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MAP-1 and IAP-1, Two Novel AAA Proteases with Catalytic Sites on Opposite Membrane Surfaces in Mitochondrial Inner Membrane of *Neurospora crassa*

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Eukaryotic AAA proteases form a conserved family of membrane-embedded ATP-dependent proteases but have been analyzed functionally only in the yeast *Saccharomyces cerevisiae*. Here, we have identified two novel members of this protein family in the filamentous fungus *Neurospora crassa*, which were termed MAP-1 and IAP-1. Both proteins are localized to the inner membrane of mitochondria. They are part of two similar-sized high molecular mass complexes, but expose their catalytic sites to opposite membrane surfaces, namely, the intermembrane and the matrix space. Disruption of *iap-1* by repeat-induced point mutation caused a slow growth phenotype at high temperature and stabilization of a misfolded inner membrane protein against degradation. IAP-1 could partially substitute for functions of its yeast homolog Yme1, demonstrating functional conservation. However, respiratory growth at 37°C was not restored. Our results identify two components of the quality control system of the mitochondrial inner membrane in *N. crassa* and suggest that AAA proteases with catalytic sites exposed to opposite membrane surfaces are present in mitochondria of all eukaryotic cells.

INTRODUCTION

The selective degradation of cellular proteins is mediated by ATP-dependent proteases, which exert dual functions within the cell (Goldberg, 1992; Gottesman and Maurizi, 1992; Schmidt *et al.*, 1999). By selectively degrading short-lived regulatory proteins, they control cellular homeostasis and allow its adaptation to altered environmental conditions. On the other hand, they ensure the removal of misfolded polypeptides, thereby preventing their accumulation and possibly deleterious effects on cellular processes. ATP-dependent proteases comprise highly conserved protein families in all kingdoms and share related ATPase domains characteristic of the AAA^+ class of ATPases (Patel and Latterich, 1998; Neuwald *et al.*, 1999). These domains exhibit chaperone-like activity, which is crucial for the breakdown of polypeptides (Gottesman *et al.*, 1997; Leonhard *et al.*, 1999; Wickner *et al.*, 1999). Increasing evidence suggests that the energy gained from nucleotide hydrolysis is required to mediate substrate unfolding and to regulate the accessiblity of the proteolytic sites (Braun *et al.*, 1999; Weber-Ban *et al.*, 1999; Groll *et al.*, 2000).

A distinct class of ATP-dependent proteases is represented by AAA proteases, which are an integral part of membranes and recognize membrane proteins as their substrates (Schumann, 1999; Langer, 2000). Members of this family of metallopeptidases have been identified in eubacteria and eukaryotic cells; however, they were analyzed only in *Escherichia coli* and the yeast *Saccharomyces cerevisiae*. In both organisms, they form large complexes in the membrane that apparently are homo-oligomeric or made up of closely related subunits (Akiyama *et al.*, 1995; Arlt *et al.*, 1996; Leonhard *et al.*, 1996). Only one AAA protease, FtsH, which is essential for cell viability in *E. coli* (Tomoyasu *et al.*, 1993; Ogura *et al.*, 1999), appears to be present in the plasma membrane of most eubacteria. In contrast, two AAA proteases have been identified in yeast that constitute a quality control system in the inner membrane of mitochondria (Pajic *et al.*, 1994; Nakai *et al.*, 1995; Pearce and Sherman, 1995; Guélin *et al.*, 1996; Weber *et al.*, 1996). The *m*-AAA protease, which is active on the matrix side of the inner membrane, is a multimeric complex composed of the related subunits Yta10 (Afg3) and Yta12 (Rca1) (Arlt *et al.*, 1996). The *i*-AAA protease, on the other hand, exposes catalytic sites into the intermembrane space (Leonhard *et al.*, 1996). It is presumably a homo-oligomeric complex made up of Yme1 subunits that are highly homologous to Yta10 and Yta12. Both proteases fulfill crucial functions during mitochondrial biogenesis. The *m*-AAA protease controls the expression of mito-

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chondrially encoded respiratory chain subunits and the posttranslational assembly of respiratory complexes and is thereby required for the maintenance of the respiratory competence of yeast cells (Paul and Tzagoloff, 1995; Arlt *et al.*, 1996, 1998). Cells lacking the *i*-AAA protease exhibit a pleiotropic phenotype. They are respiratory deficient at high temperature, show a cold-sensitive growth defect on glucose-containing medium, and extremely slow growth in the absence of mitochondrial DNA (Thorsness *et al.*, 1993). Moreover, they accumulate mitochondria with an aberrant morphology (Campbell *et al.*, 1994). Notably, inactivation of both proteases is lethal in yeast, indicating that despite their different topology in the inner membrane, they exert overlapping functions (Lemaire *et al.*, 2000; Leonhard *et al.*, 2000). A membrane protein with domains present at both membrane surfaces was indeed found to be degraded by either the *m*- or the *i*-AAA proteases if solvent-exposed domains are unfolded, suggesting a dislocation of polypeptides from the membrane for proteolysis (Leonhard *et al.*, 2000).

Although genome sequencing projects revealed the existence of several potential AAA protease subunits in various eukaryotic organisms, including humans (Juhola *et al.*, 2000), none of them has been functionally characterized. A mutation of the human mitochondrial AAA protease paraplegin causes neurodegeneration in an autosomal recessive form of hereditary spastic paraplegia (Casari *et al.*, 1998), but the function of paraplegin on the molecular level remains to be characterized. Here, we report on the identification and characterization of two novel AAA proteases in the filamentous fungus *Neurospora crassa* hat exhibit high sequence similarity to yeast and human AAA proteases.

MATERIALS AND METHODS

Cloning of Matrix AAA Protease-1 (MAP-1)

To identify genes coding for AAA protease subunits in *N. crassa*, a Blast search was performed with the use of yeast Yta10 (Afg3) as a reference (http://www.genome.ou.edu). EST4159261 (gb: AW709994) from the *N. crassa* cDNA library was identified that codes for a sequence of 145 amino acid residues homologous to Yta10. This DNA fragment was amplified by polymerase chain reaction (PCR) with the primer pair 5′-TTT GGA TCC CGT TCC GAC GGC GGC TTC AGG-3′ and 5′-TTT AAG CTT AGG ACT TGC GCT CCA GAC CGC-3′, and, after labeling with the DIG-DNA-Labeling kit (Roche, Mannheim, Germany), used as a probe for screening the *N. crassa* cosmid library pMOcosX (Orbach, 1994) by colony hybridization. A cosmid (G4:A5) was isolated and sequenced. It encoded the complete *map-1* gene. A comparison of genomic and cDNA sequences revealed the presence of a 66-bp intron at position 1959 of *map-1*.

For SP6 polymerase-driven synthesis of MAP-1 in vitro, *map-1* was amplified by PCR with the use of the primer pair 5′-AAA TTT AAG CTT TCA CCT TTG CTC CTT GCT CTC ACC-3′ and 5′-TTT AAA TCT AGA ATG GCG GTC AGA TTT CGC CAA TCG-3′ and genomic DNA as template. The PCR product was isolated, digested with *Xba*I and *Hin*dIII, and cloned into the vector pGEM4 (Promega, Madison, WI).

Cloning of Intermembrane Space AAA Protease-1 (IAP-1)

PCR was performed with the degenerate primer pair 5′-GGA CCT CCT GGT ACA GGT AAA ACT-3′ and 5′-GGC ATG TCC AGC CTC GTG GAA AGC AGT-3′ and genomic DNA of *N. crassa* as a template. A 600-bp DNA fragment was amplified that was labeled with the DIG-DNA-Labeling kit and used as a probe for screening a *N. crassa* cDNA library (kind gift of Dr. F. Nargang, University of Edmonton, Alberta, Canada) by plaque hybridization. DNA sequencing of a positive clone identified an insert of 1668 bp that shared 57% sequence homology to yeast Yme1 on the protein level but did not contain the 5′ end of the gene. To identify the full-length *iap-1* gene, the *N. crassa* cosmid library pMOcosX (Orbach, 1994) was screened by colony hybridization with the use of the 600-bp DNA fragment as a probe. A cosmid (G9:G8) was isolated that contained the complete *iap-1* gene, including the promotor region.

For repeat induced point mutation, the full-length *iap-1* gene was amplified from genomic DNA by PCR with the use of the primer pair 5′-TTA TGA ATT CAC CAT GTC TTC CCG CCA GCT CG-3′ and 5′-TTA TTG GAT CCT CAG TGA TGG TGA TGG TGG TGC GCG GGC ACA GGC GGC-3′, providing a C-terminal hexahistidine tag. The PCR product was digested with *Eco*RI and *Bam*HI and cloned into the hygromycin resistance-conferring vector pqa-2Hyg (Prokisch *et al.*, 2000), which contains the promotor of the *qa-2* gene of *N. crassa* for protein expression (Geever *et al.*, 1989).

In parallel, the PCR product was cloned into the vector pGEM4 to allow SP6 polymerase-driven expression of IAP-1 in vitro. For synthesis of IAP-1ΔN33, a PCR product, amplified from genomic DNA with the forward primer 5′-TAA TGA ATT CAC CAT GAG CAC CCA CCA GCC CG-3′ and the reverse primer as described above, was cloned as an *Eco*RI/*Bam*HI DNA fragment into the vector pGEM4. Both constructs contain a 60-bp intron at position 1776 of the full-length *iap-1* gene, which causes a premature stop after 607 amino acid residues of IAP-1 upon synthesis in reticulocyte lysate. To obtain intronless *iap-1*, the 3′ region of the genomic clone of *iap-1* in pGEM4 was removed by restriction digest with *Sac*II and *Pst*I and replaced by a corresponding cDNA fragment, which was amplified by PCR with the primer pair 5′-TTT GGA TCC GGC TTG AGC AGC ATC AAG C-3′ and 5′-TTT AAA AAG CCT TCA CGC GGG CAC AGG CGG CGG-3′.

For complementation studies in yeast, a hybrid protein consisting of the mitochondrial targeting sequence of Yme1 and the mature part of IAP-1 (IAP-1*) was expressed under the control of the endogenous *YME1* promotor in Δ*yme1* yeast cells. Exsite PCR from the plasmid pRS314-A6 (Weber *et al.*, 1996) was performed with the use of the primer pair 5′-GGG AAA TTT CAT ATG ACG TAT GAA GGA ACC TAC CTC AAG ACC-3′ and 5′-AAA CTT GGG GCA TGC AGA ATA AAA ACG GTA GAA CTT CTT TGA TC-3′. The amplificate contained the complete plasmid pRS314 (Sikorski and Hieter, 1989), the promotor region of *YME1* (bp −764–1) and the 5′ region of the *YME1* gene coding for the mitochondrial targeting sequence and two amino acid residues of mature Yme1 (amino acid residues 1–49). This DNA fragment (6.2 kb) was digested with *Nde*I and *Sph*I and ligated with a *Nde*I/*Sph*I DNA fragment (2 kb) that was obtained upon restriction digest of pGEM4/*iap-1*^{intronless} and encoded mature IAP-1 (amino acids residues 62–738). In the derived hybrid protein IAP-1*, the amino terminal portion of Yme1 is linked via two amino acid residues (AC) to mature IAP-1.

To allow SP6 polymerase-driven synthesis of the hybrid protein IAP-1* in vitro, a DNA fragment coding for IAP-1* was amplified by PCR with the use of the primer pair 5′-CCC TTT CTG CAG ATG AAC GTT TCA AAA ATA CTT G-3′ and 5′-CCC AAA GGA TCC GAG GTA GGT TCC TTC ATA CG-3′ and cloned into the vector pGEM-T (Promega).

N. crassa Strains and Growth Conditions

Standard genetic and microbiological techniques were used for growth and manipulation of *N. crassa* strains (Davis and de Serres, 1970). Hyphae were grown in Vogel's minimal medium under continuous aeration and illumination with white light at 25°C (Davis and de Serres, 1970). Race tubes contained Vogel's agar and 2% glucose or 0.3% Na-acetate as the sole carbon source. Transformation of *N. crassa* was carried out as described (Vollmer and Yanofsky, 1986; Staben *et al.*, 1989). *N. crassa* wild-type strains used

in this study were *St. Lawrence 74A* (Fungal Genetics Stock Center, Kansas City, KS) and *K93–5a* (isogenic to strain *74A*).

For repeat induced point mutation of *iap-1*, a plasmid encoding genomic *iap-1* was transformed into strain *St. Lawrence 74A*. Homokaryotic microconidia of the derived strain were isolated (Ebbole and Sachs, 1990) and used for mating with strain *K93-5a.* Sixty ascospores were isolated from this cross, germinated, and examined for the absence of IAP-1 by Western blot analysis. The *iap-1* allele of a mutant strain was amplified by PCR and sequenced. The strain was termed *iap-1^{RIP}*. Transformation of *iap-1^{RIP}* with a plasmid harboring genomic *iap-1* yielded *iap-1^{RIP}-iap-1.* For determination of expression levels of IAP-1, this strain was grown on selective medium containing 0.3% quinic acid and 0.5% glucose.

Yeast Strains and Growth Conditions

Yeast cells were grown at the indicated temperature on YP or selective medium containing 2% glucose (YPD) or 3% glycerol (YPG) according to published procedures (Sambrook *et al.*, 1989). Yeast strains used in this study are isogenic to the wild-type strain W303-1A. Yme1 was disrupted by PCR-targeted homologous recombination (Wach *et al.*, 1994) with the use of the heterologous marker *HIS3MX6* (YKC10). The complete open reading frame was replaced by the disruption cassette. Homologous recombination was verified by PCR. For complementation analysis, the plasmid pRS314 encoding IAP-1* was transformed into Δ*yme1* mutant yeast cells.

Strains lacking mitochondrial DNA (ρ^o) were obtained as previously described (Fox *et al.*, 1991) by growing wild-type, Δ*yme1*, and Δ*yme1* cells expressing IAP-1 on selective medium containing 2% glucose and 25 μg/ml ethidium bromide twice to stationary phase. Loss of mitochondrial DNA was verified by testing the ability of the strains to grow on glucose- but not on glycerol-containing medium.

Antibody Production

The synthetic peptide CKKEVERVIRGEK corresponding to amino acid residues 675–686 at the C terminus of IAP-1 was coupled to keyhole limpet hemocyanin with maleimide-activated carrier protein (Imject; Pierce, Rockford, IL) and used for generation of antibodies in rabbits. For detection of MAP-1 we used a polyclonal antiserum that is directed against the matrix-localized domain of yeast Yta10 (Arlt *et al.*, 1996) and that cross-reacted with MAP-1.

Digitonin Fractionation of Mitochondria

Mitochondria were fractionated with digitonin essentially as described (Segui-Real *et al.*, 1993). Mitochondria (100 μg) were resuspended at a concentration of 5 mg/ml in SEMK buffer [250 mM sucrose, 10 mM 3-(*N*-morpholino)propanesulfonic acid/KOH pH 7.2, 80 mM KCl, 1 mM EDTA] supplemented with varying concentrations of digitonin in the presence or absence of proteinase K (PK) (100 μg/ml) and incubated for 5 min at 4°C. For control, mitochondria were completely solubilized by adding Triton X-100 0.17% [vol/vol] final concentration). Lysis of mitochondria was stopped by diluting the samples fivefold with ice-cold SEMK. Protease digestion was allowed for additional 30 min on ice before it was inhibited by adding phenylmethylsulfonyl fluoride (PMSF; 1 mM). The samples were trichloroacetic acid-precipitated and analyzed by SDS-PAGE and immunostaining.

Gel Filtration Analysis

Mitochondria (2 mg) were resuspended at a concentration of 3 mg/ml in buffer A (1% [vol/vol] Triton X-100, 30 mM Tris-HCl pH 7.4, 150 mM K-acetate, 4 mM Mg-acetate, 1 mM PMSF), which was supplemented with 1 mM ATP when indicated. After incubation for 15 min at 4°C under vigorous mixing, mitochondrial extracts were centrifuged for 20 min at 109,000 × *g*. The supernatant was incubated for 15 min at 25°C in the presence or absence of apyrase (5 U/ml) and loaded on a Superose 6 column, which had been equilibrated with buffer A containing 0.1% (vol/vol) Triton X-100 and, when indicated, 1 mM ATP. Fractions (0.5 ml) were collected and analyzed by SDS-PAGE and immunoblotting with antisera directed against MAP-1 and IAP-1. The amount of MAP-1 and IAP-1 in the eluate fraction was determined by laser densitometry and is given as percentage of total protein in the eluate.

Protein Import into Isolated Mitochondria

Mitochondrial preproteins were synthesized in reticulocyte lysate in the presence of [^{35}S]methionine according to published procedures (Söllner *et al.*, 1991). Protein import into mitochondria isolated from *N. crassa* or *S. cerevisiae* was performed essentially as described (Wagner *et al.*, 1994; Mayer *et al.*, 1995). *N. crassa* mitochondria (30 μg) were resuspended at a concentration of 0.2 mg/ml in 250 mM sucrose, bovine serum albumin (30 mg/ml), 80 mM KCl, 5 mM $MgCl_2$, 10 mM 3-(*N*-morpholino)propanesulfonic acid/KOH pH 7.2, 2 mM ATP, 2 mM NADH, creatine phosphate (15 mg/ml), and creatine kinase (150 μg/ml), whereas mitochondria of *S. cerevisiae* were resuspended at the same concentration in 500 mM sorbitol, bovine serum albumin (30 mg/ml), 80 mM KCl, 10 mM Mg-acetate, 50 mM HEPES/KOH pH 7.2, 2 mM ATP, 2 mM NADH, creatine phosphate (15 mg/ml), and creatine kinase (150 μg/ml). Import reactions were supplemented with reticulocyte lysate (0.7%) containing [^{35}S]methionine-labeled precursor protein and incubated for 15 min at 25°C to allow import to occur. To stop protein import, samples were shifted to 4°C and the membrane potential across the inner membrane was dissipated by adding valinomycin (50 μM), antimycin A (800 μM), and oligomycin (2 mM). For control, the inhibitor mix was added before import when indicated ($-\Delta\psi$). Nonimported preproteins were digested with PK (50 μg/ml) for 15 min at 4°C. The protease was inhibited by adding 2 mM PMSF. Mitochondria were reisolated and analyzed by SDS-PAGE and autoradiography.

Proteolysis of Newly Imported Proteins

Protein import and protease digestion of nonimported preproteins was performed as described above. However, the membrane potential was not dissipated after completion of import to maintain high ATP levels in mitochondria. To examine the stability of newly imported proteins, mitochondria were resuspended after the protease treatment in import buffer at a concentration of 0.2 mg/ml and incubated at 37°C. At the time points indicated, aliquots were withdrawn and analyzed by SDS-PAGE and autoradiography.

Fluorescence Microscopy

Wild-type, Δ*yme1*, and Δ*yme1* cells expressing IAP-1* were transformed with the plasmid pVT100U-mtGFP, which allows expression under the control of the *ADH1* promotor of a hybrid protein composed of the mitochondrial targeting sequence of the F_O-ATPase subunit 9 of *N. crassa* fused to green fluorescent protein (GFP) (Westermann and Neupert, 2000). Cells were grown at 30°C overnight on agarose plates containing selective medium and glucose as carbon source. Colonies were harvested and immobilized with 0.5% (wt/vol) low melting point agarose on coverslips. Standard methods were used for fluorescence and phase contrast microscopy.

RESULTS

Identification of Two Genes, map-1 and iap-1, Encoding AAA Protease Subunits in N. crassa

Using the *YTA10* (*AFG3*) gene of *S. cerevisiae* as query sequence, available EST- and genomic databases of *N. crassa* were screened for genes encoding AAA proteases. One EST was identified to code for a sequence of 145 amino acid

residues homologous to Yta10 (amino acids 406–544 of Yta10). We isolated the corresponding full-length gene from a genomic DNA library. The gene encodes a polypeptide of 928 amino acid residues with a predicted molecular weight of 103 kDa (Figure 1). The coding sequence is interrupted by a 66-bp intron at position 1959 with respect to the translation start. The predicted polypeptide contains consensus motifs for Walker-type ATPases, Walker A and B, and a so-called second region of homology characteristic of AAA proteins (Swaffield *et al.*, 1992). Moreover, a consensus metal-binding motif, representing the proteolytic center in AAA proteases, is present carboxy terminal of the AAA domain identifying the protein as a putative AAA protease. Consistent with this, the polypeptide is highly homologous over the whole sequence to known AAA protease subunits in yeast and human. It is most closely related to Yta10 and Yta12 with sequence identities of 51.8 and 46.1%, respectively. For reasons outlined below, the protein was termed MAP-1 (for matrix AAA protease).

To identify additional homologs of this gene family in *N. crassa*, we used a PCR-based approach. Degenerate primers were designed that are complementary to sequences coding for consensus ATP- and metal-binding motifs in known AAA proteases. A 600-bp DNA fragment was amplified and used as a probe to identify the corresponding full-length gene. The gene, containing a 60-bp intron at position 1776 with respect to the translation start, codes for a polypeptide of 738 amino acid residues with a predicted molecular weight of 80 kDa, which shares 30.2% sequence identity with Yta10 (Figure 1). The presence of an AAA-domain as well as a putative proteolytic domain characterizes this protein as yet another member of the AAA protease family in *N. crassa*. Based on sequence comparisons, which revealed highest similarity to Yme1 of *S. cerevisiae* (45.9% sequence identity), and experiments described below we termed the protein IAP-1 (for intermembrane space AAA protease).

Both MAP-1 and IAP-1 Are Localized in Mitochondria

The amino terminal regions of MAP-1 and IAP-1 are enriched in positively charged and hydroxylated amino acids and lack negatively charged residues, both characteristics of mitochondrial targeting sequences. To examine a possible mitochondrial localization of both proteins, posttranslational protein import experiments were performed (Figure 2). MAP-1 and IAP-1 were synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine and incubated with energized mitochondria isolated from *N. crassa* hyphae. The radiolabeled proteins were efficiently imported into protease-protected locations (Figure 2). In either case, import was strictly dependent on a membrane potential and accompanied by proteolytic processing (Figure 2). Processing is probably catalyzed by the mitochondrial processing peptidase (MPP) in the matrix because potential MPP cleavage sites are present in the amino terminal regions of both proteins (Gavel and Heijne, 1990). Notably, removal of the amino terminal 33 amino acids of IAP-1 abrogated mitochondrial import, establishing the crucial role of the amino terminal region of this protein for mitochondrial targeting (Figure 2). These experiments demonstrate a mitochondrial localization of MAP-1 and IAP-1. In agreement with this conclusion, both proteins were identified in mitochondria by immunoblotting with specific polyclonal antisera (Figure 3).

Inverted Topology of MAP-1 and IAP-1 in the Mitochondrial Inner Membrane

Hydrophobic regions, which have the characteristics of transmembrane segments, are present in the N-terminal regions of MAP-1 and IAP-1, suggesting that both proteins, like AAA protease subunits in other organisms, are integral membrane proteins (Figure 3A). Consistently, they were found to be resistant toward alkaline extraction (Figure 3B).

Hydrophobicity blot analysis identified two hydrophobic amino acid stretches (amino acids 251–273 and amino acids 372–395) in MAP-1, whereas only one potential transmembrane segment (amino acids 208–227) is present in IAP-1 (Figure 3A). This finding points to differences in the membrane topology of both proteins. Therefore, mitochondria were subjected to digitonin fractionation in the presence of PK (Figure 3C). Fractions were analyzed by immunoblotting with polyclonal antisera directed against C-terminal parts of MAP-1 and IAP-1. Both proteins were resistant to PK in intact mitochondria (Figure 3C). This is in contrast to the mitochondrial outer membrane protein TOM22, which is clipped under these conditions (Figure 3C). On opening of the outer membrane in the presence of low concentrations of digitonin IAP-1 was degraded, as was the intermembrane space protein cytochrome *c* heme lyase (Figure 3C). In contrast, MAP-1 became accessible to PK only when membranes were completely solubilized (Figure 3C). It shared this behavior with the mitochondrial matrix protein MPP (Figure 3C). Taken together, we conclude from these experiments that both MAP-1 and IAP-1 are integral part of the inner membrane but expose large C-terminal domains with the catalytic sites to opposite membrane surfaces (Figure 3D). They thus attain a topology in the inner membrane similar to their yeast homologs Yta10, Yta12, and Yme1.

MAP-1 and IAP-1 Are Part of Two Independent Oligomeric Complexes in Inner Membrane

To determine the native molecular masses of MAP-1 and IAP-1, we performed gel filtration experiments after solubilization of mitochondrial membranes with Triton X-100 (Figure 4). Eluate fractions were analyzed by immunoblotting with the use of MAP-1- and IAP-1-specific antisera. In the presence of ATP, MAP-1 was detected in fractions corresponding to a molecular weight of ~1 MDa, demonstrating that it is part of a large oligomeric structure in the inner membrane (Figure 4A). The formation of this complex was ATP-dependent. MAP-1 was recovered exclusively as a 300-kDa complex if mitochondria were solubilized in the absence of ATP (Figure 4A). IAP-1, on the other hand, eluted in two peaks from the column in the presence of ATP, which corresponded to molecular weights >1 MDa and of ~300 kDa (Figure 4B). It is thus part of an oligomeric structure that has a similar molecular mass as the MAP-1–containing complex. Modulating the ATP-levels in mitochondria, however, provided the first evidence that MAP-1 and IAP-1 form two independent complexes in the inner membrane. At low ATP levels, a decreased but significant portion of IAP-1 eluted from the column in fractions corresponding to a molecular weight >1 MDa, whereas the large MAP-1 com-

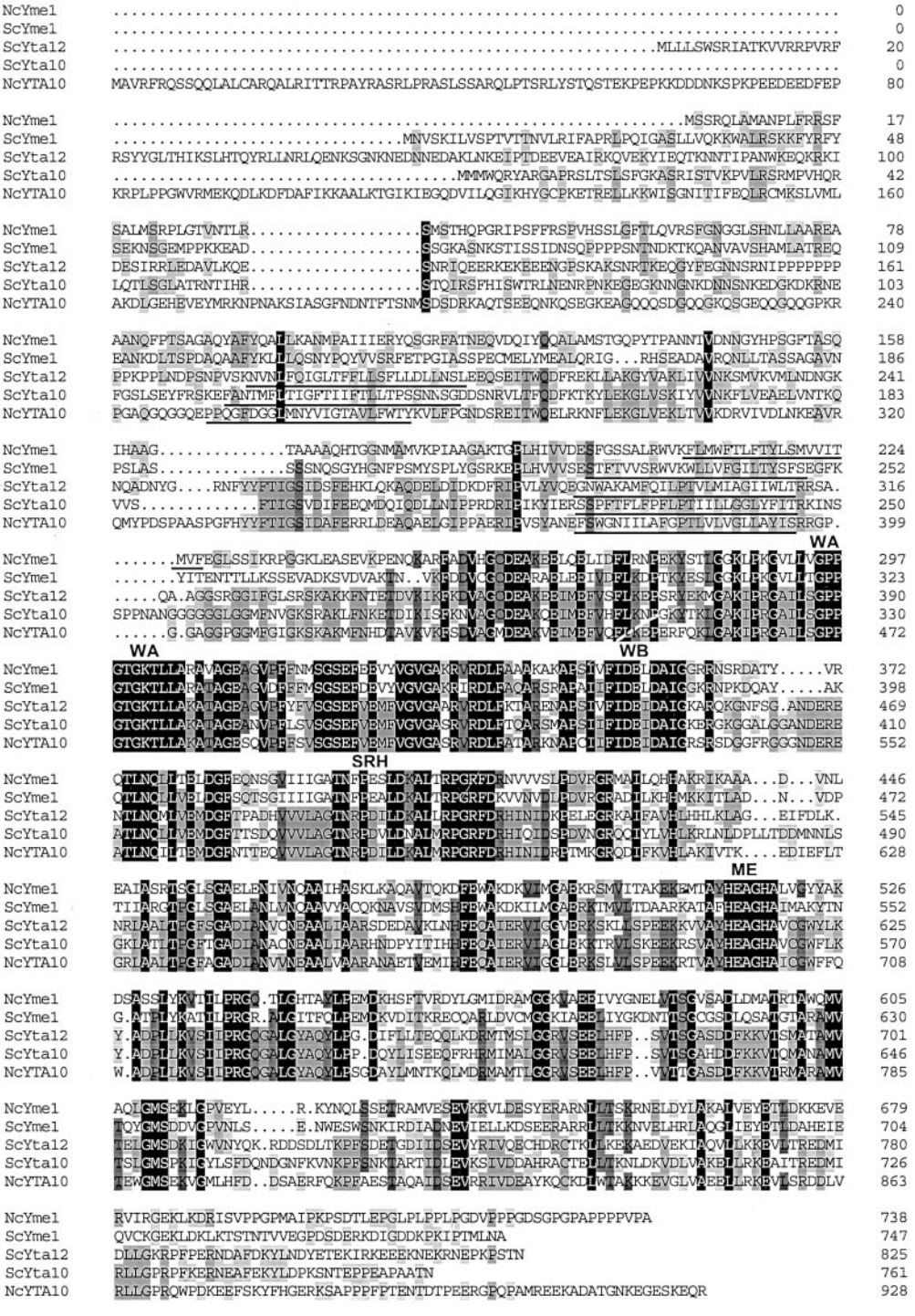

Figure 1. Sequence alignment of AAA protease subunits of *N. crassa* and *S. cerevisiae*. The sequences of IAP-1 (gb: AF323913), MAP-1 (gb: AF323912), Yme1 (gb: 809589), Yta10 (gb: 603609) and Yta12 (gb: 807972) were aligned with the use of the program ClustalW (version 1.8, gap opening penalty, 10; gap extension penalty, 0.05). Identical amino acid residues in all five sequences are printed white on black. Amino acid residues that are conserved in four or three sequences only are shaded in gray. Walker A- (WA) and Walker B-boxes (WB), the second region of homology (SRH), characteristic of the AAA family of ATPases, and consensus metal binding motifs (ME), representing the putative catalytic centers, are indicated. Predicted transmembrane domains are underlined.

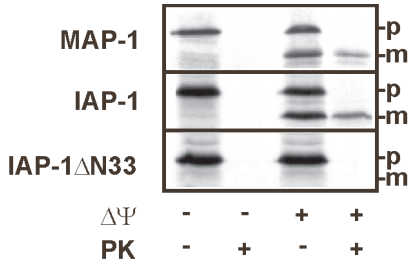

Figure 2. Import of MAP-1 and IAP-1 into *N. crassa* mitochondria. Mitochondria isolated from a *N. crassa* wild-type strain were incubated with radiolabeled precursor proteins for 15 min at 25°C in the presence or absence of a membrane potential across the inner membrane ($+\Delta\Psi$, $-\Delta\Psi$). Nonimported preproteins were digested with PK (50 μg/ml) when indicated. Mitochondria were analyzed by SDS-PAGE and autoradiography. p, precursor protein; m, mature protein.

plex was completely dissociated under these conditions (Figure 4).

To unambiguously demonstrate that MAP-1 and IAP-1 are part of two independent high molecular mass structures in the inner membrane, we generated an *N. crassa* strain lacking IAP-1 and examined the native molecular mass of MAP-1 in this strain. The *iap-1* gene was mutated by repeat-induced point mutation (RIP), a process occurring in the sexual cycle of *N. crassa* (Selker, 1990). Duplicated DNA sequences present in either of the nuclei of the mating pair are mutated by a variable number of GC-to-AT transitions. For RIP mutagenesis, a wild-type *N. crassa* strain was transformed with a hygromycin-resistance–conferring plasmid encoding the *iap-1* gene. Homokaryotic microconidia from the resulting strain containing two copies of the *iap-1* gene were mated with an isogenic wild-type strain. Ascospores were then isolated, germinated, and examined for the absence of IAP-1 by immunoblotting. The mutated *iap-1*^{RIP} allele of a mutant strain was cloned and the nucleotide sequence was determined. Several transitions characteristic of RIP mutagenesis were identified. One of them results in a premature stop after 249 amino acid residues of the IAP-1 precursor protein and thereby in a truncated IAP-1 protein that lacks both putative catalytic domains.

Mitochondria were isolated from the *iap-1*^{RIP} mutant strain, solubilized with Triton X-100, and subjected to gel filtration analysis, to determine the native molecular mass of MAP-1. Similar to our findings with the use of wild-type mitochondria, MAP-1 eluted in two peaks from the column, corresponding to molecular weights of ~1 MDa and 300 kDa (Figure 4C). Thus, the large MAP-1–containing complex does not contain IAP-1, demonstrating that both proteins form two independent complexes in the inner membrane.

Mutation of iap-1 Causes a Temperature-sensitive Growth Defect

For a further functional characterization of IAP-1, we examined the growth rate of the *iap1*^{RIP} mutant strain by measuring the linear hyphal extension rates (Figure 5A). Glass tubes were inoculated at one end of the tube with mycelia from the wild-type strain and the *iap-1*^{RIP} mutant strain. Cell growth on media containing various carbon sources was examined at different temperatures. On glucose-containing media, mutation of *iap-1* did not impair growth at any temperature (data not shown). If the medium contained glycerol or acetate instead of glucose, however, hyphae lacking IAP-1 exhibited a slow growth phenotype at 40°C but not at 12 or 20°C (Figure 5A; not shown).

To ensure that the observed growth defect is caused by alterations in the *iap-1* gene, the *iap-1*^{RIP} mutant strain was transformed with a plasmid conferring hygromycin-resistance and encoding wild-type IAP-1. Transformants were isolated and shown to contain wild-type levels of IAP-1 by immunoblotting. They displayed normal growth rates at 40°C on media containing glycerol or acetate (Figure 5A; not shown). We conclude that the observed temperature-sensitive growth defect upon RIP mutagenesis is specific for the *iap-1* gene and is solely due to the absence of the intermembrane space AAA protease.

Cells Lacking IAP-1 Exhibit Defects in Proteolysis of Misfolded Membrane Proteins but not in Mitochondrial Morphology

The presence of a consensus metal binding motif, HEXXH, characteristic of metal-dependent proteases, suggests a proteolytic function of IAP-1. We therefore examined in further experiments a potential role of IAP-1 for the proteolytic breakdown of mitochondrial inner membrane proteins. Truncated variants of *S. cerevisiae* Yme2 were used as model proteins that have been previously observed to be thermolabile in yeast (Leonhard *et al.*, 2000). Yme2 spans the inner membrane once and contains solvent-exposed domains at both sides of the membrane (Hanekamp and Thorsness, 1996). Whereas Yme2ΔC23 has only a large domain protruding into the matrix space, Yme2ΔN33 lacks the matrix domain and exposes a large domain to the intermembrane space only.

Yme2ΔN33 and Yme2ΔC23 were synthesized in reticulocyte lysate in the presence of [^{35}S]methionine and imported posttranslationally into mitochondria isolated from *N. crassa* wild-type and *iap-1*^{RIP} mutant strains. Both proteins were rapidly degraded in wild-type mitochondria upon further incubation at 37°C after completion of import (Figure 5, B and C). In *iap-1*^{RIP} mutant mitochondria, however, Yme2ΔN33 was almost completely stabilized, whereas proteolysis of Yme2ΔC23 preceded with similar kinetics as in wild-type mitochondria (Figure 5, B and C). These experiments demonstrate that IAP-1 is required for the efficient proteolytic breakdown of a misfolded inner membrane protein in *N. crassa*. However, IAP-1 appears to degrade only membrane proteins that contain solvent-exposed domains in the intermembrane space.

In *S. cerevisiae*, inactivation of Yme1 has been found to impair the morphology of mitochondria (Campbell *et al.*, 1994). This finding prompted us to investigate the mitochondrial morphology in *N. crassa* hyphae lacking IAP-1. Hyphae of the wild-type and the *iap-1*^{RIP} mutant strain were stained with the mitochondrion-specific dyes $DiOC_6$ and Rhodamine B hexyl ester (Prokisch *et al.*, 2000). We did not detect any alteration in the *iap-1*^{RIP} mutant in the distribution or morphology of mitochondria in hyphal tips or in regions of hyphal cells distant from the tip. Thus, IAP-1 is not required to maintain normal mitochondrial morphology in *N. crassa*.

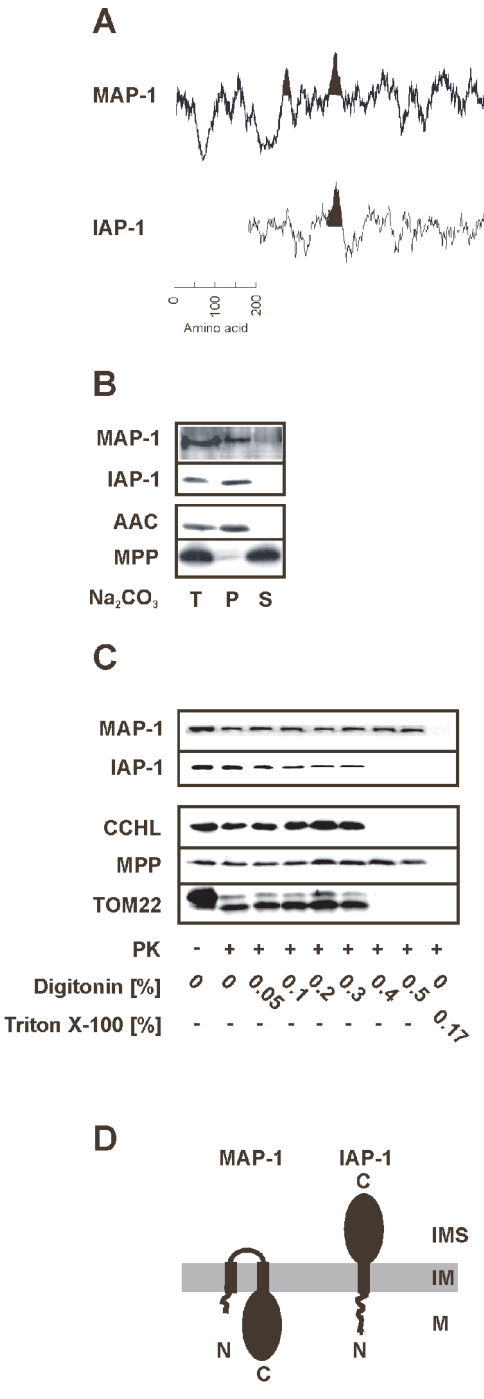

Figure 3. Submitochondrial localization of MAP-1 and IAP-1. (A) Hydrophobicity profiles of MAP-1 and IAP-1. MAP-1 and IAP-1 were analyzed with the use of the DNAMAN software (Lynnon BioSoft) (window size 15 amino acids). Hydrophobic regions that represent predicted transmembrane domains are colored in black. (B) Alkaline extraction of mitochondrial membranes. *N. crassa* mitochondria (100 μg) were resuspended in 0.1 M Na_2CO_3 at a concentration of 100 μg/ml and incubated for 30 min on ice. An aliquot was removed for control (T). Samples were then centrifuged for 30 min at 226,000 $\times$ *g*, split into pellet and supernatant fractions, and analyzed by SDS-PAGE and immunostaining with the use of antisera directed against MAP-1 and IAP-1. ATP/ADP carrier (AAC) and MPP were used as marker proteins for membrane and soluble fractions, respectively. (C) Digitonin fractionation of mitochondria. Mitochondria were incubated in the presence of increasing concentrations of digitonin or Triton X-100 as indicated and accessibility to externally added PK was examined as described in MATERIALS AND METHODS. Fractions were analyzed by SDS-PAGE and immunostaining with MAP-1– and IAP-1–specific antisera. The following marker proteins were used: the outer membrane protein TOM22, the intermembrane space protein cytochrome *c* heme lyase and matrix-localized MPP. (D) Model for the topology of MAP-1 and IAP-1 in the mitochondrial inner membrane of *N. crassa*. N, N terminus; C, C terminus; IMS, intermembrane space; IM, inner membrane; M, matrix.

IAP-1 Can Partially Substitute for Yme1 in S. cerevisiae

To examine whether IAP-1 is a functional ortholog of Yme1, we performed a complementation analysis in *S. cerevisiae*. In a first step, we analyzed the ability of the mitochondrial targeting sequence of IAP-1 to ensure the import of the protein into isolated yeast mitochondria. Radiolabeled IAP-1 and Yme1 were incubated with energized yeast mitochondria to allow posttranslational protein import. Although Yme1 was efficiently imported, only low levels of IAP-1 accumulated in its mature form within yeast mitochondria under these conditions, indicating inefficient import (Figure 6A). Therefore, a hybrid of IAP-1 and Yme1 was constructed. The first 49 amino acid residues of Yme1 containing its mitochondrial targeting sequence (amino acid residues 1–47) and two amino acid residues of mature Yme1 were fused to the predicted mature form of IAP-1 (amino acid residues 62–738). The resulting hybrid protein (IAP-1*) was radiolabeled and efficiently imported into yeast mitochondria when incubated with isolated organelles (Figure 6A). Import depended on a membrane potential across the inner membrane and was accompanied by proteolytic removal of the mitochondrial targeting sequence derived from Yme1 (Figure 6A). Newly imported IAP-1* was inserted into the inner membrane and attained a topology identical to Yme1 as indicated by subfractionation of mitochondria. These experiments demonstrate that the presequence of Yme1 is sufficient to ensure mitochondrial targeting of mature IAP-1.

For expression in yeast, we cloned the corresponding hybrid gene and the promotor of *YME1* into a centromer-based yeast expression vector and transformed the obtained plasmid into a Δ*yme1* strain of *S. cerevisiae*. Expression and mitochondrial targeting of IAP-1* was verified by immunoblotting of cell extracts. Yeast cells lacking *YME1* exhibit pleiotropic phenotypes, including impaired growth on nonfermentable carbon sources at 37°C, on glucose-containing media at 15°C, or in the absence of mitochondrial DNA (Figure 6, B–D) (Thorsness *et al.*, 1993). Expression of IAP-1* completely complemented the cold-sensitive growth defect of Δ*yme1* mutant cells (Figure 6B) and allowed growth of Δ*yme1* cells lacking mitochondrial DNA (Figure 6C), indicating functional conservation of both proteins.

However, we did not observe growth of Δ*yme1* cells expressing IAP-1* on nonfermentable carbon sources at 37°C (Figure 6D). It is conceivable that the level of IAP-1* is limiting for growth under these conditions. Therefore, the hybrid protein was expressed in Δ*yme1* cells from the con-

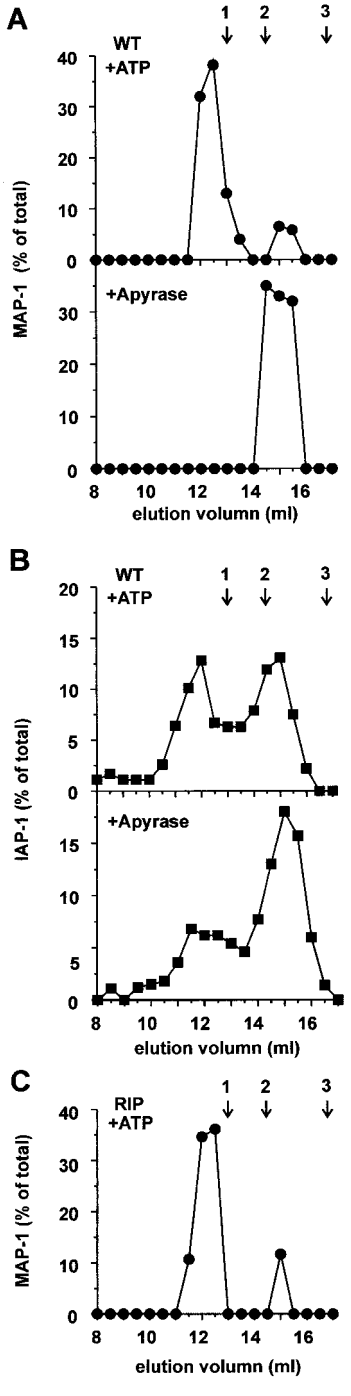

Figure 4. MAP-1 and IAP-1 form two independent high molecular mass complexes in the inner membrane of *N. crassa* mitochondria. Wild-type (A and B) and *iap-1*^{RIP} (C) mitochondria were solubilized in buffer A supplemented with apyrase (+apyrase) or ATP (ATP) as described in MATERIALS AND METHODS and subjected to Superose 6 gel chromatography. MAP-1 (A and C) and IAP-1 (B) were detected in eluate fractions by Western blot analysis. HSP60 (1; 840 kDa), apoferritin (2; 440 kDa), and alcohol dehydrogenase (3; 150 kDa) were used as standards for calibration.

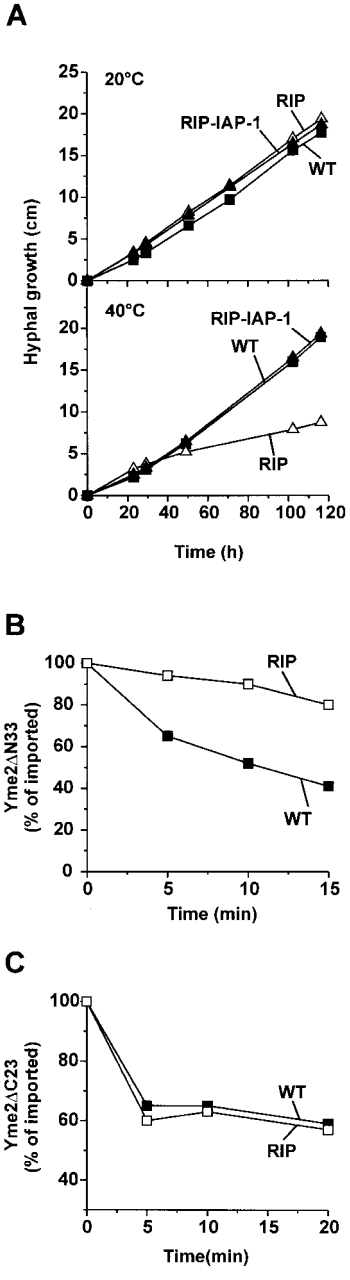

Figure 5. Inactivation of IAP-1 causes a slow growth phenotype and proteolysis defects. (A) Slow growth of *iap-1*^{RIP} hyphae under heat stress. Race tubes containing solid acetate medium were inoculated at one end of the tube with mycelia from wild-type (WT), *iap-1*^{RIP} (RIP), and *iap-1*^{RIP}*-iap-1* (RIP-IAP-1) cells and incubated at 20 or 40°C for the time points indicated. The growth distance along the agar surface was determined. Similar results were obtained on glycerol-containing medium. (B and C) Stability of inner membrane proteins in *iap-1*^{RIP} mutant mitochondria. Radiolabeled Yme2ΔN33 (B) or Yme2ΔC23 (C) were imported into mitochondria isolated from the WT or RIP cells. After protease digestion of nonimported precursor proteins mitochondria were incubated at 37°C for the time points indicated to allow proteolysis to occur. Samples were analyzed by SDS-PAGE and autoradiography. Yme2ΔN33 and Yme2ΔC23 present in mitochondria were quantified by laser densitometry and are given as percentage of total imported material.

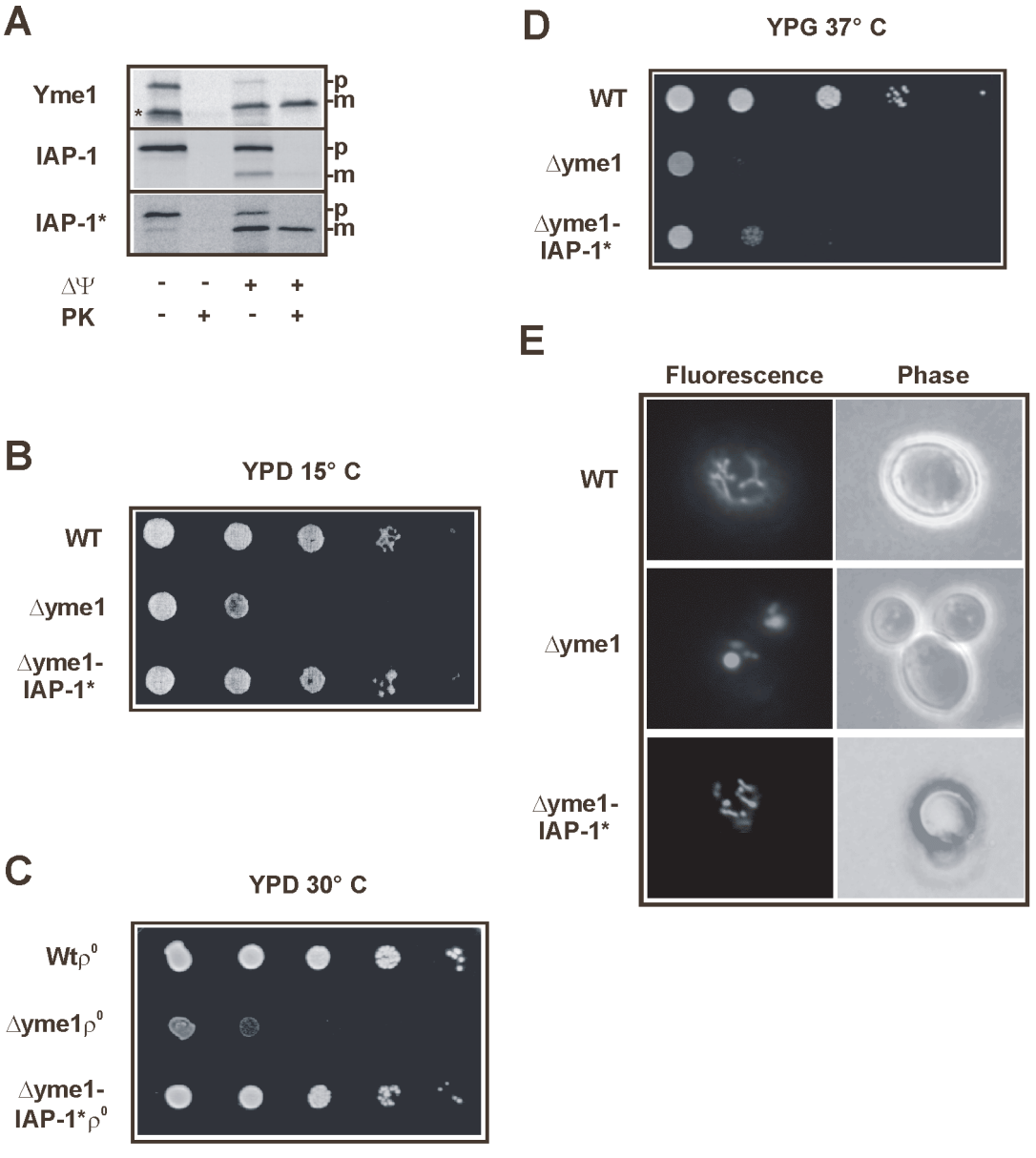

Figure 6. Substitution of Yme1 by IAP-1 in *S. cerevisiae*. (A) Import of *S. cerevisiae* Yme1 and *N. crassa* IAP-1 into isolated yeast mitochondria. Radiolabeled Yme1, IAP-1, and IAP-1* were incubated for 15 min at 25°C with yeast mitochondria in the presence or absence of a membrane potential across the inner membrane (+ΔΨ, −ΔΨ). After digestion of nonimported preproteins with PK, mitochondria were analyzed by SDS-PAGE and autoradiography. An N-terminally truncated variant of Yme1, which was synthesized in vitro from an internal start site and which migrates slightly faster than the mature form of Yme1, is marked with an asterisk. p, precursor protein; m, mature protein. (B–D) Partial suppression of growth deficiencies of Δ*yme1* yeast cells upon expression of IAP-1*. Wild-type (WT), Δ*yme1*, and Δ*yme1* cells expressing IAP-1* (Δyme-IAP-1*) were grown on YPD medium at 30°C. Tenfold serial dilutions of logarithmically growing cultures were spotted onto YP medium containing 2% glucose or 3% glycerol and incubated at 15°C (B) or 37°C (D). (C) ρ° derivatives of the yeast strains were spotted onto YPD plates as described above and grown at 30°C. (E) Mitochondrial morphology in yeast Δ*yme1* cells expressing IAP-1*. Mitochondrially targeted GFP was expressed in WT, Δ*yme1*, and Δyme-IAP-1* cells. Cells logarithmically growing on galactose-containing medium at 30°C were analyzed by fluorescence and phase contrast microscopy.

stitutive *ADH1* promotor with the use of a multicopy yeast expression vector. Although IAP-1* levels were 20-fold increased in these cells, their temperature-sensitive growth defect on glycerol-containing medium was not restored (not shown), pointing to functional differences between both proteins.

Yeast cells lacking Yme1 accumulate aberrant mitochondria that do not form a reticulated network but that are

punctate and grossly swollen in the absence of functional Yme1 (Figure 6E) (Campbell *et al.*, 1994). One or two large, spherical mitochondria and only few small organelles were detected in Δ*yme1* cells upon expression of mitochondrially targeted GFP (Figure 6E). Expression of IAP-1* significantly altered the mitochondrial morphology of these cells (Figure 6E). We did not observe giant mitochondria in Δ*yme1* cells containing IAP-1* but rather a large number of smaller mitochondria with an extended structure (Figure 6E). However, a reticulated mitochondrial network as found in wild-type cells was not formed in these cells (Figure 6E). We conclude from these observations that IAP-1* can at least partially substitute for Yme1 in maintaining normal mitochondrial morphology.

DISCUSSION

In this article, we report on the identification of two novel members of the AAA protease family in the filamentous fungus *N. crassa*, which we termed MAP-1 and IAP-1. The biochemical characterization of both proteins revealed striking similarities with the homologous proteins Yta10, Yta12, and Yme1 of *S. cerevisiae*, which represent the only eukaryotic family members characterized so far. MAP-1 and IAP-1 are part of two large protein complexes in the mitochondrial inner membrane whose assembly is nucleotide-dependent. Similar-sized proteolytic complexes are present in the mitochondrial inner membrane of yeast: the *m*-AAA protease composed of Yta10 and Yta12 subunits, which assemble in an ATP-dependent manner, and the *i*-AAA protease containing Yme1 (Arlt *et al.*, 1996, Leonhard *et al.*, 1996). Moreover, the striking membrane topology of yeast AAA proteases is conserved in *N. crassa*. Whereas IAP-1, similar to Yme1, is active in the intermembrane space, the catalytic domains of MAP-1 are exposed to the matrix, as is the case for Yta10 and Yta12. We therefore propose to name the MAP-1– and IAP-1–containing proteolytic complexes *m*- and *i*-AAA proteases, with the prefices indicating the presence of the catalytic sites in the matrix or intermembrane space, respectively.

Hydropathy analysis of IAP-1 and MAP-1 suggests the presence of one and two transmembrane domains, respectively, indicating that also the number of membrane spanning segments is conserved between *N. crassa* and yeast. Notably, potential AAA protease subunits with one or two potential transmembrane segments can be identified by screening human and *Drosophila* databases (our unpublished observation). In view of our present findings, it appears very likely that high molecular weight AAA proteases, which are active on opposite sides of the inner membrane, exist also in mitochondria of these organisms. It remains to be determined whether these proteolytic complexes are homo-oligomers or, as found for the *m*-AAA protease of yeast, are made up of closely related subunits. In *N. crassa*, IAP-1 and MAP-1 appear to form homo-oligomeric complexes because genes coding for other homologous proteins were not identified in the recently completed genome sequence of *N. crassa* (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/).

Disruption of the *iap-1* gene by repeat-induced point mutation provided first insights into the function of the *i*-AAA protease in mitochondria of *N. crassa*. IAP-1 is required for the degradation of misfolded membrane proteins and thus ensures the quality control of mitochondrial inner membrane proteins. The topology of a substrate protein appears to determine the involvement of *m*- and *i*-AAA proteases in degradation. A model substrate protein exposing a domain to the intermembrane space was efficiently degraded by the *i*-AAA protease, whereas the protease was not required for the proteolytic breakdown of a membrane protein with a large domain in the mitochondrial matrix only. A similar restriction in the ability of mitochondrial AAA proteases to recognize membrane-embedded substrate proteins was also evident in studies in yeast (Leonhard *et al.*, 2000). It is therefore an attractive hypothesis that the apparent evolutionary conservation of mitochondrial AAA proteases with catalytic sites on opposite sides of the membrane can be rationalized by the need to recognize and degrade membrane proteins with different topologies.

A comparison of deficiencies of yeast cells or *N. crassa* hyphae lacking the *i*-AAA protease reveals some parallels but, at the same time, striking differences between both organisms. In the absence of the *i*-AAA protease, growth of both organisms is impaired on nonfermentable carbon sources at high temperature, indicating an important role of the protease for mitochondrial functions under these conditions. On the other hand, *yme1*-deficient yeast cells exhibit a cold-sensitive growth phenotype (Thorsness *et al.*, 1993), which was not detected in *N. crassa*. Moreover, mitochondrial morphology is not affected in *iap-1*^{RIP} hyphae but impaired in *yme1* cells (Campbell *et al.*, 1994), pointing to differences between both fungi in the machinery maintaining the morphology of the organelle or in its regulation (Prokisch *et al.*, 2000). It therefore appears that general effects of an inactivation of the *i*-AAA protease on mitochondrial functions must be distinguished from phenotypes specific for the respective organism.

Our complementation analysis in *S. cerevisiae* established the functional conservation of *i*-AAA proteases and thereby demonstrated an overlapping substrate specificity of *N. crassa* IAP-1 and yeast Yme1. Interestingly, IAP-1 can suppress phenotypes of *yme1*-deficient cells that were not observed in *N. crassa* hyphae lacking *i*-AAA protease. The cold-sensitive growth defect of Δ*yme1* yeast cells was complemented upon expression of IAP-1. This demonstrates the proteolytic activity of IAP-1 in yeast mitochondria because the cold-sensitive phenotype was also observed in yeast cells harboring a proteolytically inactive variant of Yme1 and thus is associated with the loss of its proteolytic function (Thorsness *et al.*, 1993). Moreover, the morphology of mitochondria was at least partially restored in Δ*yme1* yeast cells containing IAP-1. These findings point to a rather degenerate substrate specificity of *i*-AAA proteases. It is conceivable that chaperone-like properties of the AAA domain (Leonhard *et al.*, 1999) enable AAA proteases to recognize and degrade a broad range of substrate polypeptides.

Interestingly, IAP-1 did not entirely substitute for functions of Yme1 in yeast mitochondria. At 37°C, the respiratory competence of Δ*yme1* yeast cells was not maintained in the presence of IAP-1. Moreover, IAP-1* was unable to promote degradation of nonassembled Cox2, a known substrate of Yme1, when expressed in yeast Δ*yme1*Δ*cox4* cells (Klanner and Langer, unpublished observation). It remains to be determined whether these observations reflect differences in

substrate selection, a low-specific activity of IAP-1 in the heterologous environment, or an impaired interaction between the *i*-AAA protease and other components of the quality control system in the inner membrane. Because overexpression of IAP-1 did not restore respiratory growth of Δ*yme1* cells at 37°C, it appears that differences in the function of *i*-AAA proteases in mitochondria of various organisms must be considered.

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