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Mcx1p, a ClpX homologue in mitochondria of *Saccharomyces cerevisiae*

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Abstract Members of the Hsp100/Clp-family of molecular chaperones form regulatory subunits of ATP-dependent Clp proteases and fulfill crucial roles for cellular thermotolerance. We have identified a Clp-like protein in *Saccharomyces cerevisiae*, Mcx1p, which shares approximately 30% sequence identity with ClpX-proteins in bacteria, plants and nematodes. Mcx1p localizes to the matrix space of mitochondria and is peripherally associated with the inner membrane. A homologue of *E. coli* ClpP protease was not identified when screening the yeast genome. We therefore propose that Mcx1p represents a novel molecular chaperone of mitochondria with non-proteolytic function.

Key words: ClpX; Hsp100; Molecular chaperone; Mitochondrion

1. Introduction

HSP100/Clp proteins comprise a ubiquitous family of molecular chaperones implicated in cellular processes as different as tolerance to stress, DNA replication, gene expression and the inheritance of prion-like factors [1,2]. Members of this family contain one or two copies of conserved Walker-type ATPase domains and are classified in subfamilies according to their structural organization and sequence similarities [1,3]. HSP100/Clp proteins form ring-like hexameric structures reminiscent of those of chaperonins [4,5].

Two members of the HSP100/Clp protein family which both belong to the ClpB subfamily have been identified in the yeast *Saccharomyces cerevisiae*. Hsp104 is located in the cytosol and is required for cellular thermotolerance [6]. It mediates the dissociation of protein aggregates formed under heat stress [7]. In addition, Hsp104 controls the inheritance of a prion-like factor [8]. The mitochondrial Hsp78 confers compartment-specific thermotolerance and is crucial for the maintenance of respiratory function under severe temperature stress [9]. Hsp78 expressed in the cytosol can substitute for Hsp104 in mediating cellular thermotolerance which suggests a conserved mode of action of both proteins [9].

ClpA and ClpX proteins form ATP-dependent, regulatory subunits of Clp proteases of *Escherichia coli* [10–12]. They associate with the structurally unrelated ClpP serine protease to form so-called self-compartmentalizing proteases which display striking structural resemblance to the eukaryotic 26S proteasome [13]. The ClpAP protease of *E. coli* is required for the proteolysis of N-end rule substrates whilst the ClpXP protease degrades starvation sigma factor and phage DNA replication components [14–16]. The ATP-dependent chaperone activity of ClpA and ClpX proteins determines the substrate specificity of the protease but may also promote unfolding of substrate polypeptides allowing their degradation [3,17]. In addition, chaperone activity independent of ClpP has been demonstrated in vivo for ClpX. In the absence of ClpP, ClpX protects the bacteriophage λO replication protein from heat aggregation and mediates the dissociation of λO protein aggregates [18]. ClpX is also involved in the disassembly of the Mu transposase-DNA complex, a prerequisite for the initiation of Mu phage DNA replication [19]. ClpX has been demonstrated to recognize C-terminal segments of some target proteins [19,20]. Its binding specificity is mediated by domains conserved within the HSP100/Clp protein family which have been proposed to be distantly related to PDZ domains, a highly degenerated 80–100-residues repeated motif mediating C-terminal protein-protein interactions [21].

We now report the identification of a third Clp protein in yeast, which we termed Mcx1p. Mcx1p was localized to the matrix space of mitochondria. As ClpP-like proteins are absent in the complete sequence of the yeast genome, Mcx1p may represent a molecular chaperone protein with non-proteolytic function in mitochondria.

2. Materials and methods

2.1. Yeast strains and genetic analysis

Yeast strains used in this study are derivatives of YPH500 [22]. Cells were grown on YEP medium (1% yeast extract, 2% peptone) or on minimal medium (0.7% yeast nitrogen base containing ammonium sulfate) that was supplemented with the auxotrophic requirements and contained glucose (2%) or glycerol (3%) as the sole carbon source.

Yeast transformation [23] and genetic analysis [24] were carried out according to published procedures. Gene disruption was performed employing the one-step method described by Rothstein [25].

2.2. Plasmids

Standard DNA manipulations were carried out as described [26]. pMCX1-4KB, which consists of a 4-kb *Eco*RI fragment containing the entire *MCX1* gene inserted in plasmid pGEM7Zf(+) (Promega), was kindly provided by B. Scherens (VUB, Brussels). pMCX1-EXO10 was originally made for sequencing by exonuclease treatment of pMCX1-4KB. It contains a 2.3-kb fragment starting 10 bp before the initiator ATG of *MCX1*. pMCX1-EXO10-CLA1 was constructed by *Cla*I digestion and religation of pMCX1-EXO10.

The disruption plasmid pMCX1-LEU2 was constructed by replacing an *MCX1*-internal 1.0-kb *Hin*dII fragment of pMCX1-EXO10-CLA1 with a 2.2-kb *Hpa*I fragment from Yep13 containing the *LEU2* gene. An *Apa*I-*Bam*HI 3.1-kb linear fragment was used to disrupt the *MCX1* gene.

To allow in vitro transcription, *MCX1* was cloned into pGEM3 (Promega) under the control of the SP6 promoter. For that purpose, pMCX1-EXO10 was digested with *Apa*I, blunt-ended with T4 DNA polymerase, and digested with *Bam*HI. The resulting fragment was cloned in the *Hin*dII and *Bam*HI sites of the vector producing pGEM-MCX1.

2.3. Import of Mcx1p into isolated mitochondria

Mcx1p precursor protein was synthesized by in vitro transcription

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and translation in the presence of [^{35}S]methionine in rabbit reticulocyte lysate (Promega) according to published procedures [27]. Import of precursor protein in isolated mitochondria was performed as described [28]. Non-imported preproteins were digested with proteinase K (50 μg/ml) for 30 min at 4°C. The protease treatment was stopped by the addition of 1 mM PMSF.

2.4. Miscellaneous

The following procedures were carried out as previously described: subfractionation of yeast cells [29]; isolation of mitochondria [30,31]; generation of mitoplasts [32]; carbonate extraction [33]; sonication of mitochondria [34]; determination of protein concentration, immunoblotting and immunostaining using the ECL chemiluminescence detection system (Amersham) [35].

Antibodies directed against Mcx1p were raised in rabbits by injecting the chemically synthesized C-terminal peptide CRDVSEEDKKL which had been coupled to activated KLH (Pierce, IL, USA).

3. Results

3.1. YBR227c encodes Mcx1p, a new member of the ClpX family

A screen of the yeast genome database led to the identification of the open reading frame YBR227c (EMBL accession number: Z36096), whose product displays striking homologies to prokaryotic and eukaryotic members of the ClpX family of ATP-dependent chaperone proteins (Fig. 1). On the basis of the subsequently elucidated cellular location of its product, YBR227c was designated *MCX1* (*M*itochondrial *C*lp*X*).

MCX1 codes for a 520 amino acid polypeptide, Mcx1p, with a predicted molecular mass of 57.9 kDa. Mcx1p contains a characteristic P-loop ATPase domain and shares 33.6%, 28.9% and 29.5% of identical residues with homologous proteins of *Escherichia coli*, *Caenorhabditis elegans*, and *Arabidopsis thaliana*, respectively (Fig. 1). The sequence similarity is highest within the ATPase domain and in the C-terminal third. The latter region of *E. coli* ClpX has recently been shown to mediate substrate binding [21]. The greatest sequence divergency, on the other hand, is found at the very N- and C-termini of the proteins: the N-terminal region of Mcx1p has characteristics of mitochondrial targeting sequences. It contains positively but not negatively charged amino acid residues and the predicted potential to form an amphipathic helix. Otherwise, the N-terminus of Mcx1p protein is shorter than in its prokaryotic and eukaryotic homologues and does not contain the C4-type zinc finger motif predicted in *E. coli* and *C. elegans* ClpX sequences (Fig. 1). Moreover, Mcx1p differs from other ClpX-homologues by the presence of additional amino acid residues at the C-terminus.

3.2. Subcellular localization of Mcx1p

To determine the subcellular location of Mcx1p, yeast cells were fractionated and microsomal, cytosolic, and mitochondrial fractions were analyzed by immunoblotting. Like the mitochondrial matrix protein Mge1p, Mcx1p was exclusively detected in the mitochondrial fraction using an Mcx1p-specific polyclonal antiserum (Fig. 2A). In contrast, Bmh2p was enriched in the cytosolic fraction while Sec61p was found associated with microsomes (Fig. 2A). To confirm its mitochon-

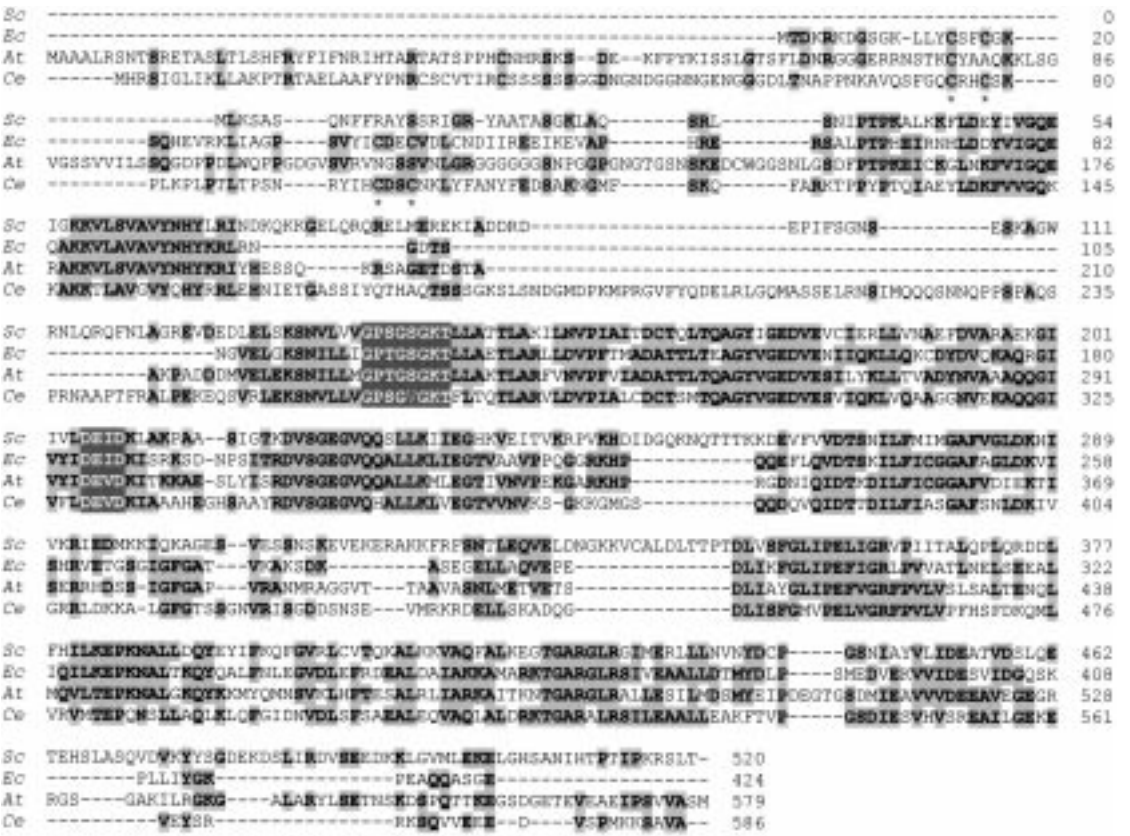

Fig. 1. Sequence alignment of ClpX homologues. Amino acid sequence of Mcx1p (*Sc*; EMBL Z36096) was aligned with ClpX sequences from *E. coli* (*Ec*; EMBL Z23278), the nematode *C. elegans* (*Cs*; EMBL Z73906) and the plant *A. thaliana* (*At*; PID G2674203) using the ClustalW 1.7 program. Identical residues are highlighted with a grey background. Walker A (P-loop) and B motifs of the ATP-binding domains are written in white and boxed. The four cysteine residues of the putative zinc-finger domain of *E. coli* ClpX are marked with an asterisk.

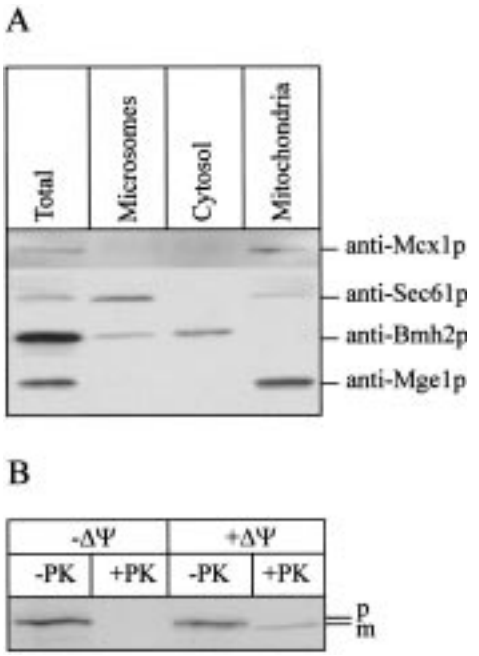

Fig. 2. Mcx1p is a mitochondrial protein. A: Subcellular localization. Cell extracts were split into microsomal (Microsomes), cytosolic (Cytosol), and mitochondrial (Mitochondria) fractions. In addition, cells (0.25 optical density units) were extracted by alkaline lysis (Total). Fractions were separated by SDS-PAGE and analyzed by Western blotting using polyclonal antibodies directed against Sec61p, Bmh2p, Mge1p and Mcx1p. Sec61p, Bmh2p and Mge1p are markers for microsomes [43], cytosol [44], and mitochondria [45], respectively. B: Import of in vitro synthesized Mcx1p precursor into isolated mitochondria. Mcx1p precursor proteins were imported for 10 min at 25°C in the presence (+ΔΨ) or in the absence (−ΔΨ) of membrane potential. Non-imported precursors were digested by incubation with proteinase K (+PK).

drial location, Mcx1p was synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine and incubated with isolated mitochondria. Mcx1p was rapidly imported into mitochondria in a membrane potential-dependent manner (Fig. 2B). As indicated by the reduced molecular weight of the imported Mcx1p species, a mitochondrial targeting sequence was cleaved off upon import. Potential cleavage sites for the mitochondrial processing peptidase can be predicted after residues 12, 20, or 33 in the Mcx1p sequence.

3.3. Submitochondrial location of Mcx1p

Hydropathy profile programs predict a transmembrane topology for Mcx1p. To examine this possibility, we analyzed the localization of Mcx1p within mitochondria (Fig. 3A). Mcx1p was protected against proteinase K treatment in mitochondria as well as in mitoplasts after osmotic disruption of the outer membrane (Fig. 3A). Solubilization of mitochondrial membranes with detergents resulted in the degradation of Mcx1p (Fig. 3A) suggesting that the protein is located either in the inner membrane or in the matrix space. Most of Mcx1p was recovered in the supernatant fraction upon alkaline extraction of mitochondrial proteins indicating that Mcx1p is not an integral part of the inner membrane.

In further experiments, mitochondria were disrupted by sonication in the presence of increasing concentrations of KCl (Fig. 3B). Soluble proteins are recovered in the supernatant fraction under these conditions even when sonication is performed in the absence of salt. Peripheral membrane proteins are recovered in the membrane fraction but are released from the membrane with increasing salt concentrations, while integral membrane proteins remain in the pellet fraction independent of the salt concentration. In these experiments Mcx1p was exclusively detected in the pellet fraction even when sonication was performed in the presence of 1 M KCl. Tim44, a peripheral component of the translocase of the inner membrane [34], was partially recovered in the supernatant fraction under these conditions (Fig. 3B). Taking together, we conclude that Mcx1p is tightly bound to the inner face of the inner mitochondrial membrane.

3.4. Disruption of the MCX1 gene

To investigate the function of Mcx1p in mitochondria, haploid cells completely lacking Mcx1p activity were generated by replacing a 1.0-kb internal fragment of *MCX1* with the *LEU2*

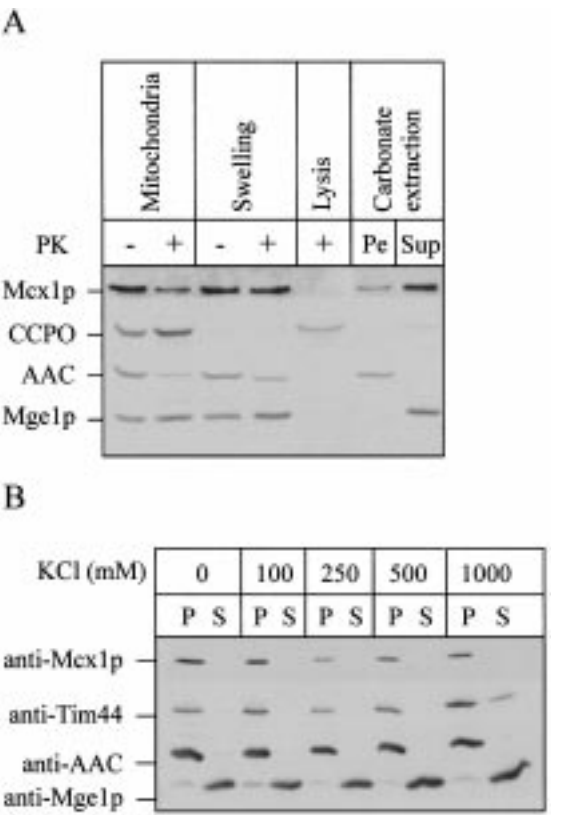

Fig. 3. Mcx1p is a peripheral membrane protein in the mitochondrial matrix space. A: Submitochondrial localization of Mcx1p. Aliquots of mitochondria (Mitochondria), mitoplast (Swelling), and solubilized mitochondria (Lysis) were treated with proteinase K when indicated (+/−PK). Another aliquot of mitochondria was incubated with 0.1 M sodium carbonate (Carbonate extraction), and split into pellet (P) and supernatant (S) fractions by centrifugation. Proteins were analyzed by immunoblotting using polyclonal antibodies directed against cytochrome *c* peroxidase (CCPO), the ADP/ATP-carrier (AAC), Mge1p and Mcx1p. CCPO, AAC, and Mge1p are markers for the intermembrane space [46], the mitochondrial inner membrane [47], and the mitochondrial matrix space [45], respectively. B: Membrane association of Mcx1p. Mitochondria were sonicated in the presence of increasing concentrations of KCl. After a clarifying spin, samples were separated in pellet (P) and supernatant (S) fractions by a 1-h centrifugation at 100 000 × *g* and 4°C. Proteins were analyzed by SDS-PAGE and immunoblotting as in A. Tim44 is a peripheral membrane subunit of the translocase of the inner membrane [34].

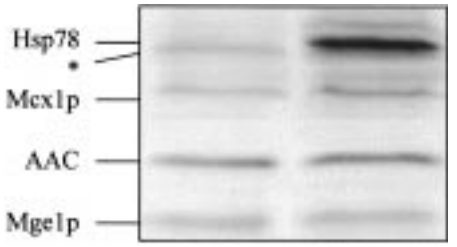

Fig. 4. Expression of Mcx1p upon heat stress. Cells were grown in glucose-containing rich medium to mid-exponential phase and heat shocked for 1 h at 42°C. Total protein extracts were analyzed by immunoblotting using polyclonal antibodies directed against Mcx1p, the heat shock protein Hsp78 [42], and the non-induced proteins AAC [47] and Mge1p [45]. The band indicated by (*) is a cross reaction of the polyclonal antibody directed against Hsp78.

gene. These cells did not exhibit any detectable phenotype under various growth conditions, i.e. on glucose-, galactose-, or glycerol-containing rich medium at 24°C, 30°C or 37°C (data not shown). Thus, Mcx1p is not essential for cell growth or respiratory competence.

Other mitochondrial proteins may exert functions overlapping with Mcx1p and thereby substitute for its activity. If Mcx1p is a subunit of a yet unidentified Clp protease in yeast mitochondria, PIM1 protease, an ATP-dependent Lon-like protease in the matrix, may compensate for a loss of Mcx1p. PIM1 has been shown to be essential for the stability of the mitochondrial genome and for the respiratory competence of yeast cells [36–38]. Disruption of *MCX1* in a *pim1* null mutant, however, did not cause any additional phenotype (data not shown). On the other hand, Hsp78, a molecular chaperone of the ClpB family in mitochondria, may substitute for Mcx1p. Similar to *mcx1* mutations, disruption of the *HSP78* gene does not cause any detectable phenotype under normal growth conditions [39]. Hsp78, however, confers compartment-specific thermotolerance to mitochondria and is crucial for maintenance of respiratory competence under severe temperature stress [9]. To examine if Mcx1p and Hsp78 perform overlapping function in mitochondria, the *MCX1* gene was disrupted in an *hsp78* null mutant. The absence of Mcx1p, however, did not result in an altered phenotype or in an increased thermosensitivity of wild-type or Δ*hsp78* cells (data not shown). Moreover, Mcx1p is only slightly overexpressed (2- to 3-fold) upon heat shock of the cells at 42°C for 1 h while expression of Hsp78 in the same conditions is induced over 35-fold (Fig. 4 and [39]).

4. Discussion

We have identified the third Hsp100/Clp-protein in *S. cerevisiae* which, in contrast to the previously described ClpB-like proteins Hsp104 and Hsp78, belongs to the ClpX-subfamily. The protein was localized to mitochondria and was therefore termed Mcx1p (for *M*itochondrial *C*lp*X*). All ClpX-homologues analyzed so far have been demonstrated to interact with ClpP-like peptidases thereby forming hetero-oligomeric ClpXP proteases. Proteins homologous to *E. coli* ClpP, however, are not present in *S. cerevisiae* whose genome has been sequenced completely. Thus, Mcx1p of *S. cerevisiae* may represent the first ClpX-like protein with exclusively non-proteolytic functions. Notably, proteins homologous to ClpP were identified in mitochondria of plants and in human but their function remains to be determined. It will be of interest to examine whether or not they functionally interact with proteins homologous to Mcx1p.

What is the physiological function of Mcx1p? *E. coli* ClpX exerts molecular chaperone activity and is thought to promote conformational alterations in associated substrate proteins thereby facilitating their degradation by ClpP. Moreover, evidence for a chaperone function of *E. coli* ClpX independent of ClpP in vivo was obtained studying its role in DNA replication of phage Mu [40,41]. It is therefore conceivable that Mcx1p acts in mitochondria as a molecular chaperone which functions independently of an associated peptidase. Similar to other molecular chaperone proteins [12], Mcx1p accumulated at 2-fold increased levels in mitochondria under heat shock conditions indicating that Mcx1p is a heat shock protein. In view of its membrane association possible substrates of Mcx1p may include membrane proteins and components of the respiratory chain complexes. Mcx1p, however, does not appear to fulfill unique functions in mitochondria under normal growth conditions suggesting that other mitochondrial chaperone proteins can substitute for a loss of Mcx1p activity. Similarly, the ClpB-homologue Hsp78 and the mitochondrial Hsp70-protein Ssc1p have been demonstrated to exert overlapping functions in mitochondria [42]. Whatever the physiological role of Mcx1p will turn out to be, mitochondria of *S. cerevisiae* provide a very useful model system to study non-proteolytic functions of a ClpX-protein under cellular conditions.

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