Autocatalytic Processing of HtrA2/Omi Is Essential for Induction of Caspase-dependent Cell Death through Antagonizing XIAP*

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A mature form of nuclear-encoded mitochondrial serine protease HtrA2/Omi is pivotal in regulating apoptotic cell death; however, the underlying mechanism of the processing event of HtrA2/Omi and its relevant biological function remain to be clarified. Here, we describe that HtrA2/Omi is autocatalytically processed to the 36-kDa protein fragment, which is required for the cytochrome c-dependent caspase activation along with neutralizing XIAP-mediated inhibition of caspases through interaction with XIAP, eventually promoting apoptotic cell death. We have shown that the autocatalytic processing of HtrA2/Omi occurs via an intramolecular event, demonstrated by incubating an in vitro translated HtrA2/Omi (S306A) mutant with the enzymatically active glutathione S-transferase-HtrA2/Omi protein. Using N-terminal amino acid sequencing and mutational analysis, we identified that the autocatalytic cleavage site is the carboxyl side of alanine 133 of HtrA2/Omi, resulting in exposure of an inhibitor of apoptosis protein binding motif in its N terminus. Our study provides evidence that the autocatalytic processing of HtrA2/Omi is crucial for regulating HtrA2/Omi-mediated apoptotic cell death.

HtrA1 (high temperature requirement A), also known as DegP, was first described as a periplasmic protein in Escherichia coli (1, 2). HtrA acts as a serine protease at high temperatures and as a chaperone at low temperatures (3–5). Most studies have shown that HtrA serine proteases exhibit endopeptidase activity by cleaving misfolded proteins or other cellular proteins as well as autocatalytic processing activity by mediating self-catalytic processing of HtrA serine proteases (4–6).

Mammalian homologues of HtrA, human HtrA (huHtrA1/L56), huHtrA2/Omi, and huHtrA3, have recently been identified (7–10). huHtrA1L56, which is the human homologue of the E. coli HtrA, was previously identified as a protein repressed in SV40-transformed fibroblasts and differentially expressed in osteoarthritic cartilage (9). The second mammalian homologue of HtrA, human HtrA2 (also known as Omi), was first identified as a protein that interacts with Mxi2, an alternatively spliced form of p38 stress-activated kinase (SAPK) (8). HtrA2 mRNAs are expressed ubiquitously in various tissues and cell types (8, 10). The full-length HtrA2 protein, which encodes 458 amino acid residues, consists of a mitochondrial target sequence in its N-terminal region, a putative transmembrane (TM) domain, an inhibitor of apoptosis protein (IAP) binding motif (IBM), a single C-terminal PDZ (post-synaptic density, discs large and zonula occludens) domain that mediates protein-protein interactions (11–13), and a conserved catalytic domain of serine proteases that contains the His198, Asp226 and Ser306 catalytic triad and GNS306GGPL motif in its conserved active site (8, 14).

Recently, HtrA2 has shown an intriguing function, contributing both to caspase-dependent and caspase-independent cell death (15–19). HtrA2 antagonizes inhibitory effect of XIAP (X-linked inhibitor of apoptosis) on caspases through interaction with the IBM of HtrA2. Moreover, HtrA2 is involved in caspase-independent cell death through regulating its serine protease activity; however, the molecular mechanism in this pathway remains to be elucidated. HtrA2 is predominantly localized to the mitochondria, from which a mature form is released into the cytosol in response to apoptotic stimuli (14–18, 20, 21). The N-terminal 133 amino acid residues seem to be processed in the mature HtrA2 protein (15–18, 22). Processing of HtrA2 exposes an internal tetrapeptide (AVPS) reaper-like motif or an IBM in its N terminus, which is conserved in Smac/DIABLO (Smac/direct IAP-binding protein with low pI) and the Drosophila death proteins Reaper, Grim, Hid, and Sickle (14, 23–30). Mature HtrA2 is involved in regulating apoptotic induction along with neutralizing XIAP-mediated inhibition of caspases through interaction with XIAP in the cytosol in response to the apoptotic stimuli (15, 31–34). These previous studies indicate that the processing of the HtrA2 precursor to the mature form is indispensable for apoptotic regulation. Nonetheless, to date no in-depth studies have examined whether HtrA2 undergoes autocatalytic processing to generate the mature form or whether it is cleaved by other

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¶ The abbreviations used are: HtrA, high temperature requirement A; TM, transmembrane; DTT, dithiothreitol; GST, glutathione S-transferase; IAP, inhibitor of apoptosis protein; IBM, IAP binding motif; Smac, second mitochondria-derived activator of caspase; XIAP, X chromosome-linked IAP; IB, immunoblot; ICAD, anti-inhibitor of the caspase-activated DNase; GFP, green fluorescent protein.
mitochondrial proteases (19, 22, 33, 35). Here, we present a detailed investigation of HtrA2 autocalytic processing, occurring through an intermolecular mechanism that was observed in an in vitro cleavage reaction in which proteolytically inactive HtrA2 served as a substrate for catalytically active HtrA2. In addition, we demonstrate that the mature HtrA2 generated by the autocalytic processing can promote caspase activation by inhibiting the XIAP activity, inducing apoptotic cell death.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Antibodies**—All chemicals were purchased from Sigma unless otherwise stated. The His-tagged HtrA2 protein was expressed in BL21 (DE3) (F’ompT hsdS2F’ rK30) gal dcm bacterial strain (Stratagene) and purified by His-Bind kits (Invitrogen). Rabbits and rats were immunized with the purified His-tagged HtrA2 protein to generate HtrA2-specific polyclonal antiserum, which was purified by protein G affinity chromatography (Koma Biotechnology) (36). Other antibodies used for immunoblot (IB) analyses were anti-FLAG (Sigma), anti-caspase-3 (Pharmingen), anti-inhibitor of the caspase-activated DNase (ICAD), anti-cytokyme c (Santa Cruz Biotechnology), anti-poly(ADP-ribose) polymerase (Pharmingen), and anti-XIAP (Transduction Laboratories) antibodies.

**Construction of Vectors for Expression of Recombinant HtrA2 and the S306A Mutant**—The N-terminally truncated HtrA2 constructs, Δ129 (truncation of amino acid residues 1–129) and Δ133 (truncation of amino acid residues 1–133), were generated by PCR amplification with Pfu DNA polymerase (Stratagene) from the pcDNA-HtrA2-FLAG as a template (37). The resulting PCR-amplified fragments were subcloned into the pGEX-4T (Amersham Biosciences) vector carrying an N-terminal GST epitope tag for purification of proteins expressed in E. coli. The QuickChange site-directed mutagenesis kit (Stratagene) was used according to manufacturer’s instructions to generate a proteolytically inactive mutant (S306A) and a putative cleavage site mutant (R/R) of HtrA2. The resulting HtrA2(S306A) and (R/R) mutants contained an alanine substitution at amino acid residues 306 and an arginine substitution at both 132 and 133 amino acid residues, respectively. Details of all plasmid constructs and all primer sequences are available upon request. The sequence integrity of all plasmid constructs was verified by DNA sequencing with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).

**Purification of Recombinant HtrA2 Expressed in E. coli**—Expression of GST-HtrA2 fusion proteins in BL21 cells was induced with 0.1 mM isopropyl-1-thio-β-D-galactoside as described previously (36, 37). The cultures were collected by centrifugation, and the bacterial pellets were resuspended in lysis buffer (50 mM Tris-HCl, 120 mM NaCl, 0.5% Nonidet P-40 (pH 8.0)) containing 7 mM dithiothreitol (DTT) and 2 μg/ml protease inhibitors, aprotinin and leupeptin. The GST-HtrA2 protein was purified from crude cell lysates under non-denaturing conditions by selective binding to glutathione-Sepharose 4B beads (Amersham Biosciences) as described previously (38–40). The bead-bound GST-HtrA2 proteins were resuspended in 1× SDS loading buffer and resolved by 15% SDS-PAGE. For purification of GST-HtrA2, the protein-bound beads were resuspended in 100 μl of elution buffer (50 mM Tris-HCl (pH 7.6), 20 mM KCl, 1 mM EDTA) containing 5 mM reduced glutathione. The GST-HtrA2 protein was eluted from glutathione beads by incubation for 5 min at 37 °C. Protein purity and concentrations were estimated by comparison with bovine serum albumin of known concentration in SDS-PAGE followed by staining of the gel with Coomassie Brilliant Blue R-250 dye.

For purification of HtrA2, the HtrA2 protein-bound beads were resuspended in 100 μl of cleavage buffer (50 mM Tris-HCl (pH 7.5), 1 mM KCl, 1 mM DTT) and incubated with 5 units of human thrombin (Amersham Biosciences) for 1 h at room temperature. The cleaved proteins were eluted from the GST-bound beads by brief centrifugation. Purified proteins were stored at −70 °C in a final glycerol concentration of 20% and 7 mM DTT and were stable for several months.

**Proteolytic Cleavage Assays and N-terminal Amino Acid Sequencing**—[35S]Methionine-labeled mutant forms of HtrA2 were prepared by in vitro transcription and translation of plasmids encoding various forms of HtrA2 as templates in the TNT T7 Coupled Reticulocyte Lysate system (Promega). The reaction mixtures were added to proteolytically active or inactive forms of Δ133 to a final concentration of 0.5 μM in 30 μl of cleavage buffer (50 mM Tris-HCl (pH 7.5), 1 mM DTT) for 6 h at 37 °C. The reaction products were analyzed by 15% SDS-PAGE, and gels were dried and exposed to x-ray film.

Amino acids at the cleavage site were identified by N-terminal se-
**Fig. 1.** **HtrA2 is autocatalytically processed in an intermolecular manner.** A, schematic representation of human full-length HtrA2 and GST-HtrA2 constructs. Full-length HtrA2 consists of distinct functional domains: mitochondrial target sequence (amino acids 1–40), TM domain (amino acids 105–123), an IBM (AVPS, amino acids 134–137), a serine protease domain (amino acids 166–342), and a PDZ domain (amino acids 364–445). Δ133 contains an IBM, and Δ129 contains 4 additional amino acid residues (MVLA, amino acids 130–133) of HtrA2. S306A indicates the catalytically inactive mutation by replacing the catalytic serine 306 residue with alanine. Subscript numbers indicate the amino acid residues of HtrA2. **B,** autocatalytic processing of HtrA2. [35S]Met-labeled Δ129 (S306A) as a substrate was incubated with proteolytically active Δ133 or inactive Δ133 (S306A). Both Δ133 proteins were expressed GST fusions and purified by selective binding to glutathione-Sepharose 4B beads (bottom panel). The reaction mixture was resolved by 15% SDS-PAGE and visualized by autoradiography (top panel). wt, wild type. C, endoproteolytic activity of Δ133 and Δ133 (S306A) was determined by the reaction with β-casein as an exogenous substrate. The reaction mixture was resolved by 15% SDS-PAGE, and the cleavage of β-casein was visualized by staining with Coomassie Brilliant Blue dye.

**RESULTS**

**HtrA2 Is Autocatalytically Processed in an Intermolecular Manner**—To identify the autocatalytic cleavage site of HtrA2, we constructed in-frame fusions of N-terminally truncated HtrA2 with GST, and GST fusion proteins expressed in *E. coli* were purified by glutathione-Sepharose 4B beads (Fig. 2). Full-length HtrA2 was unavailable for biochemical studies because it was highly susceptible to proteolytic degradation during expression in *E. coli* (37). Fortunately, we found that Δ129 is a suitable form for biochemical studies of its enzyme activity and proteolytic processing, since the Δ129 protein expressed in *E. coli* not only is as soluble as other truncated portions of HtrA2 but also contains additional amino acid residues preceding the putative cleavage site, 130MVLAVPSPP (Fig. 1A) (36).

During expression of GST-HtrA2 in *E. coli* and purification, several protein fragments of lower molecular mass were detected in the catalytically active wild-type, Δ133 and Δ129, whereas these fragments were barely detectable in the catalytically inactive mutants, Δ133 (S306A) and Δ129 (S306A) (Fig. 2A). The result suggests that in SDS-PAGE, the faster-migrating bands were due to proteolytic processing of HtrA2. To verify whether these fragments were autocatalytic processing products of HtrA2, we analyzed them by IB analysis with anti-HtrA2 (Fig. 2B) or anti-FLAG (Fig. 2C) antibodies.
Two fragments of relative molecular mass 36 and 43 kDa, which were predicted by comparison with the relative molecular mass of size marker proteins, were specific to anti-HtrA2 or anti-FLAG antibodies.

To identify the amino acid sequence in the N-terminal side of the scissile bond, we selected the 36-kDa fragment, designated p36, with a band size similar to mature HtrA2. The N-terminal amino acid sequence of this proteolytic fragment was identified as AVPSFP by N-terminal amino acid sequencing, representing that the autocatalytic cleavage site starts with alanine 134 (Fig. 3, A). Consequently, an IBM of HtrA2 is exposed by the autocatalytic Ala\textsuperscript{133}-Ala\textsuperscript{134} cleavage of it. Additionally, the presence of a larger, ~43-kDa fragment suggests that there are other cleavage sites within GST for HtrA2 endoproteolytic activity, since the 43-kDa fragment was also specific to anti-HtrA2 and anti-FLAG antibodies that recognize the C-terminal region of HtrA2 (37).

p36 was not detected from even wild-type Δ133 that had methionine and alanine residues in the junction between GST and mature HtrA2 (Figs. 2 and 3A). It is probable that the amino acid sequence GSAM in Δ133 was less susceptible to self-proteolytic processing than the amino acid sequence MVLA surrounding the cleavage site in Δ129, although both proteins consisted of the same AVPS residues in the \( P_4 \) through \( P_4' \) subsites (Fig. 3A). The results suggest that the amino acid residues in the \( P_4 \) through \( P_4' \) sites of the substrate seem to be one of the significant criteria for specificity to HtrA2 processing (5, 14, 43, 44).

p36 Serves as an Inhibitor of XIAP through Interaction with XIAP, Promoting the Activation of Caspases—To determine whether p36 interacts with XIAP, we performed in vitro binding assays with S100 extracts and the recombinant HtrA2 proteins, which were cleaved by thrombin digestion and purified by elution from the GST-bound beads (Fig. 3B). The thrombin-cleaved HtrA2 proteins were unable to interact with XIAP because extra amino acid residues preceding an IBM made them more conformationally restricted by masking a methyl group (−CH\textsubscript{3}) and the protonated amino group (−NH\textsubscript{3}+) of the N-terminal alanine of an IBM (Fig. 3, A and B) (26, 27, 30). Only wild-type Δ129 exhibited the interaction with endogenous XIAP, whereas no bands were detectable from Δ129 (S306A) as well as wild-type Δ133. The result demonstrates that p36 is essential for the interaction with XIAP (Fig. 3B).

Subsequently, we investigated whether p36 promotes cytochrome c-dependent activation of the caspases by eliminating the inhibitory effect of XIAP (Fig. 3C). To analyze this function in vitro, the thrombin-cleaved recombinant HtrA2 proteins were incubated with S100 extracts supplemented cytochrome c and dATP. In the presence of cytochrome c and dATP, procaspase-3 was proteolytically cleaved to the active forms, p20 and p17 (Fig. 3C, lane 2). However, the addition of XIAP completely abolished the activation of procaspase-3 (Fig. 3C, lane 3). p36 was observed only in wild-type Δ129 but not in Δ129 (S306A) or wild-type Δ133 (Fig. 3C, third panel). Likewise, we observed that the inhibitory effect of XIAP on the caspase activation was eliminated by incubating with wild-type Δ129, resulting in the proteolytic cleavage of procaspase-3 (Fig. 3C, lane 6).

ICAD is a caspase-3 substrate that needs to be cleaved to release active caspase-activated DNase (45, 46). To investigate whether the active caspase-3 cleaves ICAD, we analyzed IBs with anti-ICAD antibody (Fig. 3C, second panel). Activation of procaspase-3 promoted cleavage of the ICAD precursor to the processed product (~12 kDa) of ICAD. Taken together, the ability that HtrA2 enhances the activation of procaspase-3 and the enzymatic activity of caspase-3 is dependent upon exposing the IBM of HtrA2 via its autocatalytic Ala\textsuperscript{133}-Ala\textsuperscript{134} cleavage.

The Autocatalytic Ala\textsuperscript{133}-Ala\textsuperscript{134} Cleavage of HtrA2 Is Required for Enhancement of Apoptotic Cell Death through Antagonizing XIAP-mediated Caspase Inhibition—Before we characterize the role of p36 in mammalian cells, we further examined the autocatalytic processing activity of HtrA2 by the in vitro cleavage assay. Full-length \(^{35}\)S\textsuperscript{Met}-labeled HtrA2, which was generated with the cell-free expression system from the corresponding pcDNA-HtrA2-FLAG plasmids, was incubated with wild-type Δ133 (Fig. 4B). Protein fragments with molecular masses of ~42 and 36 kDa were detected in HtrA2...
To verify the autocatalytic cleavage site of HtrA2, leucine and alanine at positions 132 and 133, which are P1 and P2 subsites, respectively, were converted to arginine residues (named R/R). The 36-kDa protein fragment, which is designated p36, was almost completely abolished in the HtrA2 (R/R/S306A), indicating that HtrA2 cleaves by itself on the N-terminal side of alanine 134.

To characterize the proteolytic cleavage of HtrA2 in mammalian cells, we transiently transfected into 293 cells a plasmid encoding full-length HtrA2 with a C-terminal FLAG epitope tag (Fig. 4C). The construct encoding HtrA2 (S306A) directed the expression of one protein that was ∼50 kDa and two additional proteins with lower molecular masses of ∼42 and 36 kDa, which were specific to anti-HtrA2 or anti-FLAG antibodies. Consistent with results from the in vitro cleavage assay, the replacement of arginine residues at leucine 132 and alanine 133 in HtrA2 eliminated production of p36, whereas production of the 42-kDa fragment, designated p42, increased in comparison with that of HtrA2 (S306A) (Fig. 4C, lane 3). The results provide evidence that the mature form of HtrA2 observed in mammalian cells is predominantly generated by the autocatalytic Ala133-Ala134 cleavage event of HtrA2.

Furthermore, to investigate the role of the autocatalytic Ala133-Ala134 cleavage of HtrA2 in mammalian cells, 293 cells were transiently transfected with a plasmid encoding either full-length HtrA2 (S306A) or (R/R/S306A) (Fig. 5). Several studies show that the protease activity of HtrA2 induces caspase-independent cell death (15, 18) or promotes cytochrome c-dependent caspase activation (19). To exclude these effects of the protease activity of HtrA2, we used the catalytically inactive mutant HtrA2 (S306A). In the HtrA2 (S306A), we observed that p36 and cytochrome c were released from the mitochondria into the cytosolic in response to an apoptotic stimulus, staurosporine (Fig. 5A). The release of p36 was not observed in the autocatalytic mutant, HtrA2 (R/R/S306A). To verify that the autocatalytic Ala133-Ala134 cleavage of HtrA2 is necessary for the interaction between HtrA2 and XIAP in mammalian cells, cells transiently overexpressing HtrA2 pro-
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Fig. 4. HtrA2 is autocatalytically processed in mammalian system. A, full-length HtrA2 constructs for both mammalian and in vitro TNT expression systems. Full-length HtrA2 contains additional functional domains in its N-terminal region, which are the mitochondrial target sequence (MTS) and the TM domain. RR indicates a full-length HtrA2 construct that contains an arginine mutation at leucine 132 and alanine 133 amino acid residues. B, autocatalytic Ala133-Ala134 cleavage of full-length HtrA2. In vitro cleavage reaction, [35S]Met-labeled proteolytically inactive (S306A) or a putative cleavage site mutant (R/R/S306A) forms of full-length HtrA2 were incubated with catalytically active Δ133. The reaction mixture was separated by 15% SDS-PAGE and visualized by autoradiography. C, removal of p36 by converting the cleavage site in HtrA2. Plasmids encoding S306A or R/R/S306A mutant HtrA2 proteins were transfected into 293 cells, and expression of HtrA2 was determined by IB analysis with the anti-HtrA2 antibody (top panel). The HtrA2 proteins were immunoprecipitated (IP) with the anti-HtrA2 antibody. The immunoprecipitates were resolved by 15% SDS-PAGE, followed by IB analysis with the anti-FLAG antibody (bottom panel). The proteins were specific to anti-HtrA2 or anti-FLAG antibodies: p50, 50-kDa full-length HtrA2; p36, 36-kDa autoproteolytic product of HtrA2. The asterisk indicates endogenous mature HtrA2.

The interaction between XIAP and HtrA2 was observed in cells expressing HtrA2 (S306A) but not in HtrA2 (R/R/S306A), indicating that p36 preferentially binds to XIAP. Because endogenous mature HtrA2 was also coimmunoprecipitated with XIAP, a tiny amount of XIAP was also detected in cells that were transiently transfected with empty vector or HtrA2 (R/R/S306A). Full-length HtrA2, p50, was localized in the cytosol as well as in the mitochondria; nonetheless, the cytosolic p50 did not coimmunoprecipitated with XIAP. Thus, the autocatalytic Ala133-Ala134 cleavage of HtrA2 is significant for binding of HtrA2 to XIAP with potent caspase inhibitory activity.

To assess the role of the autocatalytic Ala133-Ala134 cleavage of HtrA2 in the induction of apoptosis, we measured the extent of apoptotic cell death by directly counting the numbers of cells with apoptotic nuclei under fluorescence microscopy (Fig. 5C). After 1 μM staurosporine treatment, HtrA2 (S306A)-transfected cells revealed significantly higher cell death than empty vector- or HtrA2 (R/R/S306A)-transfected cells (Fig. 5D). To verify the apoptotic features, we monitored the key apoptotic events in the cells. The fragmentation of the genomic DNA, a biochemical hallmark of apoptosis, was visualized by the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay (Fig. 5E, left panel). Cell surface exposure of phosphatidylserine during apoptosis was detected by annexin V binding to phosphatidylserine as an additional marker for apoptotic cells (Fig. 5E, right panel). At 12 h of exposure to staurosporine, HtrA2 (S306A)-transfected cells revealed more effective activation of caspases-3 than HtrA2 (R/R/S306A)-transfected cells, resulting in a decrease of levels of procaspase-3 along with an increase of the levels of active caspase-3 and the cleavage of poly(ADP-ribose) polymerase (PARP) (Fig. 5F). Taken together, these results support evidence that the autocatalytic Ala133-Ala134 cleavage of HtrA2 plays an indispensable role in enhancement of apoptosis.

The Autocatalytic Ala133-Ala134 Cleavage Site of HtrA2 Is Evolutionarily Conserved in Mammals—To investigate the evolutionary relationships of the autocatalytic Ala133-Ala134 cleavage of HtrA2, we performed the phylogenetic analysis (Fig. 6). Twelve aligned HtrA2 protein sequences are 515 amino acids in length, and completed sequences of HtrA2 range from 403 to 458 amino acids in length. The protease and PDZ domains of HtrA2 are well conserved among animals; however, the HtrA2 N-terminal regions that consist of 134 amino acids are conserved only in mammals. The mammalian HtrA2 protein sequences form a strongly supported monophyletic group by 100% bootstrapping supports (Fig. 6A). The same results were also obtained from the neighbor-joining analysis. The phylogenetic tree based on multiple sequence alignment also suggests that the Ala133-Ala134 sequences are conserved in the mammalian HtrA2, with the exception of a serine substitution at the 134-amino acid residue of the bovine HtrA2 (Fig. 6B). These six mammalian HtrA2 orthologs contain identical amino acid residues at the P1 and P2 sites, which are critical for the specificity to the autocatalytic processing of the human HtrA2. Therefore, the amino acid substitutions at the alanine 134 did not abolish the autocatalytic cleavage of HtrA2 between residues 133 and 134 (Fig. 6C).

DISCUSSION

We have described here that the Ala133-Ala134 cleavage of HtrA2 into the mature form, p36, is catalyzed by its own
The autocatalytic Ala<sup>133</sup>-Ala<sup>134</sup> cleavage of HtrA2 is essential for enhancement of apoptotic cell death through interaction with XIAP. A, translocation of p36 into the cytosol in response to staurosporine. 293 cells were transfected with plasmids encoding full-length HtrA2 mutants (S306A or R/R/S306A). At 24 h post-transfection, the cells were treated with (+) or without (−) 1 μM staurosporine (STS) for 5 h, collected, and fractionated as described under “Experiment Procedures.” The mitochondria-containing pellet fractions (Mito) and the cytosolic fractions (Cyto) were analyzed by IB with anti-HtrA2 or anti-cytochrome c (Cyt c) antibodies. B, interaction between XIAP and p36. Cell extracts from 293 cells, which were transfected with the indicated expression constructs, followed by treatment with 1 μM staurosporine were precipitated with the anti-HtrA2 antibody. The HtrA2-immunoprecipitated (IP) complex (top panel) and whole-cell lysates (middle panel) were analyzed by IB analysis with anti-XIAP and anti-HtrA2 antibody, respectively. The asterisks indicate the endogenous mature HtrA2. C, representative GFP and 4′,6-diamidino-2-phenylindole (DAPI) fluorescence images of cells expressing HtrA2. 293 cells were transfected with plasmids encoding full-length HtrA2 mutants (S306A or R/R/S306A) together with the pEGFP-N3 plasmid at a 3:1 ratio. At 24 h post-transfection, the cells were treated with 1 μM staurosporine for 5 h. The nuclear morphology of GFP-positive cells (green) was analyzed by 4′,6-diamidino-2-phenylindole staining (blue). The stained cells were observed under Olympus 70AX fluorescence microscopy; arrows indicate apoptotic nuclei. D, proapoptotic activity of p36. At 24 h post-transfection, the cells transfected with a plasmid encoding HtrA2 plus the pEGFP-N3 were treated with the indicated concentrations of staurosporine for 5 h and stained with 4′,6-diamidino-2-phenylindole for 5 min at room temperature. All values were expressed as the percentage of GFP-positive cells with apoptotic nucleus in total GFP-positive cells. Error bars represent S.D. derived from the mean of three independent experiments. E, representative immunofluorescence images of a terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay (left) and annexin V staining (right). F, effect of p36 on caspase-3 (cas-3) activation in mammalian cells. Cells transfected with plasmids encoding full-length HtrA2 mutants (S306A or R/R/S306A) were incubated with 1 μM staurosporine for the indicated times. Cell lysates were analyzed by IB analysis with anti-caspase-3, anti-poly(ADP-ribose) polymerase (PARP), or anti-HtrA2 antibodies.
Regardless of the amounts of XIAP that were irreversibly degraded, the activation of caspase-3 was observed only in wild-type Δ129 but not in Δ133. The difference between wild-type Δ129 and Δ133 is that Δ129 contains additional amino acid residues in the N-terminal side of the IBM (Fig. 3A). This flanking sequence permits Δ129 to be autocatalytically processed to generate p36, exposing an IBM to allow interaction with XIAP. The autocatalytic Ala133-Ala134 cleavage of HtrA2, therefore, is an essential event for regulating caspase-dependent apoptotic cell death through the interaction between XIAP and HtrA2.

The notion that the mature form of HtrA2 is generated by other endogenous proteases or its autoproteolytic activity still remains controversial because endogenous HtrA2 protein exists in the most of mammalian cells (8, 10). To overcome the technical problem that was accompanied by pre-existing mature HtrA2 in mammalian cells, we characterized the proteolytic processing event of HtrA2 by incubating an in vitro translated [35S]Met-labeled HtrA2 as a substrate and purified recombinant HtrA2 as an enzyme. Moreover, we have developed a method to identify the cleavage site of HtrA2 by using a pGEX expression and purification system, followed by an in vitro cleavage assay and the N-terminal amino acid sequencing.

When a plasmid encoding full-length HtrA2 was transfected into 293 cells, we observed three differently migrating proteins that were immunoreactive to anti-HtrA2 or anti-FLAG antibodies: the fully processed mature form, p36, the precursor form, p50, and the other proteolytic doublet between p50 and p36. The molecular mass of 42 kDa of the doublet raises the possibility that the cleavage occurs at amino acid residues close or within the TM domain of HtrA2. If so, the doublet might be generated by mitochondrial intramembrane cleaving protease (I-Clip), which cleaves within a TM domain of the substrate and liberates its biologically active form (49–52). It is likely that HtrA2 could be not only a substrate of a mitochondrial I-Clip but also serve as a protease, cleaving other membrane-anchored proteases. We presently cannot identify the N-terminal amino acid residues of the doublet by using the pGEX expression system and an in vitro cleavage assay because full-length HtrA2 was extremely susceptible to proteolysis during expression in E. coli (37).

Relatively high amino acid sequence similarities among animal HtrA2 proteins on the serine protease and PDZ domains suggest that the HtrA2 orthologs are likely to have the same evolutionary origin in the serine protease activity. However, the N-terminal regions are conserved among the mammalian HtrA2 proteins, thus implying that the functions related to this region might be conserved in the mammalian HtrA2 orthologs. The phylogenetic analysis, therefore, suggests that the autocatalytic cleavage of HtrA2 between the positions 133 and 134 is derived before the mammalian diversification and conserved in mammals.

A recent study has reported that other mitochondrial proteases may be involved in maturation of HtrA2, as demonstrated by comparing the extent of processing of the wild-type and the active site mutant of HtrA2 in mnd2 (motor neuron degeneration 2) mouse embryonic fibroblasts (53). However, further detailed investigation of the HtrA2 processing, especially by using the HtrA2 knock-out system, can provide conclusive evidence for the HtrA2 processing mechanism in mammals because mnd2 cells express transiently inactive HtrA2, which contains a cysteine substitution at serine 276.

HtrA2 might be a multifunctional protein with many different aspects, providing an endoproteolytic function for removing aberrantly unfolded or damaged proteins in the mitochondria (5, 7, 53), proapoptotic activity through an antagonizing inhib-
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The autocatalytic Ala133-Ala134 cleavage of HtrA2 is responsible for exposing an IAP binding motif that resembles the motif of IAP antagonists, such as Smac/DIABLO, Drosophila Grim, Reaper, and HID (14, 23). Even more, sequence information for the cleavage site that is targeted over, significant mode of regulation of its functions. The autocatalytic Ala133-Ala134 cleavage of HtrA2 offers insights into the processing of proteins containing an IBM. Eventually, identifying physiologically relevant substrates for HtrA2 will be important for elucidating the molecular mechanisms in regulating essential biological processes, including protein quality control and the apoptotic cell death pathway.

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