SUPPLEMENTAL INFORMATION

Supplemental Materials and Methods

Biochemicals. Restriction enzymes and DNA-modifying enzymes were purchased from Nippon Gene and Takara. Fetal calf serum and DMEM were from SIGMA Chemical Co.

Western blotting. Western blotting were performed using anti-FLAG (SIGMA), anti-LDH (SIGMA), anti-cytochrome c (Pharmingen), anti-AIF (clone E1; Santa Cruz Biotechnology), anti-Hsp70 (StressGen), anti-Tim23, anti-PARP (Promega), anti-myc (BIOMOL)、anti-smac/DIABLO (BD Bioscience), anti-HtrA2/omi (R & D Systems), and anti-Tom22 (SIGMA) primary antibodies followed by peroxidase-coupled, goat anti-rabbit or anti-mouse secondary antibodies (Biosource). Immunodetection was performed by ECL (Amersham).

Construction of AIF variants. AIF fusion constructs, alanine substitution mutants, and T7-tag-containing mutants were all created by PCR using rat AIF cDNA, pEGFP-N1 (Clontech), or their derivatives as the templates and appropriate combinations of forward and reverse oligonucleotide primers. All the constructs were confirmed by nucleotide sequencing and used for the assays.

Protein sequencing. The immuno-purified mature AIF from non-apoptotic cells was subjected to SDS-PAGE and then electro-blotted to PVDF membrane. The PVDF membrane was stained with Coomassie Brilliant Blue. The band corresponding to mature AIF (62-kDa) was electro-eluted and subjected to Edman degradation.

Supplemental Figure Legends

Figure 1. Determination of digitonin concentration for specific permeabilization of

the mitochondrial outer membrane.

(A) Immunofluorescence staining of HeLa cells. HeLa cells were treated with the indicated digitonin concentrations and then stained with antibodies to Hsp60 (matrix marker), cytochrome c (IMS marker) and HtrA2 (IMS marker). Magnification x 630; *bar* = 20 μm. **(B)** Immunoblot analysis of the supernatant and membrane fractions. Subcellular fractions prepared from HeLa cells after digitonin-treatment were subjected to SDS-PAGE followed by immunoblot analysis using the antibodies against the indicated proteins. Sup, digitonin-soluble fractions; ppt, digitonin-insoluble membrane fractions. LDH, lactate dehydrogenase (cytoplasmic marker).

Figure 2. Membrane binding properties of the endogenous AIF and exogenously expressed N120-GFP-FLAG and N120ΔH-GFP-FLAG.

Mitochondria were isolated from HeLa cells expressing N120-GFP-FLAG or N120ΔH-GFP-FLAG. They were treated with 100 mM sodium carbonate (pH 10.5) and centrifuged to separated into the supernatant (S) and membrane (P) fractions. Both fractions were subjected to SDS-PAGE and following immunoblot analysis using antibodies against FLAG (for N120-GFP and N120ΔH-GFP), AIF (for endogenous AIF), cytochrome c (IMS protein), Tim44 (matrix protein loosely associated with the inner membrane), Tim23 (inner membrane protein), and mHsp70 (matrix protein). Note that we modified standard alkali-extraction protocol [100 mM sodium carbonate (pH 11.5)] and used 100 mM sodium carbonate adjusted to pH 10.5. This is because hydrophobicity of the TMS of AIF is lower compared with other mitochondrial inner membrane proteins. In fact, AIF is released from the membrane to a significant extent by 100 mM sodium carbonate (pH 11.5) (Arnolt *et al.*, 2002; our unpublished results).

Behavior of various marker proteins indicated that the fractionation is adequately performed .

Figure 3. Permeabilization of mitochondrial outer membrane precedes mitochondrial release of AIF. (A) HeLa cells or HeLa/XL cells expressing a higher level of 6xHis-Bcl-XL, were transfected with the processing site-less mutant N95-GFP and treated with actinomycin D in the presence of zVAD-fmk. At the indicated time points, cells were treated with 25 μg/ml digitonin that specifically permeabilized plasma membranes and stained with anti-FLAG antibodies for immunofluorescence microscopy. **(B)** HeLa cells or HeLa/XL cells were permeabilized with 25 μg/ml digitionin and stained with the antibodies against mitochondrial outer membrane protein Tom22 (extruding the N-terminal segment to the cytosol). Note that the expressed protein did not interact with the externally added anti-FLAG antibody at the initial time of apoptosis **(A, upper panel)**. The number of FLAG-positive mitochondria increased as apoptosis proceeded; ~30% after 4 h, and ~75% after 7 h **(A, upper panel)**. As the control, bitopic outer mitochondrial membrane protein Tom22 could be recognized by the added antibodies **(B)**.