S1015A}). The corresponding amino acid residue Ser679 has been identified as the proteolytic active site in *E. coli* Lon protease (Amerik *et al.*, 1991; Fischer and Glockshuber, 1993). Wild-type and mutant PIM1 protease were cloned into a centromere-based yeast shuttle vector under the control of the endogenous promoter. After transformation into a diploid strain carrying one disrupted *PIM1* allele, haploid $\Delta pim1$ segregants harboring wild-type or mutant PIM1 protease were isolated by sporulation and tetrad dissection. Whilst the respiratory competence of $\Delta pim1$ cells was maintained upon expression of wild-type PIM1 ('PIM1'), $\Delta pim1$ mutant cells expressing *pim1*^{S1015A} ('*pim1*^{S1015A}') did not grow on non-fermentable carbon sources (Figure 1A). The integrity of mitochondrial DNA was analyzed in *pim1*^{S1015A} mutant cells by mating with *mit*⁻ tester strains carrying point mutations in mitochondrial genes. Diploid cells obtained were incapable of growing on non-fermentable carbon sources (data not shown). This indicates the presence of lesions in the mitochondrial DNA of *pim1*^{S1015A} mutant cells. We conclude that proteolysis by PIM1 is required to maintain mitochondrial genome integrity and respiratory competence.

Proteolytically inactive PIM1 protease accumulates in a pro-form within mitochondria

To allow purification of PIM1, a hexahistidine peptide was fused to the carboxy-terminus of wild-type and mutant forms of the protease. The corresponding DNAs were cloned into a yeast expression vector under the control of a galactose-inducible promoter and expressed in $\Delta pim1$ mutant cells. Mitochondria were isolated and analyzed by immunoblotting with a polyclonal antiserum directed against PIM1. Similar levels of wild-type and mutant PIM1 protease were present in $\Delta pim1$ mitochondria (Figure 1B). The molecular mass of proteolytically inactive PIM1, however, was significantly larger than that of the wild-type protease (Figure 1B). Thus, maturation of PIM1 protease appears to depend on its own proteolytic activity.

Wild-type and mutant PIM1 protease were isolated by metal chelate chromatography, and the amino-termini of the purified proteins were determined by protein sequencing. The amino-terminus of wild-type PIM1 was found to correspond to amino acid 99 of the precursor form (Figure 1C). Apparently, an N-terminal segment of PIM1 protease, which is significantly larger than the predicted mitochondrial targeting sequence, is cleaved off in mitochondria. The amino-terminus of the proteolytically inactive PIM1 protease, on the other hand, corresponded to amino acid 38 of the precursor form (Figure 1C). The 37 N-terminal residues of the PIM1 protease precursor have the potential to form an amphipathic helix, a characteristic of mitochondrial matrix targeting signals. The region around amino acid 37, in particular the presence of an arginine residue at position -2, displays characteristics of a typical cleavage

Fig. 1. Respiratory competence and maturation of PIM1 protease depends on PIM1-mediated proteolysis. (A) Proteolytically inactive PIM1 protease does not support growth of $\Delta pim1$ mutant cells on non-fermentable carbon sources. Wild-type (WT) and $\Delta pim1$ mutant cells harboring pRS314-PIM1 (PIM1) or pRS314-*pim1*^{S1015A} (*pim1*^{S1015A}) were grown at 30°C in synthetic medium containing 2% glucose. Ten-fold serial dilutions were spotted onto YP medium containing 3% glycerol (YPGlycerol) and incubated for 4 days at 30°C. (B) Immunoblot analysis of mitochondrial proteins. Mitochondria (50 µg) isolated from wild-type (WT), $\Delta pim1$ cells (ρ^-) and $\Delta pim1$ cells containing pYES2.0-PIM1 (PIM1)(ρ^-) or pYES2.0-*pim1*^{S1015A} (*pim1*^{S1015A})(ρ^-) were analyzed by SDS-PAGE and immunoblot analysis using a polyclonal antiserum directed against PIM1 protease. pro, pro-form; m, mature form of PIM1 protease. (C) N-terminal amino acid residues of PIM1 protease. Amino acid sequences determined by protein sequencing of PIM1^{S1015A} and PIM1 protease expressed in $\Delta pim1$ mutant cells are underlined. The arrows indicate the MPP processing site and the amino-terminus of mature PIM1.

site of the mitochondrial processing peptidase (MPP) (Brunner and Neupert, 1995). Therefore, this form is most probably generated by cleavage of newly imported PIM1 molecules by MPP.

These results suggest a two-step processing of newly imported PIM1 protease: a pro-form is generated upon removal of the matrix targeting signal by MPP and subsequently matured by further processing. To test this hypothesis, PIM1 protease was synthesized in a cell-free translation system and imported into isolated wild-type mitochondria (Figure 2A). After 5–10 min of import, a slightly smaller form was generated (Figure 2A) whose molecular mass was indistinguishable from the pro-form of endogenous PIM1 protease accumulating in *pim1*^{S1015A} mitochondria. Formation of the pro-form preceded further cleavage of newly imported PIM1 protease to the mature sized species. Maturation of PIM1 occurred only during longer periods of import and, more efficiently, upon continued incubation after completion of import (Figure 2A).

Maturation depends on pre-existing PIM1 protease and occurs autocatalytically

To investigate the role of PIM1 in its own maturation, the wild-type form was imported into mitochondria lacking PIM1 (Δ *pim1* mitochondria). PIM1 protease accumulated in the pro-form in these mitochondria (Figure 2B). The deficiency in the processing reaction might result from a disturbed energy metabolism in Δ *pim1* mitochondria which harbor defective mtDNA (Suzuki *et al.*, 1994; Van Dyck *et al.*, 1994). To exclude this possibility, processing of PIM1 protease was examined in mitochondria containing PIM1, but lacking intact mtDNA (Figure 2B). Newly imported PIM molecules were processed to the mature form in these mitochondria, demonstrating the requirement for pre-existing PIM1 protease for maturation. Surprisingly, cleavage of the pro-region of newly imported PIM1 molecules did not depend on the proteolytic activity of PIM1 present in mitochondria. Although with reduced efficiency, newly imported PIM1 was converted to the mature form when imported into *pim1*^{S1015A} mitochondria (Figure 2B).

These results establish the requirement for pre-existing PIM1 for maturation; however, another protease might cleave off the pro-region from newly imported PIM1 molecules. Therefore, the proteolytically inactive, mutant form of PIM1 protease was synthesized in a cell-free

translation system and imported into Δ *pim1* mitochondria containing the wild-type or mutant form of PIM1 protease (*PIM1*, *pim1*^{S1015A}). PIM1^{S1015A} protease was matured in Δ *pim1* mitochondria harboring the wild-type enzyme (Figure 2C). In contrast, mutant PIM1 accumulated in

the pro-form in mitochondria containing proteolytically inactive PIM1 protease (Figure 2C). These results provide compelling evidence that the pro-form of newly imported PIM1 protease is converted autocatalytically into the mature form.

Assembly of PIM1 protease is independent of autocatalytic processing

Lon-like proteases form homo-oligomeric high molecular weight complexes (Kutejová *et al.*, 1993; Goldberg *et al.*, 1994). Do newly imported PIM1 molecules assemble with pre-existing subunits and is this a prerequisite of their maturation? PIM1^{S1015A} protease was imported into isolated Δ *pim1* mitochondria and into Δ *pim1* mitochondria containing a his-tagged wild-type or proteolytically inactive form of the protease. After import, mitochondria were solubilized and extracts were analyzed by metal chelating chromatography. Newly imported PIM1 protease was retained on the Ni-NTA column (Figure 3A). This indicates assembly of imported PIM1 molecules with pre-existing subunits, as binding strictly depended on the presence of his-tagged PIM1 subunits within mitochondria (Figure 3A). After import into *pim1*^{S1015A} mitochondria, the pro-form of newly imported PIM1^{S1015A} protease was retained on the column, demonstrating that propeptide processing is not required for the oligomerization of the protease (Figure 3A).

To substantiate this conclusion further, Δ *pim1* mitochondria harboring wild-type or proteolytically inactive PIM1 were solubilized and extracts were subjected to sizing chromatography. The eluate was analyzed by immunoblotting with a polyclonal antiserum directed against PIM1. Mutant PIM1 protease, which accumulated in the pro-form within mitochondria, and the wild-type enzyme were recovered from the column in a single peak corresponding to an M_r of ~1600 kDa (Figure 3B). Thus, PIM1 protease forms a high molecular weight complex whose assembly is

independent of its proteolytic activity and the autocatalytic maturation of its subunits.

The assembly of newly imported PIM1 molecules with pre-existing protease subunits suggests that removal of the pro-region occurs after oligomerization of the protease. This was confirmed by gel filtration analysis of mitochondrial extracts following import of wild-type and mutant PIM1 protease. Newly imported PIM1 protease eluted from the column in two peaks corresponding to M_r s of

Fig. 3. Autocatalytic processing requires assembly of PIM1 protease subunits. (A) Assembly of newly imported PIM1 protease molecules with pre-existing PIM1 subunits monitored by Ni-NTA chromatography. PIM1^{S1015A} protease was imported for 20 min at 15°C in Δ *pim1* (ρ^-), PIM1 (ρ^-) and *pim1*^{S1015A} (ρ^-) mitochondria, followed by a further incubation for 30 min at 30°C. Mitochondria were lysed and subjected to Ni-NTA chromatography as described in Materials and methods. The autoradiograph of eluate fractions after TCA precipitation and SDS-PAGE is shown. Identical amounts of newly imported PIM1 were loaded onto the Ni-NTA beads. pro, pro-form; m, mature form. (B) Wild-type and proteolytically inactive PIM1 protease form high molecular weight complexes in mitochondria. Extracts of PIM1 (ρ^-) and *pim1*^{S1015A} (ρ^-) mutant mitochondria were prepared in buffer B containing 1 mM ATP and subjected to Superose 6 chromatography as described in Materials and methods. PIM1 ($\circ$) and PIM1^{S1015A} ($\blacktriangle$) were detected in eluate fractions by immunoblotting. Protein amounts were quantified by laser densitometry and are given as the percentage of PIM1 in the eluate ('total'). (C and D) Maturation of newly imported PIM1 protease occurs upon assembly with pre-existing PIM1 subunits. PIM1 (C) or PIM1^{S1015A} (D) were imported into *pim1*^{S1015A} (ρ^-) mitochondria for 20 min at 15°C, followed by a further incubation for 30 min at 30°C. Mitochondria were lysed in buffer B containing 1 mM ATP and extracts were fractionated by Superose 6 chromatography. Eluate fractions were TCA precipitated and analyzed by SDS-PAGE and autoradiography. In (C), the autoradiograph of the SDS-PAGE is shown in the upper panel. PIM1 protease recovered from the column was quantified by laser densitometry and is given as the percentage of total PIM1 in the eluate. * represents a proteolytic fragment of PIM1.

~420 and 1600 kDa (Figure 3C). Strikingly, mature PIM1 protease was detected exclusively in fractions containing assembled PIM1 protease. The same elution profile was obtained after import of the proteolytically inactive form of PIM1 protease into *pim1*^{S1015A} mitochondria (Figure 3D). Maturation of assembled PIM1 protease subunits, however, was impaired under these conditions (see Figure 2C). In summary, we conclude from these experiments that autocatalytic processing of newly imported PIM1 protease molecules occurs after their assembly with pre-existing subunits.

ATP-dependent oligomerization of PIM1 protease

To investigate if autocatalytic processing and oligomerization of PIM1 protease require an intact ATP-binding site, Lys638 within the conserved ATP-binding motif GPPGVGKT ('Walker A' motif) of PIM1 was replaced by asparagine using site-directed mutagenesis. Replacement of the corresponding lysine residue drastically reduces ATPase and proteolytic activities of *E.coli* Lon protease (Fischer and Glockshuber, 1994). *PIM1*^{K638N} was cloned into a centromere-based yeast shuttle vector under the control of the endogenous promoter and transformed into a diploid strain harboring a disrupted *PIM1* allele. The respiratory competence of a haploid Δ *pim1* strain, isolated by sporulation and tetrad dissection, was not maintained in the presence of *PIM1*^{K638N} protease, demonstrating the requirement for an intact ATP-binding motif in PIM1 for mitochondrial function (Figure 4A).

To assess the oligomeric state of *PIM1*^{K638N} protease, mitochondria were solubilized and extracts were subjected to sizing chromatography. In contrast to the wild-type enzyme, mutant PIM1 protease eluted from the column in fractions corresponding to an *M_r* of ~420 kDa (Figure 4B). This is reminiscent of the oligomeric state of newly imported PIM1 molecules (see Figure 3C) and may represent an assembly intermediate which is formed in an ATP-independent manner. These results demonstrate that oligomerization and thereby most likely the proteolytic function of PIM1 protease requires an intact ATP-binding site. This is consistent with the observation that *PIM1*^{K638N} protease accumulated in the pro-form in mitochondria when expressed in Δ *pim1* mutant cells (Figure 4B).

Studies with various nucleotide-binding proteins have

revealed a direct contact of the lysine residue in the 'Walker A' motif to the β - and γ -phosphate of the bound nucleotide (Saraste *et al.*, 1990). Replacement of Lys638 by asparagine is therefore predicted to abolish ATP binding to PIM1 protease. To examine whether binding or hydrolysis of ATP drives oligomerization of PIM1 protease, gel filtration experiments were performed after manipulation of matrix ATP levels in wild-type mitochondria. PIM1 formed a high molecular weight complex of ~1600 kDa in the presence of ATP; the majority of the protease,

Fig. 4. Oligomerization of PIM1 protease depends on an intact ATP-binding site and the presence of nucleotides. (A) Respiratory deficiency of Δ *pim1* mutant cells expressing *PIM1*^{K638N} protease. Wild-type (WT) and Δ *pim1* mutant cells harboring pRS314-*PIM1* (*PIM1*) or pRS314-*pim1*^{K638N} (*pim1*^{K638N}) were grown at 30°C in synthetic medium containing 2% glucose. Ten-fold serial dilutions were spotted onto YP medium containing 3% glycerol (YPGlycerol) and incubated for 4 days at 30°C. (B) *PIM1*^{K638N} protease does not form a high molecular weight complex in mitochondria. Extracts of wild-type (○) and *pim1*^{K638N} (■) mutant mitochondria were prepared in buffer B containing 1 mM ATP and fractionated by Superose 6 chromatography as described in Materials and methods. Eluate fractions were analyzed by SDS-PAGE and immunoblotting with a polyclonal antiserum directed against PIM1 protease, as shown in the upper panel. Protein amounts were determined by laser densitometry and are given as the percentage of PIM1 in the eluate (total). (C) Superose 6 chromatography of mitochondrial extracts in the presence of 1 mM ATP (○), 1 mM AMP-PNP (△) or in the absence of nucleotides (●). Wild-type mitochondria were lysed and subjected to gel filtration analysis as described in Materials and methods. Mature PIM1 protease in the eluate (total) was determined as in (B).

however, was recovered from the column in fractions corresponding to a native M_r of ~420 kDa, when matrix ATP levels were reduced prior to lysis (Figure 4C). PIM1 protease eluted in the same fractions from the column after replacing ATP by the non-hydrolyzable ATP analog AMP-PNP (Figure 4C). Thus, ATP binding is obviously not sufficient to stabilize the high molecular weight assembly of PIM1. Taken together, these results demonstrate that both oligomerization and autocatalytic processing occur in an ATP-dependent manner.

The presence of the pro-region is a prerequisite for sorting of PIM1 protease to mitochondria

To obtain further insights into the function of the pro-region, amino acid residues 42–92 of PIM1 protease were deleted ($PIM1^{\Delta 42-92}$). $pim1^{\Delta 42-92}$ was expressed in a diploid strain carrying one disrupted *PIM1* allele, and a haploid $\Delta pim1$ strain harboring $PIM1^{\Delta 42-92}$ protease was isolated by sporulation and tetrad dissection ($pim1^{\Delta 42-92}$). The respiratory competence of these cells was strongly impaired when $PIM1^{\Delta 42-92}$ protease was expressed from a centromere-based yeast plasmid under the control of the endogenous promoter (Figure 5A). This points to a crucial role of the pro-region during the biogenesis of PIM1 protease. Interestingly, the growth rate on non-fermentable carbon sources increased significantly in $\Delta pim1$ cells expressing $PIM1^{\Delta 42-92}$ from a multicopy plasmid under the control of a galactose-inducible promoter (Figure 5A). Thus, when present at high levels, $PIM1^{\Delta 42-92}$ protease can substitute for functions of wild-type PIM1 protease in mitochondria.

To determine the cellular localization of $PIM1^{\Delta 42-92}$ protease, extracts were prepared from *PIM1* and $pim1^{\Delta 42-92}$ cells, separated into a mitochondrial and cytosolic fraction and analyzed by immunoblotting (Figure 5B). A similar molecular mass of wild-type and $PIM1^{\Delta 42-92}$ subunits was observed, indicating processing of $PIM1^{\Delta 42-92}$ protease (Figure 5B). Surprisingly, $PIM1^{\Delta 42-92}$ protease accumulated in the cytosolic fraction (Figure 5B). The protease was present only at reduced levels, most probably due to degradation of the missorted protein. In contrast, wild-type PIM1 was recovered exclusively in the mitochondrial fraction (Figure 5B). Thus, the pro-region is required to ensure correct sorting of PIM1 protease to mitochondria. In agreement with the observed growth phenotype of $pim1^{\Delta 42-92}$ cells, however, low amounts of $PIM1^{\Delta 42-92}$ protease expressed from a multicopy plasmid were found to accumulate within mitochondria (data not shown). Thus, the propeptide of PIM1 protease is apparently not essential for targeting to mitochondria, but ensures the efficiency of the sorting process.

The propeptide might act as an inhibitor of the proteolytic activity of PIM1 protease. Premature autocatalytic processing of the $PIM1^{\Delta 42-92}$ precursor in the cytosol may result in the removal of the mitochondrial targeting signal, thereby impairing sorting to mitochondria. To examine whether missorting of PIM1 protease lacking the pro-region depends on its proteolytic activity, the catalytic site serine residue 1015 was replaced by alanine ($pim1^{\Delta 42-92/S1015A}$). The mutant protein was expressed in $\Delta pim1$ cells. An uncleaved precursor form of proteolytically inactive $PIM1^{\Delta 42-92}$ protease was observed, indicating that proteolytically active $PIM1^{\Delta 42-92}$ protease,

Fig. 5. The propeptide is crucial for sorting of PIM1 protease to mitochondria. (A) The respiratory competence of $pim1^{\Delta 42-92}$ mutant cells is impaired. $\Delta pim1$ mutant cells harboring pRS314-*PIM1* (*PIM1*), pRS314- $pim1^{\Delta 42-92}$ ($pim1^{\Delta 42-92}$) or pYES2.0- $pim1^{\Delta 42-92}$ ($pim1^{\Delta 42-92} \uparrow \uparrow$) were grown at 30°C in synthetic medium containing 2% glucose. Ten-fold serial dilutions were spotted onto YP medium containing 3% glycerol (YPGlycerol) and incubated for 4 days at 30°C. (B) Subcellular localization of mutant variants of PIM1 protease in $\Delta pim1$ cells harboring pRS314-*PIM1* (*PIM1*)(p⁺), pRS314- $pim1^{\Delta 42-92}$ ($pim1^{\Delta 42-92}$)(p⁻) or pRS314- $pim1^{\Delta 42-92/S1015A}$ ($pim1^{\Delta 42-92/S1015A}$)(p⁻). Cellular extracts were prepared and subjected to differential centrifugation as described in Materials and methods. Total soluble proteins after lysis of the cells (300 µg; Total), mitochondrial proteins (50 µg; Mito) and the post-mitochondrial fraction containing cytosolic proteins (300 µg; Cytosol) were analyzed by SDS-PAGE and immunoblotting with polyclonal antibodies directed against PIM1, Bmh2p (brain modulosignalin homolog; Van Heusden *et al.*, 1992) and Mge1p (mitochondrial GrpE homolog). Bmh2p and Mge1p are markers for the cytosolic and mitochondrial fraction, respectively. pre, precursor; m, mature form of PIM1 protease.

presumably after oligomerization, was processed autocatalytically to the mature form in the cytosol (Figure 5B). Subcellular fractionation revealed a cytosolic localization of unprocessed, proteolytically inactive $PIM1^{\Delta 42-92}$ protease (Figure 5B). We conclude from these experiments that missorting of PIM1 protease lacking the pro-region to the cytosol is not caused by premature processing of preproteins in the cytosol, but rather reflects the requirement for the propeptide for efficient sorting to mitochondria.

Discussion

We demonstrate here that mitochondrial PIM1 protease is synthesized as a preproprotein and matured by two-step processing in mitochondria. First, the mitochondrial processing peptidase removes the N-terminal 37 amino acid residues which comprise the matrix targeting signal

and thereby generates a pro-form of the protease. Second, an N-terminal propeptide consisting of 61 amino acid residues is cleaved off autocatalytically following ATP-dependent assembly of PIM1 protease subunits.

The propeptide is crucial for sorting of the cytosolic preprotein to mitochondria and thereby for the biogenesis of PIM1 protease. How does the propeptide determine the sorting process? The pro-region might act as a potent autoinhibitor of PIM1 protease and protect the cell from precocious activation of the precursor protein in the cytosol. While a possible inhibitory effect of the pro-region on the degradation of substrate proteins remains to be determined, the observed missorting of proteolytically inactive PIM1 protease lacking the propeptide cannot be explained by premature autocatalytic processing of the preprotein. Similarly, it seems rather unlikely that deleting large parts of the pro-region interferes indirectly with the functionality of the mitochondrial targeting information. Therefore, additional functions of the propeptide have to be envisaged. Although not yet demonstrated for any other mitochondrial protein, the propeptide may contain targeting information which, in addition to the classical matrix targeting sequence of PIM1, is required to ensure sorting of the protease to mitochondria. A targeting function of the pro-region would be reminiscent of vacuolar proteins, which carry an N-terminal propeptide harboring the vacuolar sorting signal (Valls *et al.*, 1990; Van Voorst *et al.*, 1996; Westphal *et al.*, 1996). Pro-sequences of serine and aspartic proteinases are known to act as intramolecular chaperones facilitating folding to the native conformation (Baker *et al.*, 1993; Shinde and Inouye, 1993; Eder and Fersht, 1995). Along this line, the propeptide of PIM1 protease may maintain the precursor protein in a translocation-competent conformation in the cytosol, either by serving as an intramolecular chaperone shielding hydrophobic regions of the protein or by providing a binding site for other cytosolic proteins. This may also result in the exposure of the mitochondrial targeting sequence and thereby allow efficient binding of the PIM1 precursor to receptor proteins at the mitochondrial surface. Similarly, it is also conceivable that the pro-region facilitates unfolding of native-like PIM1 precursor proteins at the mitochondrial surface and thereby ensures efficient translocation across the mitochondrial membranes.

PIM1 protease forms a high molecular weight, presumably homo-oligomeric complex in the mitochondrial matrix space. Assembly of PIM1 protease, and thereby most probably its proteolytic activity, depends on ATP hydrolysis. While an effect of nucleotides on the assembly state of *E. coli* Lon protease has not been reported, other ATP-dependent proteases, e.g. the eukaryotic 26S proteasome, Clp- and AAA proteases, form high molecular weight complexes in a nucleotide-dependent manner (Coux *et al.*, 1996; Gottesman, 1996; Hilt and Wolf, 1996; Hochstrasser, 1996; Langer and Neupert, 1996; Baumeister and Lupas, 1997). However, nucleotide binding rather than ATP hydrolysis appears to promote the assembly of Clp proteases and the mitochondrial *m*-AAA protease (Thompson *et al.*, 1994; Arlt *et al.*, 1996). Oligomerization of PIM1 protease subunits is a prerequisite for autocatalytic propeptide processing. The observed maturation of newly imported PIM1 in the presence of proteolytically inactive PIM1 protease points to an intramolecular cleavage event,

suggesting that an inactive pro-form of PIM1 protease is converted into an active form upon assembly with pre-existing subunits. On the other hand, autocatalytic processing can also occur intermolecularly, as demonstrated by the efficient maturation of proteolytically inactive PIM1 protease subunits by pre-existing, proteolytically active protease molecules.

The mutational analysis establishes the requirement for both catalytic activities of PIM1 protease for maintenance of mitochondrial function. The respiratory competence of the cells is impaired if PIM1 protease is mutated at the proteolytic site or the ATP-binding site. Although the respective mutant strains display similar growth phenotypes, the biogenesis of PIM1 is affected differently by mutations at the two sites: oligomerization of PIM1 protease depends on its intrinsic ATPase activity and thereby on an intact ATP-binding site, but is not impaired by mutations of the proteolytic site.

In addition, our results shed new light on the recent finding that overexpression of proteolytically inactive PIM1 protease can suppress the respiratory deficiency of yeast cells lacking the *m*-AAA protease, whereas suppression is not observed when the ATP-binding site was mutated (Rep *et al.*, 1996b). This has been taken as evidence for a chaperone function of PIM1 protease in the assembly of mitochondrial protein complexes which is independent of its proteolytic activity (Rep *et al.*, 1996b). As endogenous wild-type PIM1 protease was present in these cells, however, the formation of mixed oligomers composed of endogenous wild-type and overexpressed mutant PIM1 protease subunits is likely. The intermolecular autocatalytic cleavage of the propeptide of PIM1 protease observed upon oligomerization demonstrates the proteolytic activity of such complexes. Overexpression of proteolytically inactive PIM1 subunits results, therefore, in an increased number of proteolytically active complexes in mitochondria. This is consistent with the observation that the degradation of newly imported, misfolded polypeptides is not affected in wild-type mitochondria containing high levels of proteolytically inactive PIM1 protease (A.Savel'ev and T.Langer, unpublished observations). As oligomerization of PIM1 protease subunits depends on their intrinsic ATPase activity, the requirement for an intact ATP-binding site for suppression can be explained by the impaired assembly with wild-type subunits. Thus, defects in mitochondrial biogenesis of $\Delta pim1$ mutant cells as well as the observed suppression of deficiencies in the *m*-AAA protease upon overexpression of PIM1 can be explained satisfactorily by and attributed to proteolytic functions of PIM1. The existence of a chaperone-like activity of PIM1 protease, which may be required for maintaining substrate polypeptides in a conformation susceptible for degradation, however, remains an attractive possibility.

Materials and methods

Cloning and site-directed mutagenesis of PIM1

PIM1 was amplified by PCR employing the primer pair GGG GGT ACC CGG GCA TGC TAA GAA CAA GAA CC (5'-primer) and GGG GTC GAC TTA GTC CTT TTC CTT TTT (3'-primer). The PCR product was digested with *KpnI* and *SalI* and cloned into pGEM4 (Promega), allowing the *in vitro* synthesis of the protein (pGEM4-PIM1). To express PIM1 protease carrying a carboxy-terminal hexahistidine tag in

Saccharomyces cerevisiae, *PIM1* was first amplified by PCR using the 5'-primer described above and the 3'-primer GGG GTC GAC TTA ATG GTG ATG GTG ATG GTG GTC CTT TTC CTT TTT AGC which codes for the 3' end of *PIM1* and six histidine residues. The PCR product was cloned as a *KpnI*-*SalI* DNA fragment (3.4 kb) into pYES2.0 (Invitrogen) (pYES2.0-*PIM1*). An internal *BamHI*-*BglII* DNA fragment of pGEM4-*PIM1* and pYES2.0-*PIM1* was replaced by the corresponding genomic DNA, and the remaining DNA segments at the 5' and 3' end of *PIM1* were verified by DNA sequencing. To allow expression of *PIM1* from the endogenous promoter in Δ *pim1* mutant cells, a 5.3 kb DNA fragment was isolated by restriction digest of pSK⁺-*PIM1* (Van Dyck *et al.*, 1994) with *XhoI* and *NotI* and cloned into the corresponding sites in pRS314 (pRS314-*PIM1*) (Sikorski and Hieter, 1989).

For construction of *pim1*^{S1015A}, *PIM1* was mutagenized by two-step PCR (Perrin and Gilliland, 1990) employing the following primers: 5'-primer AAA GAT GGT CCA GCC GCA GGT GTC ACT (first reaction), and 3'-primer GGG GTC GAC TTA GTC CTT TTC CTT TTT and 5'-primer GAA GAC AAG GTT AAG ATT GCC GAG CAG (second reaction). After restriction digest with *XbaI* and *BglII*, the 1 kb DNA fragment was used to replace the corresponding segment in pSK⁺-*PIM1* (pSK⁺-*pim1*^{S1015A}). To allow synthesis of *PIM1*^{S1015A} in reticulocyte lysate, an internal 3 kb DNA fragment was isolated by restriction digest with *BamHI* and *BglII* and cloned into the corresponding sites in pGEM4-*PIM1* (pGEM4-*pim1*^{S1015A}). For expression in *S. cerevisiae*, a 5.3 kb DNA fragment harboring *pim1*^{S1015A} and the endogenous promoter was isolated by restriction digest of pSK⁺-*pim1*^{S1015A} with *XhoI* and *NotI* and inserted in pRS314 (pRS314-*pim1*^{S1015A}). For overexpression in yeast, an internal 3 kb DNA fragment was isolated by restriction digest of pSK⁺-*pim1*^{S1015A} with *BamHI* and *BglII* and cloned into the corresponding sites in pYES2.0-*PIM1* (pYES2.0-*pim1*^{S1015A}).

pim1^{K638N} was generated by PCR with the primer pair AAA TCA TAT GTT TCG TAG GAC CCC CGG GTG TTG GTA ACA CGT CCA TAG GTA AAT CT (5'-primer) and GGG GTC GAC TTA GTC CTT TTC CTT TTT (3'-primer). The PCR product was cleaved with *NdeI* and *XbaI* and inserted into pGEM4-*PIM1* digested with *NdeI* and *XbaI* (pGEM4-*pim1*^{K638N}). Then, a 1.4 kb *NcoI*-*BglII* DNA fragment was used to replace the corresponding segment in pSK⁺-*PIM1* (pSK⁺-*pim1*^{K638N}). For expression in yeast, a 5.3 kb *XhoI*-*NotI* DNA fragment was subcloned into pRS314 (pRS314-*pim1*^{K638N}).

Amino acids 42–92 of the pro-region of *PIM1* protease were deleted in two steps. First, a *BamHI* site was introduced by PCR-based mutagenesis replacing the base triplet AAA (coding for K94) by TCC (*pim1*^{K94S}). Second, a 140 bp DNA fragment coding for the 41 N-terminal amino acid residues was amplified by PCR employing the primer pair CCG GGG GTA CCC GGG CAT GCT AAG AAC AAG AAC C (5'-primer) and CCC GGA TCC CAA GGT CGA TGC GAA CCT (3'-primer) bearing a *KpnI* and a *BamHI* site, respectively. The PCR product was cloned as a *KpnI*-*BamHI* DNA fragment into pGEM4-*PIM1* (pGEM4-*pim1*^{Δ42–92}). The gene obtained codes for *PIM1* protease lacking amino acid residues 42–92. An internal 3 kb DNA fragment was isolated by restriction digest with *PstI* and used to replace the corresponding DNA segment in pSK⁺-*PIM1* (pSK⁺-*pim1*^{Δ42–92}). For expression of *PIM1*^{Δ42–92} protease in yeast under the control of the endogenous *PIM1* promoter, a 2.5 kb *XhoI*-*NcoI* DNA fragment of pSK⁺-*pim1*^{Δ42–92} was cloned into pRS314-*PIM1* (pRS314-*pim1*^{Δ42–92}). For overexpression, a 420 bp DNA fragment of pSK⁺-*pim1*^{Δ42–92} was isolated by restriction digest of pSK⁺-*pim1*^{Δ42–92} with *KpnI* and *HindIII* and cloned into corresponding sites of pYES2.0-*PIM1* (pYES2.0-*pim1*^{Δ42–92}).

To generate *pim1*^{Δ42–92/S1015A}, a 1.4 kb DNA fragment was isolated from pRS314-*PIM1*^{S1015A} by restriction digest with *NcoI* and *BglII* and cloned into the corresponding sites of pRS314-*pim1*^{Δ42–92} (pRS314-*pim1*^{Δ42–92/S1015A}).

Yeast strains and growth conditions

All yeast strains are derivatives of the wild-type strain YPH500 (Sikorski and Hieter, 1989). For disruption of *PIM1*, a 1.8 kb *BamHI* DNA fragment harboring *HIS3* was inserted into pSK⁺-*PIM1* (Van Dyck *et al.*, 1994) which had been digested with *BamHI* and *BglII*. The linearized construct was transformed into the yeast strain YPH500 (Δ *pim1*). Disruption of *PIM1* was monitored by PCR. Yeast plasmids carrying wild-type or mutant variants of *PIM1* were transformed into Δ *pim1* mutant cells (*PIM1*, *pim1*^{S1015A}, *pim1*^{K638N}, *pim1*^{Δ42–92}, *pim1*^{Δ42–92/S1015A}). For determination of the growth phenotype of *PIM1* mutants, *PIM1* was disrupted in the diploid strain YPH501 which subsequently was transformed with plasmids harboring *PIM1* variants. Haploid Δ *pim1* segregants containing plasmid were isolated by sporulation and tetrad dissection. Cells were grown on selective medium containing 2% glucose

at 30°C according to published procedures. To induce expression from the *GAL1* promoter of pYES2.0, the same medium was supplemented with 0.1% galactose.

Ni-NTA chromatography

For protein sequencing, *PIM1* and *PIM1*^{S1015A} protease were purified under denaturing conditions. Cells were resuspended in buffer A (7 M urea, 50 mM Tris-HCl pH 7.4) (50 OD/ml). After addition of glass beads (Ø0.5 mm, two-thirds of the total volume), cells were lysed by vigorous vortexing for 30 s and chilled on ice for 30 s. This procedure was repeated four times. Glass beads and intact cells were removed by centrifugation for 3 min at 1000 g. Extracts were incubated with Ni-NTA-agarose (Qiagen) for 90 min at 4°C. After washing with buffer A, proteins were eluted with buffer A containing 300 mM imidazole, analyzed by SDS-PAGE and transferred onto a PVDF membrane. Protein bands corresponding to *PIM1* or *PIM1*^{S1015A} protease were sequenced by Edman degradation.

To assess oligomerization of *PIM1* protease subunits after import, mitochondria (0.5 mg) were resuspended at a concentration of 2 mg/ml in buffer B [0.1% (w/v) Triton X-100, 30 mM HEPES-KOH pH 7.4, 150 mM KOAc, 4 mM MgOAc, 1 mM phenylmethylsulfonyl fluoride (PMSF)] containing 1 mM ATP and incubated for 15 min at 4°C. After a clarifying spin for 10 min at 30 000 g, the supernatant was diluted 2-fold with lysis buffer and imidazole was added to a final concentration of 10 mM. Mitochondrial extracts were incubated with Ni-NTA-agarose for 90 min at 4°C. After repeated washing with lysis buffer containing 20 mM imidazole, bound material was eluted by incubation of the Ni-NTA beads for 30 min at 4°C in lysis buffer containing 300 mM imidazole. Eluted proteins were analyzed by SDS-PAGE and autoradiography.

Gel filtration analysis

Mitochondria (0.5 mg) were lysed in buffer B containing 1 mM ATP as described above and extracts were centrifuged for 10 min at 30 000 g. The supernatant was loaded onto a Superose 6 column (Pharmacia) equilibrated with the same buffer. Fractions (0.5 ml) were collected and analyzed by SDS-PAGE. Eluted proteins were detected by autoradiography or immunoblotting using the enhanced chemiluminescence detection system (Amersham) and quantified by laser densitometry. The following standards were used for size calibration: Hsp60 (840 kDa; 13.2 ml), thyroglobulin (669 kDa; 13.7 ml), apoferritin (443 kDa; 14.5 ml), β -amylase (200 kDa; 15.7 ml), alcohol dehydrogenase (150 kDa; 16.5 ml).

To examine the role of ATP in *PIM1* assembly, mitochondria were lysed in buffer B in the presence of AMP-PNP (10 mM) or in the absence of nucleotides. The Superose 6 column was equilibrated with lysis buffer containing AMP-PNP (1 mM) or no nucleotides. When the oligomeric state of *PIM1* was analyzed in the presence of AMP-PNP, mitochondria were depleted of matrix ATP prior to lysis. For that purpose, mitochondria (0.5 mg) were resuspended at a concentration of 1 mg/ml in SHKCl buffer and incubated for 10 min at 25°C in the presence of apyrase (20 U/ml) and oligomycin (20 μ M).

Cell fractionation

For subcellular localization of *PIM1* protease variants, cells were incubated with zymolyase (3 mg/g cells) at 30°C. Spheroplasts were harvested by centrifugation for 5 min at 2500 g, washed with 20 mM K phosphate pH 7.4, 1.2 M sorbitol, resuspended in 0.6 M sorbitol, 20 mM HEPES-KOH pH 7.4, 1 mM PMSF (3 ml/g cells) and chilled on ice. Then, spheroplasts were homogenized using a Dounce homogenizer (10 strokes). Extracts were centrifuged for 5 min at 3000 g. Mitochondria were isolated from the supernatant fraction ('Total') by centrifugation for 12 min at 12 000 g and resuspended in SHKCl buffer ('Mito'). The post-mitochondrial supernatant contained cytosolic proteins ('Cytosol').

Miscellaneous

Standard procedures were used for isolation of mitochondria (Herrmann *et al.*, 1994; Zinser and Daum, 1995), protein synthesis in rabbit reticulocyte lysate (Söllner *et al.*, 1991) and protein import into isolated mitochondria (Wagner *et al.*, 1994). *PIM1* protease carrying a carboxy-terminal hexahistidine tag was purified from yeast by Ni-NTA chromatography and used for generation of a polyclonal antiserum in rabbits. Goat anti-rabbit antibodies conjugated to horseradish peroxidase (Sigma) and a chemiluminescence kit (Amersham) were used for immunodetection.

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