Mature DIABLO/Smac Is Produced by the IMP Protease Complex on the Mitochondrial Inner Membrane

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DIABLO/Smac is a mitochondrial protein that can promote apoptosis by promoting the release and activation of caspases. To do so, DIABLO/Smac must first be processed by a mitochondrial protease and then released into the cytosol, and we show this in an intact cellular system. We propose that the precursor form of DIABLO/Smac enters the mitochondria through a stop-transfer pathway and is processed to its active form by the inner membrane peptidase (IMP) complex. Catalytic subunits of the mammalian IMP complex were identified based on sequence conservation and functional complementation, and the novel sequence motif RX5P in Imp1 and NX5S in Imp2 distinguish the two catalytic subunits. DIABLO/Smac is one of only a few specific proteins identified as substrates for the IMP complex in the mitochondrial intermembrane space.

INTRODUCTION

Programmed cell death is a means whereby metazoans can remove unwanted cells, with failure of programmed cell death enabling cancer and autoimmune disease, and inappropriate cell death contributing to neurodegenerative disease. Several of the proteins that regulate cell death, including Bcl-2 family members, signal-transduction receptors, effector proteases and DIABLO/Smac, are specifically enclosed within subcellular structures allowing precise control over the commitment of a cell to die. Understanding where and how these key regulators are localized is crucial in understanding their normal biological function and their role in the pathogenesis of malignant diseases.

A family of intracellular proteases called caspases implement programmed cell death (Ekert et al., 1999). The activity of caspases is regulated by a family of inhibitor of apoptosis proteins (IAPs) that bind and neutralize active caspases (Deveraux and Reed, 1999). For example, the inhibitor MIHA/XIAP/hILP/BIRC4 can bind and neutralize active caspases 3, 7, and 9 (Duckett et al., 1996; Liston et al., 1996; Uren et al., 1996; Deveraux et al., 1997, 1998). This damping of caspase activity provides a layer of regulation over the cell death-promoting activities of this family of effector proteases.

In mammalian cells, signals for cell death can lead to rupture of the mitochondrial outer membrane, and under these conditions the inhibition of caspases can be antagonized by the mitochondrial proteins DIABLO/Smac (Du et al., 2000; Verhagen et al., 2000) and HtrA2/Omi (Suzuki et al., 2001; Martins et al., 2002; Verhagen et al., 2002). Structural studies (Chai et al., 2000; Liu et al., 2000; Wu et al., 2000) have shown that DIABLO is a homodimer (Chai et al., 2000), and each DIABLO dimer can bind to the BIR domains of inhibitor proteins via contacts made to the N-terminal residues of DIABLO (Liu et al., 2000; Wu et al., 2000). The avid interaction DIABLO makes with the inhibitor MIHA competes the inhibitor away from active caspase 9, freeing caspase 9 to proteolytically activate downstream caspases (Ekert et al., 2001).

Mouse DIABLO is translated as a 237-residue precursor protein (preDIABLO) with an N-terminal presequence that must be cleaved to generate the mature form with the amino-terminal sequence A34VPI (Du et al., 2000; Verhagen et al., 2000). Here, we provide evidence that the N-terminal presequence of DIABLO precursor is a bipartite, stop-transfer type targeting signal. We suggest that the precursor form of DIABLO is recognized and translocated through the mitochondrial outer membrane via the translocase in the outer mitochondrial membrane (TOM) complex and in the intermembrane space is transferred to the translocase in the inner mitochondrial membrane (TIM23) complex. The stop-transfer sequence is cleaved by the IMP complex, an oligomeric inner membrane peptidase. We have identified metazoan homologues of the catalytically active subunits of the IMP complex, hitherto only identified in yeast, and show the

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Abbreviations used: IAP, inhibitor of apoptosis protein; IMP, inner membrane peptidase; TIM23, translocase of the inner mitochondrial membrane; TOM, translocase of the outer mitochondrial membrane.
mammalian orthologues function to process, and thereby activate, DIABLO/Smac in mitochondria.

MATERIALS AND METHODS

Plasmids, Yeast Strains, and Media

DNA fragments corresponding to MmImp1 (AK015978) and MmImp2 (AF396934) were amplified by PCR by using primers that generated in-frame restriction sites. PCR products were subcloned in front of green fluorescent protein (GFP)-S65T under the control of the MET25 promoter (George et al., 1998) for analysis by confocal microscopy or into pYADE4 under the control of the ADH1 promoter (Brunelli and Pall, 1996) for the complementation assays. FLAG-tagged MmImp1 and MmImp2 cDNAs were generated by introducing an in-frame XhoI restriction site into the 3′ end of the coding regions by PCR, effectively removing the stop codon of each cDNA.

To generate yeast mutants lacking the IMP1 gene or the SOM1 gene, PCR-mediated gene disruption (Wach et al., 1997) was used with the plasmid pFA6a-HIS3MX6 as template. For the knockin strain, MmImp1 was first cloned into a vector in front of a HIS3 gene marker. This vector was then used for PCR-mediated gene disruption. Yeast plasmids Caspase-3-LacZ, pADH2-(TRP1)-MHLA, and pADH2-(HIS3)-DIABLO1-237 and empty vector controls have been described previously (Hawkins et al., 2001). pGALL-(HIS3)-DIABLO1-237 was generated by subcloning full-length mouse DIABLO with XhoI and NotI into pGALL-(HIS3).

Strains of Saccharomyces cerevisiae were grown at 30°C on YPAD [2% (wt/vol) glucose, 1% (wt/vol) yeast extract, 2% (wt/vol) peptone supplemented with adenine sulfate], grown until late log phase, and harvested by centrifugation; or grown on solid media containing 2% agar in YPAD or YPEG [2% (vol/vol) ethanol, 2% (wt/vol) glycerol, 1% (wt/vol) yeast extract, and 2% (wt/vol) peptone] or YPGal [2% (wt/vol) galactose, 1% (wt/vol) yeast extract, and 2% (wt/vol) peptone supplemented with adenine sulfate].

To determine expression levels of the DIABLO constructs, cytosolic extracts of transformed yeast were prepared according to Cartwright et al. (1997) and analyzed by SDS-PAGE and immunoblots.

To rupture the mitochondrial outer membrane in vivo, yeast cells were cultured transiently on media containing 120 mM acetic acid. Acetic acid is not metabolized by glucose-repressed yeast cells, enters cells in the protonated form, but, if the extracellular pH is lower than the intracellular pH, not metabolized by glucose-repressed yeast cells, enters cells in the protonated form, and 2% (wt/vol) peptone, 1% (wt/vol) yeast extract, and 2% (wt/vol) peptone supplemented with adenine sulfate. To determine expression levels of the DIABLO constructs, cytosolic extracts of transformed yeast were prepared according to Cartwright et al. (1997) and analyzed by SDS-PAGE and immunoblots.

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Preparation of Mitochondria and Protease Sensitivity

Mitochondria were isolated according to Daum et al. (1982). Osmotic shock treatment, to produce rupture the outer membrane in purified mitochondria, was as described by Glick et al. (1992b), and trypsin treatments were performed as described previously (Beilharz et al., 1998). Samples of mitochondrial protein (100 μg) were separated by Tris-glycine SDS-PAGE, and Western blots were carried out according to published methods (Lithgow et al., 1994; Beilharz et al., 1998; Sambrook and Russell, 2001).

Prediction of the Mitochondrial Targeting Sequence in DIABLO

The mitochondrial targeting sequences of hundreds of proteins are now known and are usually rich in positively charged residues and form two to three turns of a helix with amphipathic character (von Heijne, 1986). Predotar (http://www.inra.fr/predotar/) is a neural network predictor trained to find such extensions and predicts a high score (0.984) for the likelihood that the amino terminus of DIABLO is a mitochondrial targeting sequence. Mitoprot II (ftp://ftp.ens.fr/pub/molbio) calculates a probability for a protein being mitochondrial based on physicochemical properties, including a mesohydrophobicity score (Claro et al., 1995; Claro and Vincenzi, 1996). Mitoprot scores DIABLO as 98.99% likely to be a mitochondrial protein. We recently showed that a combined prediction from both Predotar and Mitoprot II is an excellent indicator for mitochondrial location (Lucattini et al., 2004).

RESULTS

The precursor Form of DIABLO Is Targeted to Mitochondria by Its Amino Terminus

Sequence analysis suggested that the previously reported mitochondrial localization of DIABLO in mammalian cells was due to the presence of a mitochondrial targeting sequence at the amino terminus of the protein. This would imply that DIABLO also should be targeted to mitochondria in nonmammalian cells. To determine whether this was true, three GFP reporter proteins were constructed for expression in yeast (Figure 1). GFP was fused to the full preDIABLO sequence [DIABLO(1-237)GFP], to DIABLO(1-53)GFP, or DIABLO(54-237)GFP were cotained with the fluorescent dye MitoTracker Red and viewed by confocal microscopy. Filters selective for the green fluorescence of GFP (left) or the red fluorescence of MitoTracker Red (middle) were used. Green and red fluorescence pictures merged are shown (right) (V, cell vacuole).

Fluorescence Microscopy

For fluorescence microscopy, cells were visualized directly or after staining with MitoTracker (MitoTracker Red CM-H2X(Ros) according to the standard protocol from Molecular Probes (Eugene, OR). All fluorescence images were captured using an MRC1024 confocal scanning laser microscope (Bio-Rad, Hercules, CA) mounted on an Axioskop (Carl Zeiss, Jena, Germany).

Figure 1. DIABLO is targeted to mitochondria in yeast. Yeast cells expressing preDIABLO [DIABLO(1-237)GFP], DIABLO(1-53)GFP, or DIABLO(54-237)GFP were cotained with the fluorescent dye MitoTracker Red and viewed by confocal microscopy. Filters selective for the green fluorescence of GFP (left) or the red fluorescence of MitoTracker Red (middle) were used. Green and red fluorescence pictures merged are shown (right) (V, cell vacuole).

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Isolation of mitochondria from yeast cells expressing preDIABLO showed that much of the precursor form of DIABLO had been processed (Figure 2). As in mammalian cells expressing DIABLO (Du et al., 2000; Verhagen et al., 2000), some unprocessed preDIABLO was still partially exposed on the mitochondrial surface, because it was degraded when mitochondria were incubated with trypsin. The processed form, however, was protected from trypsin cleavage within the outer membrane, as is an apparent processing intermediate. Consistent with this conclusion, when the outer membrane was ruptured by osmotic shock, processed DIABLO was...
degraded by trypsin, as was the intermembrane space protein cytochrome (Cyt) $b_2$. Matrix-located proteins, such as the mitochondrial 70-kDa heat-shock protein (mtHSP70) remained protected from trypsin by the inner mitochondrial membrane, even after the outer membrane was ruptured (Figure 2). The small fraction of DIABLO that is not degraded in this sample remains resistant to protease even in the presence of Triton X-100 (our unpublished data) that solubilizes the inner membrane, suggesting this fraction of the protein is aggregated into a protease inaccessible form. These experiments show that DIABLO is targeted by its amino terminal targeting sequence to the mitochondria, where the targeting peptide is removed liberating DIABLO in the intermembrane space.

**Processing of preDIABLO Is Processed by the IMP Complex**

Mature DIABLO can antagonize the caspase inhibitory properties of MIHA in yeast (Hawkins et al., 2001), and we exploited this cellular system to explore the impact of the amino terminal region of DIABLO on its ability to antagonize IAP activity. Yeast tolerated expression of either the precursor or mature forms of DIABLO (Figure 3A, lanes 1 and 2). Expression of autoactivating caspase 3 was toxic (lane 3), unless inhibited by coexpression of MIHA (lane 4). Coexpression of the mature form of DIABLO (corresponding to DIABLO[54-237]) killed the cells due to liberation of active caspase 3 from MIHA (Figure 3A, lane 5).

To be certain that the death of yeast cells is directly a result of the DIABLO-IAP interaction and requires the correctly processed N terminus of DIABLO, we designed and tested a mutant form (IVPG) of DIABLO. In the crystal structure of the DIABLO-IAP complex (PDB accession 1G73; the N-terminal residues are $A^{54}$, $V^{55}$, $P^{56}$, and $I^{57}$), residues $A^{54}$ and $I^{57}$ are buried at the interface with the IAP BIR3 domain, in small and large pockets, respectively. The $V^{55}$ and $P^{56}$ side chains largely project out and out of the DIABLO-IAP interface. The mutations $A^{54}L$ and $L^{57}G$ were made on the basis that the leucine residue would have difficulty packing in the small pocket in BIR3 that accommodates the small alanine residue, and the presence of a glycine will eliminate all the favorable packing that was contributed by the large isoleucine residue in the large pocket of the IAP. In addition, the mutant sequence IVPG at the N terminus of DIABLO should favor the formation of a type-II beta-turn at VPGA$^{56}$, which should further destabilize the interaction with IAP. These otherwise conservative mutations, $A^{54}L$ and $L^{57}G$, do not affect the expression of the mutant protein as judged by Western blotting of cytosolic extracts (our unpublished data), but the mutant DIABLO (IVPG) is ineffective at promoting the caspase-mediated death of yeast cells (Figure 3B, lane 6).

Yeast cells expressing the precursor form of DIABLO, which is targeted to mitochondria (Figure 1), together with autoactivating caspase 3 and MIHA survived (Figure 3B, lane 9) because the mature, active form is compartmentalized (within the mitochondrial intermembrane space) from MIHA and caspase 3. Nevertheless, rupturing the outer membrane of mitochondria by treatment of intact cells with 120 mM acetic acid (Ludovico et al., 2002) was able to antagonize the antiapoptotic effect of MIHA (Figure 3B, lanes 11 and 12). Because inhibition of MIHA requires the correctly processed AVPI amino terminus of DIABLO (Figure 3A, lane 6), preDIABLO must have been cleaved, in vivo, at the same processing site in yeast as it is in mammalian cells.

**PreDIABLO Is Processed by the IMP Complex**

In yeast, few proteins have been identified that are proteolytically processed for release into the intermembrane space. The three best characterized, Cyt$b_2$, Cyt$c$, and Mcr1 (Gakh et al., 2002), each first dock with the TIM23 complex and are then phosphorylated by the IMP complex. The IMP complex is a hetero-oligomer integrated in the mitochondrial inner membrane, and deletion of any one subunit leads to destabilization and loss of the other subunits (Nunnari et al., 1993; Gakh et al., 2002). Two catalytic subunits, Imp1 and Imp2, are similar in sequence but seem to have nonoverlapping substrate specificities (Nunnari et al., 1993; Gakh et al., 2002). A third noncatalytic subunit, Som1 has been shown to assist substrate recognition by the catalytic Imp1 subunit (Esser et al., 1996; Jan et al., 2000). The oligomeric IMP complex processes mitochondrial precursors releasing mature proteins to the intermembrane space. To determine whether DIABLO is a substrate of the IMP complex, purified mitochondria of yeast strains expressing preDIABLO, but lacking either of the three subunits of the Imp complex, were assayed in Western blots for the presence or absence of the mature form of DIABLO (Figure 4).

The model substrate Cyt$b_2$ is processed in two steps: initially by the matrix peptidase MPP to generate an intermediate (i-Cyt$b_2$; Figure 4), and this intermediate is then substrate for the IMP complex. In wild-type or in $\Delta som1$ mutants, processing to the mature form of Cyt$b_2$ occurred. The Imp1 subunit is primarily responsible for Cyt$b_2$ processing (Nunnari et al., 1993), and Figure 4 shows processing of Cyt$b_2$ is blocked completely in $\Delta imp1$ cells. Because yeast $\Delta imp2$ mutants express decreased levels of the Imp1 subunit (Nunnari et al., 1993), Cyt$b_2$ processing also is also blocked in $\Delta imp2$ cells. Analogously, processing of preDIABLO was partially compromised in $\Delta imp2$ mutants and failed to occur at all in either the $\Delta imp1$ and $\Delta som1$ mutants. We conclude the IMP complex processes preDIABLO to its mature form, and it is likely the Imp1 catalytic site at which this occurs.
Identification of Mouse Homologues for Yeast Imp1 and Imp2 Proteases

An IMP complex has not yet been functionally characterized in organisms other than *S. cerevisiae*. However, iterative BLAST analyses revealed genes predicted to encode proteins similar to Imp1 and Imp2 in all animals and fungi for which substantial genome sequence data exist (Figure 5). The catalytic serine and lysine residues and the structurally important arginine and aspartate residues from the yeast Imp1 and Imp2 (Chen *et al.*, 1999) are conserved across all species (Figure 5, stars). In addition, we found a sequence motif that distinguishes Imp1(RX5P) from Imp2(NX5S). The sequence motif defining the Imp1 and Imp2 subunits sits close by structurally important aspartate residues thought to stabilize the shape of the substrate-binding cleft in the bacterial leader peptidase (Paetzel *et al.*, 1998).

*Mm*Imp1 and *Mm*Imp2 Function as Catalytic Subunits of an IMP Complex

The cDNAs encoding the putative mouse proteases were cloned and expressed in yeast cells to determine their intracellular localization. Both *Mm*Imp1 (*Mm*Imp1 refers to the *Mus musculus* form of Imp1 according to the nomenclature of Pfanner *et al.* (1996) and *Mm*Imp2 localize to mitochondria (Figure 6A). Like yeast Imp1, *Mm*Imp1 could be stably expressed in /H9004imp1 yeast (Figure 6A) but not in wild-type (i.e., Imp1+) yeast cells (our unpublished data). The cytochrome substrates of the IMP complex are key components of the mitochondrial electron transport chain, so that neither Δimp1 mutants nor Δimp2 yeast mutants can grow on nonfermentable carbon sources. We exploited this phenotype to test for complementation, which would imply functional homology by the proposed mouse counterparts of the yeast IMP genes.

The *Mm*Imp2 subunit restored activity in the Δimp2 strain, allowing it to process cytochromes and grow on the nonfermentable carbon sources ethanol and glycerol (Figure 6B). Based on sequence similarities, correct mitochondrial location and functional complementation, we suggest an IMP complex exists in mammalian cells for the processing and activation of proteins such as preDIABLO. That *Mm*Imp1...
The presequences of Cytb2 and Cytc1 residues shown. Imp1-specific residues (RX 5P) are colored pink and Imp2-specific residues (NX 5S) are colored blue. Catalytic residues in CeL. Burri et al.

...catalytic helix that might direct the protein to the TIM23 complex, providing access to the IMP complex for Imp1 and Imp2 (analogous to Ser 90 and K145 of Schizosaccharomyces pombe, Ce, Mm, Dm, C. elegans; Ha, Homo sapiens; Mm, M. musculus). Asterisks designate a possible C-terminal extension to Cytb1. Amino acid residues conserved across at least six species are highlighted, and the total number of residues shown. Imp1-specific residues (RX 5P) are colored pink and Imp2-specific residues (NX 5S) are colored blue. Catalytic residues in Imp1 and Imp2 (analogous to Ser90 and K145 of E. coli leader peptidase) are designated with stars.

**DISCUSSION**

In healthy cells, mature DIABLO is encapsulated within mitochondria, where it cannot interact with IAPs. Here, we have shown that after preDIABLO is translated in the cytosol and transported across the mitochondrial outer membrane, it is processed by the IMP complex in the intermembrane space to produce the potent, mature form. Activated DIABLO remains in the intermembrane space until an apoptotic signal is received (Figure 7A). DIABLO then remains in the intermembrane space until an apoptotic signal is received (Figure 7A).

The loss of Imp2 destabilizes the IMP complex so that Δimp2 yeast cells process preDIABLO only poorly. However, this impairment was reversed by expression of MmImp2 in the Δimp2 yeast (Figure 6C). We conclude that preDIABLO is proteolytically activated to DIABLO by the IMP complex and that the mammalian Imp1 and Imp2 sequences represent functional homologues of the yeast IMP complex subunits.

**Other Substrates of the Mammalian IMP Complex**

A number of proteins that might potentiate apoptosis are released from mitochondria (van Gurp et al., 2003; Saelens et al., 2004). None of these have the hallmarks that would suggest activation by the IMP complex. A candidate substrate, AIF is a peripheral component of the mitochondrial inner membrane (Arnoult et al., 2002) made as a precursor protein, with a presequence of 101 residues processed after by the IMP complex, making its import pathway into mitochondria (Figure 7A) analogous to the stop-transfer pathway traveled by Cytb2 and Cytc1 (Glick et al., 1992a,b).

Imp1 and Imp2 are members of the signal peptidase family of proteases. In Escherichia coli, a signal peptidase removes the presequence of proteins translocated across the bacterial membrane (Paetzel et al., 2002). Two proteins related to the IMP subunits are present in all animals and fungi where complete genome information is available, and a diptych of amino acid residues corresponding to the motif RX 5P in Imp1 and NX 5S in Imp2 seems to be diagnostic for Imp1 or Imp2. The RX 5P motif of Imp1 and its surrounding residues are conserved in the E. coli leader peptidase (Figure 5), and as part of a thorough examination of the structure of crystallized leader peptidase, Paetzel et al. (1998, 2002) suggested that arginine in (the RX 5P motif) is important in stabilizing the active site region and positioning of surrounding amino acids. We note from their data that the proline residue seems to be involved in another structurally important position, in a turn between two beta-sheets (Strahm and Lithgow, unpublished observations). The distinguishing motif in Imp2(NX 5S) and the context provided by surrounding residues might influence the structure around the active site enough to broaden the range of substrates that can be processed by the IMP complex. That the RX 5P and NX 5S sequence motifs are each so widely conserved through evolution shows they are fundamentally important for IMP complex activity and makes them the diagnostic motif in distinguishing Imp1 from Imp2.
Import into mitochondria (Susin et al., 1999). It is not clear yet which protease is responsible for AIF processing, but the presequence of AIF shows no obvious similarity to a stop-transfer sequence, and the processing site has none of the residues suggested to be important for recognition by the IMP complex (Gakh et al., 2002).

Cytochrome c is imported into the intermembrane space via the TOM complex without the participation of the TIM complex and without any proteolytic processing (Diekert et al., 2001; Wiedemann et al., 2003). HtrA2/Omi is targeted to the intermembrane space of mitochondria, but it is processed autocatalytically in mammalian cells (Seong et al., 2004) and in yeast (Verhagen and Silke, unpublished results), and its role in apoptosis might be secondary to its role as a molecular chaperone for other mitochondrial proteins (Vaux and Silke, 2003).

Endonuclease G (EndoG) is a mitochondrial protein, released during cell death, that contributes to nuclear DNA fragmentation in the terminal stages of apoptosis (Li et al., 2001). EndoG is encoded in the nucleus, translated as an ~33-kDa precursor in the cytosol, translocated across the mitochondrial membranes with the presequence and then cleaved to yield the ~28-kDa mature nuclease (Schaefer et al., 2004). EndoG had been tentatively suggested to be located in import into mitochondria (Susin et al., 1999). It is not clear yet which protease is responsible for AIF processing, but the presequence of AIF shows no obvious similarity to a stop-transfer sequence, and the processing site has none of the residues suggested to be important for recognition by the IMP complex (Gakh et al., 2002).

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Figure 6. MmImp1 and MmImp2 are mouse subunits of the IMP complex. (A) GFP was fused to the C terminus of MmImp1 and MmImp2 and the fusion proteins expressed in Δimp1 or wild-type yeast cells. Cells were costained with the fluorescent dye MitoTracker Red and visualized by confocal microscopy. Z-sections through representative cells are shown. Green fluorescence of GFP in groups of cells (left), red fluorescence (middle), and the merged pictures of green and red fluorescence (right). (B) Wild-type yeast cells and the Δimp1 or Δimp2 mutants were transformed with the respective mouse homologue to be tested for complementation of the growth defect on the nonfermentable carbon sources glycerol and ethanol (YPEG). In addition, the open reading frame in the yeast gene encoding Imp1 (YMR150c) was directly replaced with the MmImp1 open-reading frame through homologous recombination (KI MmImp1). (C) Mitochondria expressing preDIABLO isolated from wild type, yeast mutant lacking Imp2, and yeast mutant lacking Imp2 but expressing MmImp2 were compared after SDS-PAGE separation of proteins. Amounts of DIABLO and the control protein Tom70 were compared by Western blot analysis.

Figure 7. A stop-transfer pathway for the import and activation of DIABLO by mitochondria. (A) The precursor form of DIABLO (black) is synthesized in the cytosol and the N-terminal presequence recognized by the TOM complex and thereby transferred across the outer membrane. Engaged in the TIM23 complex, DIABLO might be processed by the matrix-located peptidase, such as mitochondrial processing peptidase, and is processed by the IMP complex composed of Imp1, Imp2, and Som1. Release of processed DIABLO into the intermembrane space allows for its assembly into a dimer and its availability for release into the cytosol only if the outer membrane is ruptured. (B) The presequences of DIABLO (top schematic) and Cyt b2 (bottom schematic) show similar regions corresponding to a basic amphipathic helix with the matrix targeting information (gray) and the intermembrane sorting sequence, containing a core of hydrophobic residues (dark gray) preceded by a cluster of charged residues (black) important for recognition of the sorting sequence. For DIABLO the IMP1 processing site is designated between C53 and A54 and for Cyt b2 between N80 and E81.
the intermembrane space (Ohsato et al., 2002), but EndoG must be located in the mitochondrial matrix for it to generate the primers needed for mitochondrial DNA replication (Cote and Ruiz-Carrillo, 1993) and would therefore be processed by matrix-located proteases (Gakh et al., 2002).

Discrete defects in mitochondrial protein import and sorting can lead to human disease. Mohr–Tranenberg syndrome, a deafness-dystonia disorder, results through mutations that effect assembly of the Tim8/Tim13 complex, thereby inhibiting protein sorting to the TIM22 complex (Roesch et al., 2002; Binder et al., 2003). Chromosomal mapping of patients suffering from Gilles de la Tourette syndrome, another neurological condition, has revealed that one of the genes located at a breakpoint region (7q31) that is associated with symptom development encodes the human ortholog of the protein we designate here as MfnImp2 (Petek et al., 2001; Gakh et al., 2002). Speculation, based on sequence similarity the authors noted to the yeast Imp2, suggested that defects in respiratory chain complexes might impact on the etiology of Tourette syndrome and other neuropsychiatric disorders (Petek et al., 2001; Gakh et al., 2002). Our findings on the role of the IMP complex in processing non-mitochondrially imported proteins and their targeting sequences. Eur. J. Biochem. 273, 35268–35272.

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