Cargo Sequences Are Important for Som1p-dependent Signal Peptide Cleavage in Yeast Mitochondria*

Haobo Liang, Wentian Luo, Neil Green, and Hong Fang‡

From the Department of Microbiology and Immunology, School of Medicine, Vanderbilt University, Nashville, Tennessee 37232-2363

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The inner membrane protease (IMP) has two catalytic subunits, Imp1p and Imp2p, that exhibit nonoverlapping substrate specificity in mitochondria of the yeast Saccharomyces cerevisiae. The IMP also has at least one noncatalytic subunit, Som1p, which is required to cleave signal peptides from a subset of Imp1p substrates. To understand how Som1p mediates Imp1p substrate specificity, we addressed the possibility that Som1p functions as a molecular chaperone, which binds to specific substrates and directs them to the catalytic site. Our results show that cargo sequences attached to the signal peptide are important for Som1p-dependent prescission cleavage; however, no specific cargo sequence is required. Indeed, we show that a substrate normally destined for Imp2p is cleaved in a Som1p-dependent manner when the substrate is directed to Imp1p. These results argue against the notion that Som1p is a molecular chaperone. Instead, we propose that the cargo of some Imp1p substrates can assume a conformation incompatible with prescission cleavage. Som1p could thus act through Imp1p to improve cleavage efficiency early during substrate maturation.

Mitochondria consist of two hydrophobic compartments, the outer and inner membranes, and two hydrophilic compartments, the matrix and intermembrane space, which hold proteins that perform important cellular functions including oxidative phosphorylation, tri-carboxylic acid cycle, and fatty acid oxidation (1). Among the hundreds of proteins that reside within the mitochondrial compartments, only 13 (in human) or 2 (in yeast) are encoded by the mitochondrial genome. All other proteins are encoded within the nucleus and translated in the cytoplasm. To achieve mitochondrial targeting, many proteins have N-terminal signal peptides that must be cleaved following membrane translocation. The mitochondrial processing peptidase (MPP) cleaves the signal peptides from most of these proteins (2). A smaller set of mitochondrial proteins has a signal peptide required for sorting to the intermembrane space and inner membrane following mitochondrial targeting and translocation. This signal peptide is cleaved by the inner membrane peptidase (IMP).

Study of the IMP in the yeast Saccharomyces cerevisiae has revealed three distinct subunits, Imp1p, Imp2p, and Som1p (3–5). Imp1p and Imp2p are catalytic subunits and members of the type I signal peptidase family, which includes endoplasmic reticulum (ER) signal peptidase, chloroplast thylakoid processing peptidase, and many eubacterial signal peptidases (6). Imp1p and Imp2p exhibit nonoverlapping substrate specificity (4). Imp1p cleaves signal peptides from nuclearly encoded cytochrome b2 precursor (pre-cyt b2), the precursor to NADH-cytochrome-b2 reductase (pre-cyt b2-red), the precursor to glycerol-3-phosphate dehydrogenase (pre-Gut2p) and mitochondrially encoded pre-cytochrome c oxidase subunit 2 (pre-Cox2p) (3, 7–9). Imp2p cleaves the signal peptide from nuclearly encoded cytochrome c1 precursor (p-cyt c1) (4). Nonoverlapping substrate specificity derives in large part from the fact that Imp1p does not follow the “−3, −1 rule” in which small uncharged amino acids are required at the P1 and P2 positions in signal peptide cleavage sites (10). Instead, Imp1p tolerates a variety of amino acid residues at the P1 position and requires a negatively charged residue at the P2 position in its substrates (11, 12).

The third IMP subunit, Som1p, binds to Imp1p and is required for signal peptide cleavage from two Imp1p substrates, pre-cyt b2-red and pre-Cox2p (5, 13). Other known Imp1p substrates, p-cyt b2 and pre-Gut2p, can be cleaved in the absence of Som1p (5, 9). This behavior indicates that noncatalytic subunit Som1p exerts a novel level of IMP substrate specificity, one that differentiates between Imp1p substrates. Here, we consider whether Som1p is a molecular chaperone that binds to specific substrates and delivers them to Imp1p.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, Media, and Antibodies—Yeast strains used in this study were BY4741 (MATa his3Δ1 leu2Δ30 met15Δ0 ura3Δ0) (Invitrogen), HY501 (MATa som1::LEU2 his3Δ1 leu2Δ30 met15Δ0 ura3Δ0) (prepared for this study), CYT101 (MATa imp2Δ1 HIS3 ura3Δ2 leu2Δ3 his3Δ12 trp1Δ901 sec2Δ901) (11), and JNY34 (MATa imp2Δ1 ura3Δ2 trp1Δ901) (11). Plasmid pX3C carries a mutated IMP2 gene (11). This mutation results in a S41A substitution that inhibits Imp2p catalytic activity but allows Imp2p to be expressed and bind to Imp1p. Binding of Imp2p to Imp1p is needed to stabilize Imp1p in yeast cells (4). Media used to support yeast cell growth have been described previously (14). Anti-HA high affinity rat monoclonal antibody and protein G-conjugated agrose beads (Roche Applied Science) were used for immunoprecipitation.

Disruption of SOM1 Gene—Oligonucleotides containing sequences flanking the SOM1 and the LEU2 genes were used to amplify the LEU2 gene using PCR. PCR product was introduced into strain BY4741 with selection for Leu-positive colonies. Recombination of the LEU2 gene into the SOM1 chromosomal locus was then confirmed by diagnostic PCR. The disruption removed the entire SOM1 open reading frame from the genome of strain BY4741.

Construction of HA-tagged Proteins—A DNA segment encoding three consecutive HA epitopes (15) followed by a stop codon (TAA) was inserted between the EcolI and KpnI restriction sites of plasmids pHF454 (2µ, TRP1, ADH1 promoter) and pHF455 (2µ, URA3, ADH1 promoter) (16). Genes encoding mutant and fusion proteins without

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‡ To whom correspondence should be addressed. Tel.: 615-343-2233; Fax: 615-343-7392; E-mail: hong.fang@vanderbilt.edu

1 The abbreviations used are: MPP, mitochondrial processing peptidase; IMP, inner membrane protease; ER, endoplasmic reticulum; cyt, cytochrome; red, reductase; pre-Gut2p, glycerol-3-phosphate dehydrogenase precursor; pre-Cox2p, pre-cytochrome c oxidase subunit 2; HA, hemagglutinin.
translational stop codons were synthesized using two-step PCR (16). The genes were then inserted into the above mentioned plasmids between the ADH1 promoter (17) and the DNA segment encoding the triple HA epitopes. Sequences of oligonucleotides used in this study are available on request.

Pulse Labeling and Immunoprecipitation—Methods similar to those described previously were used (14). Briefly, cells were grown at 30 °C to log phase (A600 = 1.5) in appropriate media. Cells were then shifted to media lacking methionine and cysteine for 1 h and pulse-labeled for 10 min with 60 μCi/ml [35S]Met (PerkinElmer Life Sciences). Cells were harvested and broken by glass beads. Total proteins were precipitated with 10% trichloroacetic acid, resuspended in 20 μl of SDS-PAGE sample buffer, and placed in a boiling water bath for 5 min. This mixture was diluted with 1 ml of phosphate-buffered saline/Triton X-100 (0.1%), and cell debris was removed by centrifugation. The protein solution was incubated overnight with 1.6 μg/ml anti-HA antibodies at 4 °C. This incubation was followed by incubation (2 h) with 20 μl of protein G-agarose beads (Roche Applied Science). Protein G beads were washed twice with cold phosphate-buffered saline/Triton X-100 (0.1%) and then twice with distilled water. 20 μl of SDS-PAGE sample buffer was added to the protein-bead slurry. The mixture was placed in a boiling water bath (5 min) and loaded onto an SDS-gel.

RESULTS

Cargo Sequences Influence Som1p-dependent Presequence Cleavage—Som1p is needed for Imp1p cleavage of pre-cyt b2-red but not p-cyt b2 (5). Based on this difference, we reasoned that pre-cyt b2-red has a sequence, which is not present in p-cyt b2, that is responsible for Som1p-dependent cleavage. To determine whether the putative sequence is located within the presequence or cargo of pre-cyt b2-red, we constructed a chimera that contained the cargo of pre-cyt b2-red fused to the presequence of p-cyt b2. The p-cyt b2 presequence is actually composed of two signal peptides fused together in tandem (18). MPP cleaves a mitochondrial targeting signal peptide to generate i-cyt b2 and then Imp1p cleaves the second signal peptide to generate mature cyt b2. The p-cyt b2/cyt b2-red chimera, tagged with three HA epitopes at its C terminus, was expressed in strains BY4741 (wild type) and HFY501 (∆som1). Yeast strain genotypes can be found under “Experimental Procedures.” Cells were then subjected to a 10-min pulse with radiolabeled methionine, and proteins were precipitated from cell extracts with anti-HA antibodies (see under “Experimental Procedures”). As shown in Fig. 1, cleavage of i-cyt b2 signal peptide from i-cyt b2/cyt b2-red was Som1p-dependent. Because this signal peptide can be cleaved from the cyt b2 cargo independently of Som1p (5), the data support the notion that amino acid sequences located within the cargo of pre-cyt b2-red are important for Som1p-dependent cleavage.

Som1p Facilitates Imp1p Substrate Recognition without Requirement for a Specific Substrate Sequence—We next sought to identify specific cargo sequences involved in presequence cleavage from the p-cyt b2/cyt b2-red chimera. To this end, we introduced distinct internal and C-terminal deletions into the cyt b2-red cargo (Fig. 2A) and expressed the constructs in strains BY4741 (wild type) and HFY501 (∆som1). Cells were then examined by pulse labeling as described above. To our surprise, the mutated forms of i-cyt b2/cyt b2-red were cleaved in a Som1p-dependent manner (Fig. 2B). This result suggests that no specific cargo sequence is required for Som1p-dependent cleavage. Cleavage efficiency, however, was reduced when a region immediately flanking the Imp1p cleavage site was removed from the chimera (Fig. 2B, lanes 3 and 4). To examine this region in more detail, we designed a new set of constructs containing smaller internal deletions (10 or 11 amino acids) (Fig. 3A). As shown by pulse labeling, Som1p clearly was needed to cleave signal peptides from this new set of mutant chimeras (Fig. 3B). These results thus suggest that no specific cargo sequence is required for Som1p-dependent substrate cleavage, although it is plausible that deletion of cargo residues located near the cleavage site affect the conformation of the cleavage site and thus partially inhibit presequence cleavage in some protein contexts.

Next, we examined the role of cargo sequences using p-cyt b2-red, a natural Imp1p substrate. This protein is identical to the above-mentioned chimera with respect to the cargo sequence but differs, of course, in presequence composition. A series of internal and C-terminal deletions was constructed in the p-cyt b2-red cargo (Fig. 4A), and Imp1p cleavage was examined in cells that have or lack Som1p. Although most of the mutations did not prevent Som1p-dependent cleavage (Fig. 4B), residues located close to the cleavage site were important (Fig. 4B, lanes 3 and 4). These residues were nonessential for presequence cleavage in the chimeric protein context (Fig. 2B), suggesting that cargo residues located near the cleavage site can affect presequence cleavage in some protein contexts. Taken together, our data suggest that no specific cargo sequence is required for Som1p-dependent presequence cleavage.

p-Cyt b2-red Can Be Directed to Both the Mitochondria and ER—Fig. 4B depicts protein bands (*) with molecular weights that are greater than that of p-cyt b2-red. These bands disappear when yeast cells are treated with tunicamycin prior to the pulse (data not shown). Tunicamycin inhibits Asn-linked glycosylation in the ER (19), indicating that the bands with greater molecular mass represent p-cyt b2-red that has been directed to the ER and glycosylated. This conclusion is supported by our inspection of the p-cyt b2-red sequence, which identified four consensus Asn-linked glycosylation sites. We also observed differently sized glycosylated protein bands with different cargo deletions (Fig. 4B, compare lane 5 with lane 7). This result is consistent with deletion of one or more of the consensus glycosylation sites. The data presented in Fig. 4B thus demonstrate that p-cyt b2-red can be targeted both to mitochondria and the ER. Our data further suggest that the p-cyt b2-red presequence but not the p-cyt b2 presequence exhibits this novel dual targeting, because glycosylated cyt b2-red cargo was not seen when the presequence of p-cyt b2 was used to target the p-cyt b2/cyt b2-red chimera to mitochondria (Fig. 2B). The p-cyt b2-red presequence has a rather long hydrophobic stretch consisting of ~20 amino acid residues (8). This unusually long hydrophobic stretch is probably responsible for ER targeting, whereas distinct uncharacterized sequences in the p-cyt b2-red presequence are likely to be responsible for mitochondrial targeting. We do not know whether ER targeting occurs naturally in yeast cells that express wild type p-cyt b2-red, and it has not been reported previously. However, our overexpression of p-cyt b2-red using the ADH1 promoter to facilitate protein detection (see under “Experimental Procedures”) may contribute to the ER targeting seen in this study.

A Mutant Form of p-Cyt c1 Can Be Cleaved in a Som1p-dependent Manner—Previous work from our laboratory showed that Imp2p substrate p-cyt c1 could be switched to Imp1p when
Fig. 2. Partial deletion of cyt b5-red cargo does not block the Som1p-dependent signal peptide cleavage of p-cyt b2/cyt b5-red. A, design of internal and C-terminal deletions of cyt b5-red cargo are shown. Dashed lines represent the deleted regions. B, HA-tagged p-cyt b2/cyt b5-red constructs were expressed in strains BY4741 (WT, wild type) (odd-numbered lanes) and HFY501 (∆som1) (even-numbered lanes), and strains were subjected to pulse labeling as described in Fig. 1. Proteins were resolved on a SDS-10% gel (lanes 1–10) or a SDS-12% gel (lanes 11–12).

Fig. 3. Deletion of cargo residues next to the Imp1p cleavage site in the p-cyt b2/cyt b5-red chimera does not prevent Som1p-dependent cleavage. A, design of short internal deletions of cyt b5-red cargo is shown. B, HA-tagged p-cyt b2/cyt b5-red constructs were expressed in strains BY4741 (WT, wild type) (odd-numbered lanes) and HFY501 (∆som1) (even-numbered lanes), and strains were subjected to pulse labeling as described in Fig. 1. Proteins were resolved by a SDS-10% gel.
the P₁, P₂, and P₃ residues of p-cyt b₅-red were changed to Ile, Asn, and Glu (INE), respectively (12). The presequence of p-cyt c₁ consists of a bipartite signal peptide (20). MPP cleavage of p-cyt c₁ generates i-cyt c₁, and IMP cleavage of i-cyt c₁ generates cyt c₁. To determine whether Som1p is needed for cleavage of i-cyt c₁ when it is switched to Imp1p, we prepared a DNA construct encoding a truncated form of p-cyt c₁ (p-cyt c₁(INE)), lacking its C-terminal 87 residues, a region that includes the transmembrane segment of i-cyt c₁ (20). We employed this truncation to facilitate detection of the protein, because the truncated protein was expressed at higher levels than full-length p-cyt c₁ in yeast mitochondria (data not shown).

The p-cyt c₁(INE) was expressed in strains BY4741 (wild type), XCY101 (Δimp1), HFY501 (Δsom1), and JN34 (Δimp2)/pXC3. Plasmid pXC3 carries a mutation that results in a S41A substitution in the Imp2p protein. This mutation inhibits Imp2p catalytic activity but allows Imp2p to bind to and stabilize Imp1p (11). As such, strain JN34/pXC3 has Imp1p activity but no Imp2p activity. As shown by pulse labeling (Fig. 5), i-cyt c₁(INE) was processed by Imp1p but not by Imp2p (Fig. 5, lanes 2 and 3). This result is consistent with our previous study, which showed that full-length i-cyt c₁ could be switched from Imp2p to Imp1p by incorporating INE residues at the cleavage site (12). Importantly, when Imp1p cleaved i-cyt c₁(INE), it did so in a Som1p-dependent manner (Fig. 5, compare lane 2 with lane 4). As Imp2p does not need Som1p to cleave wild type i-cyt c₁ (5), it is highly unlikely that i-cyt c₁(INE) would have a sequence that interacts with Som1p only when Imp1p cleaves the precursor artificially as a result of mutation at the presequence cleavage site. Thus, taken to-
gether with data presented above, our results strongly argue against a model in which Som1p directly interacts with an Imp1p substrate to facilitate its cleavage.

To further support our conclusion that cargo sequences are important for Imp1p substrate cleavage, we designed a fusion protein that contained the bipartite presequence of p-cyt c$_1$(INE) fused to the cyt b$_2$ cargo. The signal peptide of i-cyt c$_1$(INE) is cleaved in a Som1p-dependent manner (Fig. 5), whereas the signal peptide of i-cyt b$_2$ is cleaved in a Som1p-independent manner (5). We reasoned that, if cargo sequences dictate Som1p-dependence, the i-cyt c$_1$(INE)/cyt b$_2$ chimera would be cleaved independently of Som1p. To test this idea, the chimera was expressed in strains BY4741 (wild type), JN34 (Δimp2)pXC3, XCY101 (Δimp1), and HFY501 (Δsom1), and cells were subjected to pulse labeling. As shown in Fig. 6, the signal peptide of i-cyt c$_1$(INE)/cyt b$_2$ was indeed cleaved in a Som1p-independent manner (lanes 2 and 4). These results support our argument that cargo sequences play a critical role in determining whether an Imp1p substrate needs Som1p to be cleaved.

DISCUSSION

Som1p is one of three known subunits, the others being Imp1p and Imp2p, of the yeast IMP. Imp1p and Imp2p are catalytic subunits with nonoverlapping substrate specificity (4), and both subunits are members of the type I signal peptidase family (6). Although the function of Som1p remains largely unknown, previous studies (5, 9, 13) have shown that Som1p has a Som1p-binding site used only when it is normally cleaved by Imp2p in a Som1p-independent manner, but when the substrate is switched to Imp1p, the substrate could be cleaved in a Som1p-dependent manner (i.e., those substrates that require Som1p for signal peptide cleavage) can be processed only when Imp1p operates with high efficiency. Because our study clearly demonstrates that cargo sequences play the major role in dictating Som1p dependence, cargo sequences may interfere with premature cleavage. Interference could occur when the cargo folds into a conformation that renders the cleavage site inaccessible following membrane translocation of the precursor polypeptide. In the presence of Som1p, Imp1p would, according to our interpretation, be able to recognize the precursor and cleave its precursor early during the folding process. It then follows that the conformations of different precursors would influence the Som1p requirement differently. This model thus suggests that, rather than binding substrates directly, Som1p acts through Imp1p to ensure cleavage of a structurally diverse set of protein substrates.

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