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Mitochondrial import of Omi: The definitive role of the putative transmembrane region and multiple processing sites in the amino-terminal segment

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Abstract

The mitochondrial serine protease Omi/HtrA2 has a proapoptotic role in mammalian cells. However, neither the topology nor the processing of Omi in mitochondria is clearly understood. To determine the topology of Omi in the mitochondrial IMS, EGFP fusions were expressed with the entire N-terminal segment of full-length Omi (FL-Omi) (133-EGFP), and that without the transmembrane region (ΔTM-EGFP) in the cells. Immunocytochemical staining and alkaline extraction experiments revealed that the TM determines the topology of Omi in the IMS and anchors the pro form into the inner membrane. As a result, the protease and the PDZ domains are exposed to the IMS. Mature Omi largely exists in the IMS as a soluble form. The processing sites of the precursor protein were examined by *in vitro* import experiments. The import of the processing mutants revealed importance of Arg80, Arg91, and Arg93 residues for the processing of the N-terminal segment of FL-Omi. These results suggest that the N-terminal segment of FL-Omi contains multiple processing sites processed by matrix processing proteases.

Keywords: Omi/HtrA2; Processing; Matrix processing protease

Most mitochondrial proteins are synthesized in the cytosol as precursors with N-terminal presequences. The presequence is proteolytically removed by matrix processing proteases during import [1,2]. In particular, the mitochondrial processing peptidase (MPP) is the best-characterized matrix processing protease. The MPP exists in the mitochondrial matrix. Four kinds of MPP processing site motifs have been identified for the mitochondrial proteins [2]. The R-2 motif is xRx↓x(S/x) and the R-3 motif is xRx(Y/x)↓(S/A/x)x. The R-10 motif is xRx↓(F/L/I)xx(S/T/G)xxxx↓ and is cleaved sequentially by the MPP and the mitochondrial intermediate peptidase (MIP). In addition, the R-none motif is also present (xx↓x(S/x)).

The human mitochondrial serine protease Omi/HtrA2 consists of a mitochondrial targeting sequence (MTS) in its N-terminus, a putative transmembrane region (TM), a conserved serine protease domain, and a single C-terminal PSD-95/Dlg/ZO-1 homology (PDZ) domain [3,4]. The mature Omi localizes in the IMS after the processing of the precursor [5–7].

Following apoptotic stimuli, the mature Omi is released from the mitochondria into the cytosol, and it executes apoptosis in a caspase-dependent manner [5–8]. The mature Omi antagonizes the inhibitory effect of inhibitor of apoptosis proteins (IAPs) on caspases through interaction with the IAP-binding motif and causes proteolytic degradation of bound IAPs, thereby activating caspases

Abbreviations: AIF, apoptosis-inducing factor; EGFP, enhanced-green fluorescence protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IM, inner membrane; IMS, intermembrane space; MPP, mitochondrial processing peptidase; MTS, mitochondrial targeting sequence; PDZ, PSD-95/Dlg/ZO-1 homology; TIM, translocase of the inner membrane of mitochondria; TM, transmembrane region.

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indirectly [5,6,8–10]. In addition, the mature Omi triggers apoptotic cell death in a caspase-independent manner and this function depends on its serine protease activity [5].

Although recent studies have focused on the biological importance of the Omi protein, the topology and processing of Omi in mitochondria have not yet been sufficiently characterized. This study was designed to characterize the localization of Omi in the IMS and, for the first time, determine the processing steps of the precursor protein in the matrix. The TM of full-length Omi (FL-Omi) determines the topology of the protein toward the IMS. The pro form Omi is anchored to the IM and exposes the protease and the PDZ domains to the IMS. A large proportion of the mature Omi remains in the IMS in a soluble form. Moreover, the Arg residues in the N-terminal region of FL-Omi that are necessary for multiple processing by matrix processing proteases have been identified.

Materials and methods

Subcellular fractionation. The cells were suspended in ice-cold 1% Triton X-100 lysis buffer (10 mM Hepes–KOH, pH 7.4, 150 mM NaCl, 10% glycerol, and 1% Triton X-100). After sonication, the suspension was centrifuged at 15,000*g* for 10 min at 4 °C, and the supernatant was used as whole cell extract. For digitonin fractionation, the cells were suspended in 0.2 mg/ml digitonin buffer (10 mM Hepes–KOH, pH 7.4, 150 mM NaCl, 10% glycerol, and 0.2 mg/ml digitonin) as described previously [11]. To separate the soluble and the particulate fractions, the suspension was centrifuged at 15,000*g* for 10 min at 4 °C. The whole cell extracts and the fractions were analyzed by immunoblotting, and the blots were developed using enhanced chemiluminescence reagents.

Fractionation of isolated mitochondria. Isolation of mitochondria from HeLa-S3 cells was done as described previously [12]. The isolated mitochondria were sub-fractionated with the Na_2CO_3 buffer [13]. Briefly, the mitochondria were sonicated in 0.1 M Na_2CO_3 buffers (pH 11.5 or pH 10.5) or in the high-salt buffer (10 mM Hepes–KOH, pH 7.4, 0.2 mM EGTA, 0.5 M NaCl), and then the samples were incubated for 30 min on ice. To separate the soluble and the membrane fractions, the samples were centrifuged at 100,000*g* for 1 h at 4 °C. The fractions were analyzed by immunoblotting.

In vitro import into isolated mitochondria. The plasmids, pGEM-3Zf(+)-human ornitthine transcarbamylase precursor (pOTC)-GFP [14], pTNT-133-EGFP and 133-EGFP processing mutants were used for the templates of *in vitro* transcription reactions. The ^{35}S-labeled translation products in the reticulocyte lysate (10 μl) were imported into the isolated rat liver mitochondria (100 μg of protein) in 50 μl of reaction mixture at 25 °C [15]. The reaction was stopped by adding 0.1 mM dinitrophenol [15]. The mitochondria were reisolated by centrifugation and then subjected to SDS–PAGE followed by fluorography. Relative intensities of the labeled bands were quantitated by imaging plate analysis using a BAS2500 image analyzer (Fuji Photo Film Co., Ltd.).

Results

Subcellular localization of the expressed 133-EGFP derivatives in COS-7 cells

To confirm the subcellular localization of Omi, the expression plasmids for 133-EGFP derivatives were constructed. The 133-EGFP has the N-terminal segment of the full length-Omi (FL-Omi) and EGFP. MTS-EGFP has the mitochondrial targeting sequence (MTS) only. ΔTM-EGFP lacks the putative transmembrane region (TM), while ΔMTS-EGFP lacks the MTS (Supplementary Fig. 1A). These plasmids were transfected into COS-7 cells (Supplementary Fig. 1B). In the cells expressing 133-EGFP, ΔTM-EGFP, and MTS-EGFP, the particulate fluorescence of GFP was observed and it overlapped with the localization of mitochondria. In contrast, in the cells expressing ΔMTS-EGFP and EGFP, the fluorescence was distributed throughout the cells. These observations indicate that the N-terminal MTS of FL-Omi is sufficient for mitochondrial targeting and the deletion of putative TM does not affect the mitochondrial localization. Next, COS-7 cells transiently expressing 133-EGFP, ΔTM-EGFP, and EGFP were fractionated with digitonin and analyzed by immunoblotting (Fig. 1). The 32 kDa and the 28 kDa products of 133-EGFP and endogenous mitochondrial proteins, Omi, apoptosis-inducing factor (AIF), and Hsp60, were almost completely recovered in the particulate fraction. The majority of the 30 kDa product of ΔMTS-EGFP was also recovered in the particulate fraction. In contrast, more of the 28 kDa EGFP protein and an endogenous cytosolic protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were recovered in the soluble fraction than in the particulate fraction. These results indicate that 133-EGFP and ΔTM-EGFP proteins are localized in the mitochondria.

The putative TM of FL-Omi is necessary for mitochondrial IMS localization

The localization of Omi fusion proteins in the mitochondria was then examined. The cells expressing 133-EGFP and ΔTM-EGFP were treated with 0.4 mg/ml digitonin to permeabilize the outer membrane of mitochondria or with 2.0 mg/ml digitonin for permeabilizing the outer and the inner membranes of mitochondria. The permeabilized cells were analyzed by immunocytochemical staining

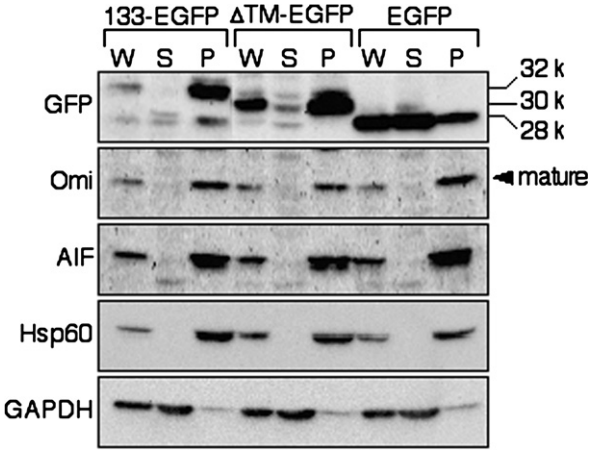

Fig. 1. Fractionation of the transfected cells with 0.2 mg/ml digitonin. The fraction were separated by SDS–PAGE, and subjected to immunoblot analysis. The arrowhead indicates the position of mature Omi. W, whole cell extract; S, soluble fraction; P, particulate fraction.