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Som1, a third component of the yeast mitochondrial inner membrane peptidase complex that contains Imp1 and Imp2

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Abstract The mitochondrial inner membrane peptidase Imp is required for proteolytic processing of the mitochondrially encoded protein Cox2, the nucleus-encoded Cyt b_2, Mcr1, and Cyt c_1, and possibly other proteins, during their transport across the mitochondrial membranes. The peptidase contains two catalytic subunits, Imp1 and Imp2. The small protein Som1 was previously shown to affect the function of Imp1, but the precise role of Som1 remained unknown. Using mutants deleted for *IMP1*, *IMP2* and *SOM1*, we show here that the Som1 protein is absent in the *imp1*Δ mutant, whereas the level of the Imp1 subunit of the peptidase is only slightly reduced in the *som1* null mutant. The Som1 protein is not essential for proteolytic processing of Cyt b_2, while the two other known Imp1 substrates, Cox2 and Mcr1, are not processed in the absence of Som1. Proteolytic processing of Cyt c_1 by the Imp2 subunit, and of Ccp by an as yet unidentified peptidase, is not impaired in the *som1* deletion mutant. By crosslinking and co-immunoprecipitation assays we demonstrate that the Imp1 and Som1 proteins physically interact. We conclude from our results that stabilisation of Som1 and correct Imp1 function is mediated by a direct interaction between the Imp1 and Som1 proteins, suggesting that Som1 represents a third subunit of the Imp peptidase complex.

Key words *Saccharomyces cerevisiae* · Mitochondria · Protein export · Inner membrane peptidase Imp · *SOM1*

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Introduction

The mitochondrial inner membrane peptidase (Imp) complex of *Saccharomyces cerevisiae* has been described as comprising two different catalytic subunits, Imp1 and Imp2. Imp2 was shown to stabilize the Imp1 subunit, probably by forming the heterodimeric complex (Nunnari et al. 1993; Pratje et al. 1994; Schneider et al. 1994). The two subunits are thought to be associated with the inner mitochondrial membrane via N-terminal membrane-spanning domains, while their larger C-terminal segments face the intermembrane space. As shown by sequence comparisons, Imp1 and Imp2 are related to each other and to prokaryotic and eukaryotic signal peptidases (Behrens et al. 1991; Dalbey and von Heijne 1992; Van Dijl et al. 1992), which are involved in cleaving signal sequences of organellar and secretory proteins during their transport across membranes.

The *IMP1* and *IMP2* genes were initially identified by functional complementation of the respective mutants, which exhibit respiratory deficiency, and are defective in proteolytic maturation of certain mitochondrial proteins that are translocated across mitochondrial membranes (Pratje and Guiard 1986; Behrens et al. 1991; Nunnari et al. 1993). The two catalytic subunits of the inner membrane peptidase appear to possess separate, non-overlapping substrate specificities (Nunnari et al. 1993): the Imp1 subunit is known to be responsible for proteolytic cleavage of three mitochondrial proteins, one of which, the mitochondrially encoded cytochrome *c* oxidase subunit 2 (Cox2), is synthesized by mitochondrial ribosomes as a precursor with a transient N-terminal presequence (Pratje et al. 1983). During targeting a large part of the Cox2 protein is exported across the inner mitochondrial membrane and the presequence of only 15 amino acid residues is removed by the Imp1 peptidase. The second substrate, nucleus-encoded cytochrome b_2 (Cyt b_2), is synthesized in the cytosol with a bipartite N-terminal signal sequence which is processed in two steps during import into mitochondria (Daum

et al. 1982), firstly by the soluble matrix processing peptidase, and subsequently by Imp1, causing release of the soluble mature Cyt b_2 into the intermembrane space. The third known Imp1 substrate, the nucleus-encoded NADH-cytochrome b_5 reductase (Mcr1) exists in two isoforms in yeast mitochondria: the 34-kDa form is localized in the outer membrane, but due to inefficient anchorage during import a large number of molecules are translocated to the inner membrane and cleaved by Imp1, resulting in the soluble 32-kDa form in the intermembrane space (Hahne et al. 1994; Haucke et al. 1997).

The Imp2 subunit is reported to be required for proteolytic maturation of the nucleus-encoded cytochrome c_1 (Cyt c_1) (Nunnari et al. 1993). Cyt c_1 is synthesized with a bipartite targeting sequence similar to that of Cyt b_2, processed in two stages by the matrix processing peptidase and by Imp2, respectively, and released into the intermembrane space.

Two additional genes have been identified, that are involved in proteolytic processing of Cox2 (Pratje et al. 1997). The inner membrane protein Oxa1 is required for export and assembly of mitochondrially encoded proteins across the inner membrane. The *oxa1* mutants are respiration deficient, and the proteolytic processing of Cox2 by the Imp1 peptidase is prevented due to impaired transport (Bauer et al. 1994; Bonnefoy et al. 1994; He and Fox 1997; Hell et al. 1997; Herrmann et al. 1997; Meyer et al. 1997). The small Som1 protein represents another component required for proteolytic processing of Cox2 by the Imp1 subunit of the inner membrane peptidase (Esser et al. 1996, 1998). An interaction between Imp1 and Som1 is suggested by the following observations: the *SOM1* gene acts as a high-copy-number suppressor of the *imp1* mutant phenotype, Som1 plays a role in processing of pCox2, and Som1 is localised on the outer face of the inner mitochondrial membrane (Esser et al. 1996; Bauerfeind et al. 1998). The protein contains a conserved pattern of four cysteine residues, possibly involved in protein-protein interaction or metal ion binding.

To gain more insight into the function of the Som1 protein, we systematically investigated various null mutants, the proteolytic processing of substrates in these mutants, and protein-protein interactions, using genetic and biochemical approaches. In this study we report evidence for direct interactions between the proteins Som1 and Imp1. This interaction is essential for Imp function and Som1 stabilisation, suggesting that the Som1 protein represents a third subunit of the Imp peptidase complex.

Materials and methods

Chemicals and enzymes

DNA-modifying enzymes were purchased from Boehringer Mannheim, Pharmacia, Gibco BRL, and MBI Fermentas. Radiochemicals ([^{35}S]Met and [α-^{35}S]dATP) were from Amersham and Hartmann Analytics. Oligonucleotides were synthesized by MWG Biotech.

Strains and plasmids

The following *S. cerevisiae* strains were used: JRY-675 (*MAT***a**, *MAT*α or the corresponding diploid, *his4-519 ura3-52* Δ*leu2*; J. Rine, unpublished), JRYΔ*3* (*MAT*α *his4-519 ura3-52* Δ*leu2 som1*:: *LEU2*; Esser et al. 1996), JRYΔ*pet1402* (*MAT*α *his4-519 ura3-52* Δ*leu2 oxa1*:: *URA3*; Bauer et al. 1994), Sc167 pet ts2858 (*MAT*α *ade1 imp1*; Michaelis et al. 1982), KN79 Δ*imp1* (*MAT*α *trp1-1* Δ*leu2 imp1*::*LEU2*), GM103-1B Δ*imp1* (*MAT*α *ura3* Δ*leu2 his4 imp1*::*LEU2*), GM103-4D Δ*imp1* (*MAT*α *ura3* Δ*leu2 trp1 imp1*::*LEU2*), EP9-4B (*MAT***a** *leu2 ura3 imp2*), the double deletion strain GM109-1A Δ*3* Δ*imp1* (*ura3 his4* Δ*leu2 som1*::*LEU2 imp1*::*LEU2*), and progenies from crosses between these strains. Two-hybrid analysis was performed using the Clontech strains PJ69-4A (*MAT***a**, *trp1-901 leu2-3,112 ura3-52 his3-200 gal4*Δ *gal80*Δ *GAL2-ADE2 LYS2*::*GAL1-HIS3 met2*::*GAL7-lacZ*; James et al. 1996) and Y187 (*MAT*α *trp1-901 leu2-3,112 ura3-52 his3-200 ade2-101 gal4*Δ *gal80*Δ *met*$^-$ *URA3*::*GAL1UAS-GAL1TATA-lacZ*; Harper et al. 1993).

The *Escherichia coli* strains used were DH5α (Hanahan 1983) and RR1ΔM15 (Bolivar et al. 1977). Subcloning was performed in the shuttle vectors YEp351, YEp352 (Hill et al. 1986), YCp50 (Rose et al. 1987) and pRS416 (Sikorski and Hieter 1989), and in the *E. coli* vector pUC19 (Yanisch-Perron et al. 1985).

For two-hybrid analysis we used the vectors pACT2 and pGBT9 (Clontech), and a yeast cDNA library constructed in the pACT2 vector (S. Elledge, unpublished).

Media

Yeast cells were grown on standard media at 23, 28, and 36 °C as described by Pratje et al. (1983). Media for two-hybrid analysis were prepared as described in the Clontech manual. *E. coli* was grown on LB media containing 100 mg/l ampicillin to select for plasmid maintenance.

Screening of specific clones and yeast cDNAs in the two-hybrid system

Two-hybrid analysis was carried out using the Clontech system with minor modifications. *IMP1*, *IMP2*, and *SOM1* sequences were fused in frame with the sequence encoding the DNA-binding domain of GAL4 (GAL4BD) in the two-hybrid vector pGBT9, or with the coding sequence for the GAL4 transcription activation domain (GAL4AD) in the two-hybrid vector pACT2. In the case of the *IMP1* and *IMP2* constructs, truncated sequences lacking the first 26 codons of the genes were used; these codons are thought to encode the transmembrane domains, which could prevent possible interactions of the proteins when they are not correctly localized in the inner mitochondrial membrane. The *SOM1* construct contained the entire coding sequence. The constructs were designated pGBT9:IMP1-26, pACT2:IMP1-26, pACT2:IMP2-26, and pGBT9:SOM1, respectively.

For screening we used a library containing yeast cDNA cloned downstream of GAL4AD in pACT2. The plasmids were either introduced into the strain PJ69-4A by cotransformation, or combined by crossing Y187 transformants carrying a pGBT9 bait plasmid with PJ69-4A cells containing the pACT2 library plasmids. Crosses were performed as follows: a 20-ml culture of Y187 transformants, grown in synthetic medium lacking tryptophan, was mixed with 0.5 ml of PJ69-4A containing the pACT2 library; then 20 ml of rich glucose medium was added, and the culture was incubated overnight, and subsequently plated on synthetic medium lacking leucine, tryptophan and adenine. Positive clones were transferred to synthetic medium containing 5 mM 3-amino-triazole, and lacking leucine, tryptophan and histidine. The plasmids

contain the *LEU2* or *TRP1* marker gene, and the two-hybrid strain PJ69-4A carries the *ADE2* gene under the control of the *GAL2* promoter and the *HIS3* gene under control of the *GAL1* promoter. Growth on medium without leucine and tryptophan indicates successful transformation of the cells with the plasmids pGBT9 and pACT2, growth on medium without adenine or histidine indicates an interaction between the fusion proteins containing the GAL4BD and the GAL4AD domains, respectively.

Positive yeast colonies containing both plasmids were first subjected to an analytical PCR, using either plasmid DNA isolated from yeast or whole yeast cells as a template. The PCR with yeast cells was performed as described by Esser et al. (1999), using the primers MMamp1 (5′-CTATTCGATGATGAAGATACCCCAC-CAAACC-3′) and MMamr2 (5′-GTGAACTTGCGGGGTTTTT-CAGTATCTACG-3′), which bind directly upstream and downstream, respectively, of the cloned cDNA sequences in the multiple cloning site of the two-hybrid vector pACT2.

The pACT2 plasmids were isolated from positive colonies, analysed by restriction mapping, and then reanalysed in a second two-hybrid assay. The cDNA sequences of positive clones were determined by the dideoxy-chain termination method (Sanger et al. 1977) using the T7 sequencing kit (Pharmacia). Computer analysis of the nucleotide and amino acid sequences was carried out with the PC-Gene program and using databases available on the Internet.

Construction of the myc-tagged *IMP1* gene

A fusion that attaches three myc epitopes to the Imp1 protein was constructed by PCR, using the *IMP1* gene region cloned in the plasmid YEp352 as template, and the Ex Taq polymerase (Takara) to avoid errors during PCR. We used the oligonucleotides 5imp-sal (5′-CTTGTCGACTGATCTTGTGTCGTGAGC-3′) and 3imp-myc (5′-GGTGAATTCTCAATTCAAGTCTTCTTCAGAAATA-AGTTTTTGTTCATTCAAGTCTTCTTCAGAAATAAGTTT-TTGTTCATTCAAGTCTTCTTCAGAAATAAGTTTTTGTT-CGTTGCTCTTAGCCTGCAC-3′), which were designed for amplification of a 960-bp PCR product, containing the *IMP1* gene with its own promoter region. Binding of the 5′ primer allowed amplification of 277 bp of the upstream untranslated region, and an Imp1 protein with additional three copies of the myc epitope (EQKLISEEDLN) was created by the sequence of the 3′ primer. The PCR product was ligated into the vectors YEp352 and pRS416, using the *Sal*I and *Eco*RI restriction sites introduced into the PCR product by the primers, and the respective clones were used for transformation of the deletion strains GM103-1B Δ*imp1* and GM109-1A Δ*3* Δ*imp1*.

Crosslinking experiments

Isolated mitochondria were washed twice with SHE buffer (0.6 M sorbitol, 20 mM HEPES-KOH pH 7.4, 2 mM EDTA) and suspended in the same buffer (at a concentration of 2.5 mg/ml). The crosslinking reagent sulfo-*m*-maleimidobenzyl-N-hydroxysuccinimide ester (s-MBS) was added to a final concentration of 2.5 mM and incubated for 2 h at 4 °C. The reaction was stopped by adding 0.285 M TRIS-HCl pH 8.0, and the mitochondria were washed twice with SHE buffer before they were lysed in PAGE sample buffer and fractionated by SDS-gel electrophoresis. Lanes loaded with the crosslinked samples contain twice the amount of protein added to the other lanes.

Immunoprecipitation

The procedure is based on the protocol of Horst et al. (1995), which was modified in several respects. Mitochondria were isolated from a 30-ml yeast culture as described by Pratje and Michaelis (1977), and solubilized in 100 µl of MTE buffer [0.6 M mannitol, 25 mM TRIS-HCl, 1 mM EDTA pH 7.2, containing half a tablet of the Complete protease inhibitor (Boehringer Mannheim) in 15 ml of MTE (to give a final concentration of approximately 2.7 mg/ml)]. Samples (50 µl) of this preparation were collected by centrifugation and dissolved in TNET buffer [0.5% (v/v) Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM TRIS-HCl pH 8.0, and Complete protease inhibitor at approximately 2.7 mg/ml] for 10 min on ice. After removal of insoluble proteins by centrifugation, the supernatant was subjected to unspecific preadsorption with 100 µl of Sepharose 4B in 1 ml TNET for 2 h.

At the same time preadsorption of the antibody was performed in a separate reaction by adding 25 mg of Protein A-Sepharose and 5 µl of a 0.4 mg/ml anti myc solution [Anti myc (Boehringer Mannheim); dissolved in 0.9% NaCl, 10 mM sodium phosphate, pH 7.2) or 30 µl of anti-Som1 antiserum, respectively, to 1 ml of TNET and incubating for 2 h.

The antibodies bound to Protein A-Sepharose were collected by centrifugation, mixed with the supernatant of the unspecific preadsorption reaction, containing the mitochondrial proteins, and incubated for 16 h at 4 °C.

After centrifugation, the unbound proteins in the supernatant were precipitated with TCA, solubilized, and small aliquots were analysed by SDS-PAGE as a control.

The Protein A-Sepharose pellet was washed twice, and subsequently the antibody-bound protein was eluted for 20 min by adding 35 µl of 0.1 M citrate (pH 2–3) on ice. The eluate, usually about 40 µl, was neutralised by addition of 5 µl of 2 M TRIS (pH 9.8), then mixed with SDS-sample buffer, incubated at 95 °C, and analysed by SDS-PAGE.

Other methods

Standard methods for transformation of *E. coli*, plasmid purification, DNA restriction, DNA ligation, agarose gel electrophoresis of DNA, and Western transfer were performed essentially according to Sambrook et al. (1989). For protein transfer we used either NC membranes (Schleicher and Schüll BA83) or PVDF membranes (Millipore Immobilon and Pall FluoroTrans W). Detection of membrane-bound proteins was performed using the Tropix chemiluminescence system or directly on the membrane using horseradish peroxidase- or alkaline phosphatase-conjugated antibodies (Boehringer Mannheim).

Extraction of total protein from yeast was performed as described by Yaffe and Schatz (1984), PCR as in Innis et al. (1990) and transformation of yeast as described by Gietz et al. (1995). Total yeast DNA was isolated according to Holm et al. (1986), plasmid DNA was recovered from yeast using the protocol of Nasmyth and Reed (1980) and yeast mitochondria were purified as previously reported (Pratje and Michaelis 1977). SDS-PAGE followed the method of Laemmli (1970).

Results

The Imp1 subunit of the signal peptidase has an additional function in stabilizing the Som1 protein

In the study by Nunnari et al. (1993) the Imp2 subunit of the Imp peptidase complex was shown to stabilize the Imp1 subunit, probably by forming a heterodimeric complex with it. We first checked whether the Som1 protein is similarly stabilized by Imp1. Western analysis using a specific Som1 antiserum revealed no detectable Som1 protein in mitochondria isolated from the *imp1* deletion mutant, or from the temperature-sensitive point mutant pet ts2858 when grown at the non-permissive temperature (Fig. 1A). Deletion of *OXA1*/*PET1402*,

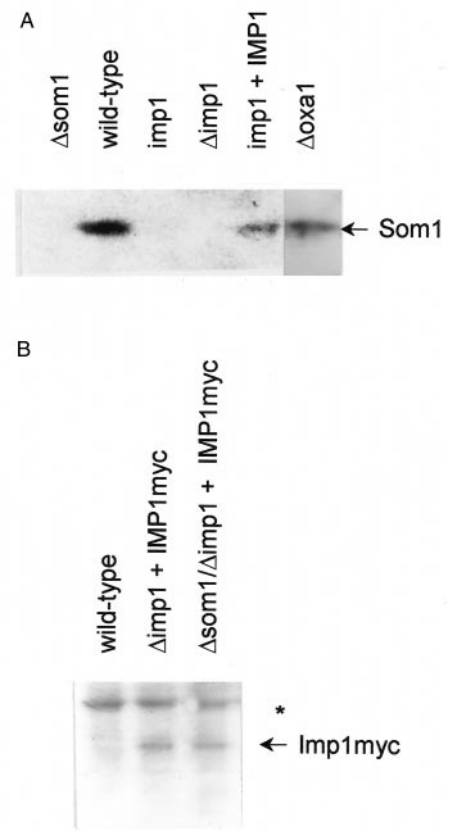

Fig. 1A, B The Som1 protein is undetectable in *imp1* mutants. **A** A wild-type strain, the mutants Δsom1, Δimp1, Δoxa1, pet ts2858 (*imp1*), and the pet ts2858 mutant, carrying a copy of the *IMP1* gene on a plasmid (imp1 + IMP1), were grown in galactose-containing medium and incubated for 14 h at 36 °C. Mitochondria were isolated and analysed by SDS-PAGE. The Som1 protein was detected immunologically on a Western blot. **B** A wild-type strain, the mutant Δimp1 carrying a copy of the myc-tagged *IMP1* gene on a plasmid (Δimp1 + IMP1myc), and the double deletion mutant Δsom1/Δimp1 transformed with the same plasmid (Δsom1/Δimp1 + IMP1myc), were grown in galactose-containing medium, and mitochondria were isolated and analysed by SDS-PAGE. The myc-tagged Imp1 protein was detected using an anti-myc antiserum (Boehringer Mannheim). An unspecific band (*asterisk*) at 32 kDa illustrates that comparable amounts of proteins were loaded in each lane

another gene essential for proteolytic processing of Cox2, did not significantly affect the stability of Som1.

In a second experiment, we investigated whether the Som1 protein mediates stabilisation of Imp1 and other components that are essential for correct proteolytic processing of Cox2. To facilitate detection of the Imp1 protein, we constructed the double deletion strain GM109-1A Δ*3* Δ*imp1*, which was transformed with a myc-tagged *IMP1* allele on a plasmid. Such cells should be equivalent to a *som1* deletion mutant. Mitochondria isolated from this strain contained slightly reduced but significant amounts of the Imp1 protein (Fig. 1B), suggesting that the Som1 protein mediates stabilisation of Imp1 only to a certain degree. In the *som1* mutant, as in *imp1* mutants, the amounts of Imp2 and Oxa1/Pet1402 proteins are not reduced significantly (not shown). These results, and those reported earlier (Nunnari et al. 1993), suggest that, in the Imp peptidase complex, the components Imp1 and Som1 are stabilized by interaction with Imp2 and Imp1, respectively.

Proteolytic processing of two Imp substrates is blocked in the *som1*Δ strain

The results of the experiments described above show that loss of Som1 does not result in complete loss of Imp1, and raise the question to what extent the Imp1 peptidase activity is reduced in *som1* deletion mutants. As previously reported, the Imp2 activity is not abolished in an *imp1* deletion mutant (Nunnari et al. 1993), demonstrating the non-overlapping substrate specificities of the two known catalytic Imp subunits. To investigate the influence of Som1 on Imp1 function, we analysed the processing of the known substrates of the inner membrane peptidase in the *som1* deletion mutant by Western analysis (Fig. 2). As described previously, the presequence of the Cox2 protein is not proteolytically removed in *imp1* and *som1* mutants (Behrens et al. 1991; Esser et al. 1996). In the case of Cyt b2, we initially reported the level of the protein to be strongly reduced in the *som1* deletion strain (Esser et al. 1996). Analysis of different preparations of mitochondrial protein, however, revealed that the mature form of Cyt b_2 is present in reduced but detectable amounts (Fig. 2A), indicating a residual function of Imp1 in the absence of the Som1 protein. In *imp1* mutants proteolytic processing of Cyt b_2 is abolished, resulting in accumulation of the intermediate form of Cyt b_2 (Pratje and Guiard 1986). The proteolytically processed 32-kDa form of Mcr1 (the third known Imp1 substrate) in the intermembrane space is not detectable in the *imp1* and *som1* mutants (Fig. 2B), but is clearly detectable in mitochondria from the wild type. According to Hahne et al. (1994) the 32-kDa form represents about two-thirds of the total Mcr1 protein. Proteolytic processing of the mitochondrial proteins Cyt c_1 by the Imp2 subunit and Ccp by an as yet unknown peptidase is not impaired in the *som1* or *imp1* mutant (Fig. 2C, D).

Two-hybrid analysis

In order to identify proteins that interact with the inner membrane peptidase, a two-hybrid screen was carried out, using Imp1 and Som1 proteins as bait; their respective coding sequences were ligated downstream of GAL4BD in the vector pGBT9. The *SOM1* clone included the entire coding sequence, while for the Imp1 bait a truncated sequence was used, that lacks the 26 N-terminal codons thought to specify the transmembrane domain –which could interfere with protein folding and possible interactions when not localized in the membrane. For screening, the pACT2 yeast library was introduced into transformants carrying the bait plasmids. From several positive clones the respective library plas-

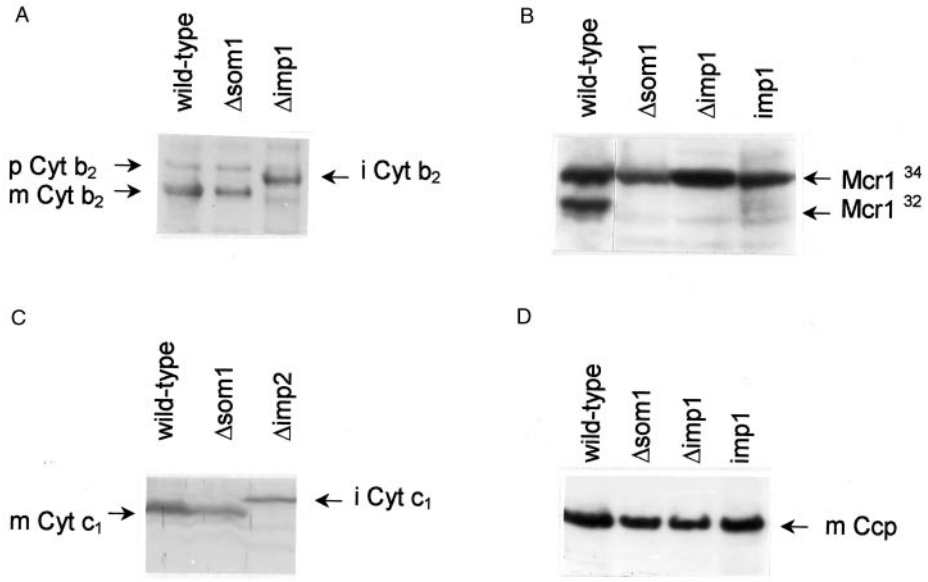

Fig. 2A–D Proteolytic processing of mitochondrial proteins. A wild-type strain and the mutants Δsom1, Δimp1, Δimp2, and pet ts2858 (*imp1*) were grown in galactose-containing medium. The mutant strain pet ts2858 was subsequently incubated for 14 h at 36 °C. Mitochondria and total yeast proteins were isolated and analysed for maturation of the indicated proteins by SDS-PAGE and Western analysis using specific antisera. **A** Western analysis using total protein extracts and an antiserum raised against cytochrome b_2 (Cyt b_2). The mature Cyt b_2 is present in the *som1* deletion strain but cannot be detected in the *imp1* deletion mutant. **B** Western analysis using mitochondria and an antiserum against NADH cytochrome b_5 reductase (Mcr1). The 32-kDa form of Mcr1 is found only in the wild-type strain. **C** Western analysis using mitochondria and an antiserum against cytochrome c_1 (Cyt c_1). Processing of the intermediate form of Cyt c_1 requires Imp2. **D** Western analysis using total protein extracts and an antiserum against cytochrome *c* peroxidase (Ccp). Processing of Ccp is not impaired in any of the strains investigated

mids were recovered and characterized in order to identify the interacting proteins. None of the interacting proteins was identical to Imp1, Imp2, Oxa1 or Som1. Positive clones included *HSP60* and an ORF of unknown function. A direct two-hybrid assay, performed with the two bait plasmids and the *IMP1* and *SOM1* sequences, fused to GAL4AD in the pACT2 plasmid, yielded no growth, indicating that an interaction between these two mitochondrial inner membrane proteins cannot be demonstrated in this system with the plasmid constructs used.

Som1 interacts with Imp1: evidence from crosslinking and co-immunoprecipitation

To test whether the Som1 protein interacts with other proteins, crosslinking experiments were performed using the water-soluble crosslinker sulfo-*m*-maleimidobenzyl-N-hydroxysuccinimide ester (s-MBS). Isolated mitochondria were crosslinked for 2 h at 4 °C. A crosslinked product with an apparent molecular weight of about 30 kDa was detected with an anti-Som1 antibody (raised against a synthetic peptide; Esser et al. 1996) in wild-type mitochondria and in a strain carrying a high-copy-number plasmid bearing the *SOM1* gene (Fig. 3A). This band is absent in the *som1* deletion strain that lacks the Som1 protein and in the samples not treated with crosslinker. This result suggests that Som1 makes a close contact with a protein of about 20 kDa. Such a size is expected for the Imp1 peptidase. To demonstrate the presence of Imp1 in the crosslinked 30-kDa band, the experiment was repeated with a strain carrying a high-copy-number plasmid bearing the *IMP1* gene fused, upstream of and in frame, with the sequence encoding three myc epitopes. The crosslinked bands were visualized with the Som1 antibody. A new shifted band of about 35.5 kDa (33.9 kDa expected) was observed (not shown); however, the 30-kDa band, although reduced, did not disappear. Further studies are necessary to show whether this residual 30-kDa band results from degradation of the Imp1-myc tag, from Imp2 or some other 20-kDa protein crosslinked to Som1.

To obtain independent evidence for a direct interaction between Som1 and Imp1, we performed co-immunoprecipitation experiments. To facilitate detection of the Imp1 protein, the plasmid carrying the *IMP1* gene tagged with three myc epitopes was transformed into an *imp1* deletion strain. The Imp1 protein function was not severely impaired by the tag, as shown by functional complementation of the *imp1* deletion strain with the construct inserted in high- or low-copy-number vectors. Mitochondrial proteins were isolated from this strain, solubilized and subjected to coprecipitation assays, using anti-myc antiserum. Precipitated proteins and the supernatants were analysed by immunoblotting and subsequent immunological detection using the specific Som1 antiserum. As shown in Fig. 3B, the Som1 protein coprecipitated with the myc-tagged Imp1 protein. The specificity of the coprecipitation assay was confirmed by

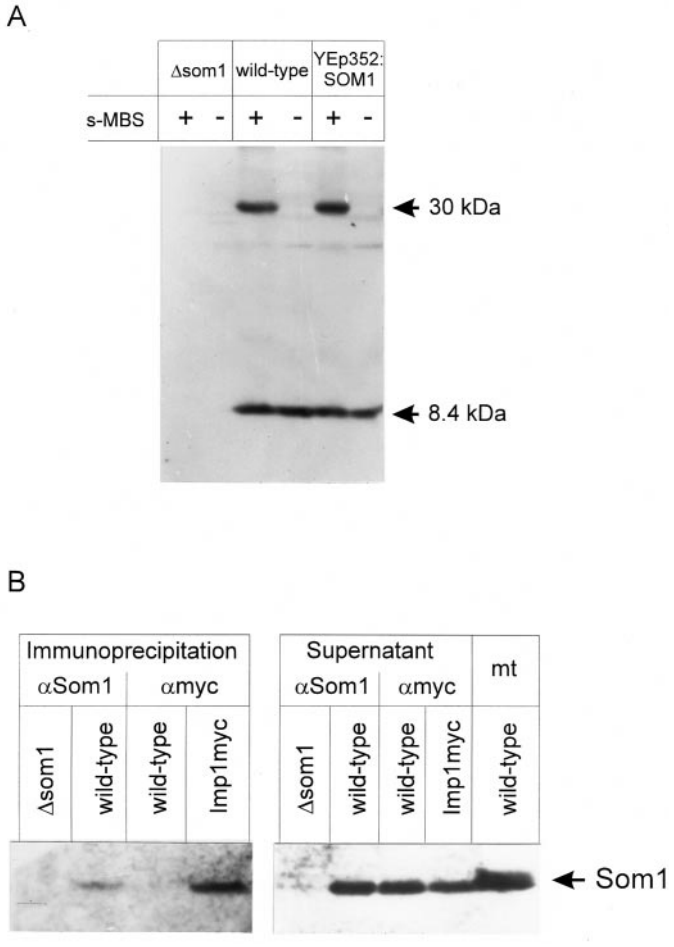

Fig. 3A, B Interaction of Imp1 and Som1 proteins. **A** Crosslinking analysis. Mitochondrial proteins were solubilized with SHE buffer (see Materials and methods), and subjected to SDS-PAGE either directly (−) or after incubation with the crosslinker s-MBS (+). An antiserum specific for Som1 was used for Western analysis. Mitochondria of the following strains were used: Δsom1 (*SOM1* gene deleted), wild-type, and YEp352:SOM1 (wild-type with *SOM1* present on a high-copy-number plasmid). **B** Coimmunoprecipitation. Mitochondrial proteins were solubilized with 0.5% Triton X-100 and incubated with Protein A- Sepharose-bound antibodies directed either against the myc tag (αmyc) or against Som1 (αSom1). Aliquots of the bound protein (Immunoprecipitation) and unbound protein (Supernatant) were analysed by SDS-PAGE and subsequent Western analysis using the specific anti-Som1 antiserum. As a control, an aliquot of a crude mitochondrial preparation (mt) was also analysed. Mitochondria of the following strains were used: Δsom1 (*SOM1* gene deleted), wild-type (non-transformed wild-type strain), and Imp1myc (the *imp1* deletion mutant expressing *IMP1* with a C-terminal myc tag from a multicopy plasmid)

parallel controls, using mitochondria from a non-transformed wild-type strain for coprecipitation, and by direct precipitation of Som1 with the Som1 antibody. The supernatants of the precipitation assays, except that obtained from the *som1* deletion strain, always contained detectable amounts of the Som1 protein, which therefore was not quantitatively removed by coprecipitation. In the control experiment with mitochondria from a non-tagged wild-type strain, no indications for non-specific copurification of the Som1 protein were obtained. The results described in this section clearly demonstrate a specific interaction between the Som1 and Imp1 proteins.

Discussion

The experiments presented here demonstrate that the Som1 protein physically interacts with the Imp1 subunit of the mitochondrial inner membrane peptidase: (1) the Som1 protein is undetectable in an Imp1 deletion strain; (2) Som1 can be crosslinked, yielding an adduct of 30 kDa – exactly the apparent molecular weight expected for a Som1-Imp1 product, and (3) Som1 can be coprecipitated with Imp1 protein. These results are consistent with our previous studies, since the *SOM1* gene was initially identified as a high-copy-number suppressor of an *imp1* point mutation (Esser et al. 1996), the gene product was shown to affect proteolytic processing by the Imp1 peptidase, and the Som1 protein was localized in the same compartment as the Imp peptidase itself, at the outer side of the inner mitochondrial membrane (Fig. 4; Bauerfeind et al. 1998). The protein interactions described could not be demonstrated by screening of a yeast cDNA library or by direct use of the corresponding genes in the yeast two-hybrid system. However, the specific interaction of the Imp1 and Imp2 subunits, which was shown previously in independent experiments (Nunnari et al. 1993; Schneider et al. 1994), also could not be detected in the two-hybrid assay. The protein interactions may be dependent on the correct membrane topology and structure of Imp1 and Imp2, which are probably not able to interact when not correctly localized in the mitochondrial membrane. In addition, we observed that the respiration deficiency of the *som1* deletion mutant was rescued after transformation with the Som1 fusion construct in the two-hybrid plasmid pGBT9, indicating that at least a portion of the fusion protein is imported into mitochondria, where it is unable to activate expression of the reporter genes. Therefore a significant interaction between Som1 and Imp1, or Imp1 and Imp2 in the two-hybrid system may be easier to achieve using constructs containing partial sequences or domains.

Our Western analyses using the mutants, and the results reported earlier (Nunnari et al. 1993), suggest that, either during assembly or in a functional Imp peptidase complex, the two catalytic subunits have an additional function in stabilizing another component – probably by protein-protein interaction – without their own stability being dependent on this interaction. The Imp1 protein is absent in *imp2* mutants, and Som1 is absent in *imp1* mutants, whereas the Som1 is not required to stabilise Imp1, and Imp1 is not required for Imp2 stability. Deletion of *OXA1*, another gene essential for proteolytic processing of pCox2, did not affect the stability of Som1. The presence of very low residual amounts of the proteins destabilized in *imp1* and *imp2* mutants, however, cannot be excluded. This possibility

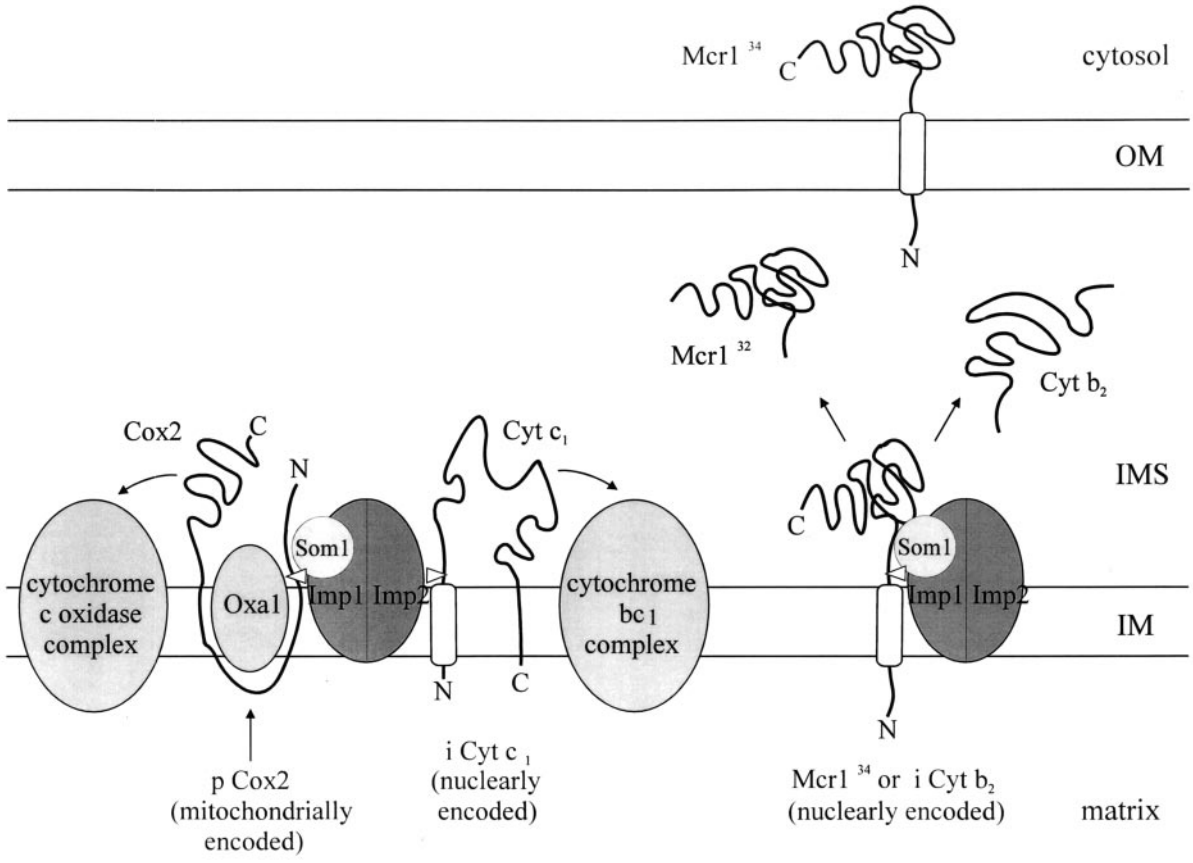

Fig. 4 Location of the mitochondrial inner membrane peptidase complex (Imp1/Imp2/Som1), its substrates and products. The mitochondrially encoded subunit 2 of cytochrome oxidase (Cox2) is translated on mitochondrial ribosomes as a precursor (pCox2) with an N-terminal presequence, which is removed by the mitochondrial inner membrane peptidase (Imp1 and Som1 subunits) during export to the outer face of the mitochondrial inner membrane (IM). The inner membrane protein Pet 1402/Oxa1 is required for the export and assembly of Cox2. Other substrates for the inner membrane peptidase are encoded in the nucleus, and have to be imported into mitochondria. Cytochrome b_2 (Cyt b_2) and cytochrome c_1 (Cyt c_1) are synthesized as precursors with bipartite signal sequences. During their import into mitochondria the N-terminal mitochondrial targeting sequences are removed by the matrix processing peptidase (not shown). The second part of the signal sequence is removed by the Imp1 or Imp2 subunit of the peptidase, releasing the mature proteins into the intermembrane space (IMS). The mitochondrial NADH cytochrome b_5 reductase (Mcr1) is synthesized as a 34-kDa form, which is localized in the outer membrane (OM). Due to incomplete arrest during mitochondrial import, some of the protein is transferred to the inner membrane, were it is processed by Imp1/Som1 to generate the soluble 32-kDa form of Mcr1 in the intermembrane space

is supported by the observation that in the *imp1* mutant pet ts2858 at high temperature no Som1 protein was detectable, but when the *SOM1* gene is overexpressed and acts as high copy suppressor of the mutant, a small amount of the Som1 gene product sufficient to restore Imp1 function must exist under these conditions. Similarly, very small amounts of the Som1 protein, which are not detectable by Western analysis, are sufficient for functional complementation of the phenotype of a *som1* deletion mutant (unpublished results).

Mutation of the conserved residue S_{41} to A in the Imp2 protein results in a lack of Cyt c_1 maturation, but, interestingly, causes no respiration deficiency of the cells (Nunnari et al. 1993), suggesting that stabilisation of the Imp1 subunit by Imp2 and/or correct processing of an Imp1 substrate may be decisive for respiratory function. A processing event that is important for respiratory function might involve an unknown new substrate of the peptidase rather than pCox2, because it seems that pCox2 can be assembled into a functional enzyme complex (Michaelis et al. 1982), and processing of the other known substrates of the inner membrane peptidase is not essential for respiratory function. In addition, the extent to which the phenotype of *imp1* and *imp2* mutants is dependent on the loss of Som1 function requires further investigation.

Analysis of processing of the substrates of the inner membrane peptidase revealed that the Som1 protein is important for the Imp1-mediated proteolytic processing of Cox2 and Mcr1, but not essential for maturation of Cyt b_2 by Imp1 or of Cyt c_1 by the Imp2 subunit of the peptidase (Esser et al. 1996, and this study). Therefore, so far Som1 must be regarded as Imp1 specific. In addition, the presence of only the mature form of Cyt b_2 in reduced but detectable amounts in the *som1* deletion indicates at least a residual proteolytic activity of Imp1 in the absence of the Som1 protein, and a function for Som1 in substrate recognition. As proteolytic cleavage by Imp1 is known to occur between N and D (in pCox2)

or N and E residues (in iCyt b_2 and the 34 kDa form of Mcr1) (Pratje and Guiard 1986; Hahne et al. 1994), specificity of Som1 function in facilitating proteolytic processing at one or other of the two possible cleavage sites can also be excluded. Moreover, these cleavage sites are assumed to form only one of several determinants for specific proteolytic processing of substrates by the Imp peptidase (Dalbey and von Heijne 1992; Poyton et al. 1992).

Because the study by Schneider et al. (1991) showed that the Imp1 peptidase requires divalent cations for activity, and the characteristic cysteine pattern in the Som1 protein could possibly have a function in metal ion binding, we added different concentrations of Mg^{2+} and EDTA to the coimmunoprecipitation assays, but no significant influence on the amounts of the coprecipitated proteins Som1 and Imp1 was detectable. It is conceivable that the cysteine pattern in the Som1 protein has a function in protein-protein interaction. A similar motif of four cysteine residues is present in several other small *S. cerevisiae* proteins: Tim8, Tim9, Tim10, Tim12, and Tim13. Their human homologues Ddp1 and Ddp2 contain a twin CX_3C motif (Koehler et al. 1999) and in the small chloroplast protein CP12 of higher plants a similar cysteine pattern is found, that is thought to mediate protein interactions by forming peptide loops via disulfide bonds between flanking cysteine residues (Wedel et al. 1997). The precise function of Som1 remains to be established; nevertheless, the independent genetic and biochemical approaches presented demonstrate an interaction between the Imp1 and Som1 proteins. This interaction is a prerequisite for correct Imp1 peptidase function, and therefore we conclude that Som1 represents an additional subunit of the inner membrane peptidase Imp in yeast mitochondria.

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