Inhibitor of Apoptosis Proteins are substrates for the Mitochondrial Serine Protease Omi/HtrA2*

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Running title: IAPs are degraded by Omi/HtrA2.

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ABSTRACT

The mature serine protease Omi/HtrA2 is released from the mitochondria into the cytosol during apoptosis. Suppression of Omi/HtrA2 by RNA interference in human cell lines reduces cell death in response to TRAIL and etoposide. In contrast, ectopic expression of mature wildtype Omi/HtrA2, but not an active site mutant, induces potent caspase activation and apoptosis. In vitro assays demonstrated that Omi/HtrA2 could degrade inhibitors of apoptosis proteins (IAPs). Consistent with this observation, increased expression of Omi/HtrA2 in cells increases degradation of XIAP while suppression of Omi/HtrA2 by RNA interference has an opposite effect. Combined, our data demonstrate that IAPs are substrates for Omi/HtrA2, and their degradation could be a mechanism by which the mitochondrially-released Omi/HtrA2 activates caspases during apoptosis.
INTRODUCTION

The genes for inhibitors of apoptosis proteins (IAP) were originally identified in the genome of baculoviruses based on the ability of their gene products to protect infected host cells from virus-induced apoptosis (1). Cellular homologues of the viral IAPs have been identified in insects, nematodes, yeast and mammals (2,3). All IAPs contain one or more conserved domains, referred to as baculovirus IAP repeats (BIRs), that are essential for inhibition of apoptosis (reviewed in (4,5)). The BIR domains and the linker regions between them bind directly to caspases and inhibit their activity. Some IAPs, such as human XIAP, c-IAP1 and c-IAP2, and Drosophila DIAP1 and DIAP2 also contain C-terminal RING domains. The RING domain is important for ubiquitination and proteosome degradation of IAPs and IAP-associated proteins (reviewed in (5,6)).

The anti-apoptotic activity of IAPs is regulated by a group of proteins that bind to the BIR domains of IAPs via a N-terminal conserved 4-residue IAP-binding motif (IBM) (reviewed in (5,7)). In Drosophila melanogaster, five IBM-containing proteins known as Reaper, Hid, Grim, Sickle and Jafra2 have been identified as direct IAP-binding proteins (5,8). These proteins promote caspase activation by disrupting caspase-IAP complexes and/or inducing auto-ubiquitination and degradation of IAPs, thus preventing IAPs from inhibiting caspases (4-7).

In mammals two functional homologues of the Drosophila proteins, known as Smac/Diablo (9,10) and Omi/HtrA2 (11-15) have been identified. Both Smac/Diablo and Omi/HtrA2 are synthesized as precursor proteins with N-terminal mitochondrial localization signal peptides that are removed during maturation in the mitochondria to expose their N-terminal IBM. During apoptosis both proteins are released from the inter-membrane space of the
mitochondria into the cytoplasm and promote caspase activation and apoptosis by binding to the BIR3 domain of XIAP (11-15).

Unlike Smac/Diablo, Omi/HtrA2 is a serine protease with a high degree of homology to the bacterial heat inducible serine protease HtrA/DegP (16,17). In bacteria DegP aids in the degradation or refolding of misfolded or unfolded proteins in the periplasmic space. The high degree of similarity between bacterial HtrA/DegP and mammalian Omi/HtrA2, suggests that the later might also have a similar function in the mitochondria. However, very little is known about the protease activity or substrates of Omi/HtrA2 after its release from the mitochondria during cell death. In this report we provide evidence demonstrating for the first time that IAPs are substrates for Omi/HtrA2 and their degradation could be a mechanism by which Omi/HtrA2 activates caspases and induces cell death.

EXPERIMENTAL PROCEDURES

Cell Culture—Cells were cultivated either in Dulbecco’s Modified Eagle Medium (DMEM) (HeLa cells) or DMEM/F12 (293T cells) or RPMI 1640 (MCF-7 cells) (Invitrogen-LifeTechnologies), supplemented with 10% fetal bovine serum, 200 mg.ml\(^{-1}\) penicillin and 100 mg.ml\(^{-1}\) streptomycin sulfate.

Expression constructs—Expression constructs for Omi/HtrA2 variants and IAPs were described before (11).

RNA interference—Cells were seeded in 6-well or 12-well plates and then transfected with non-specific siRNA duplex (UGU UGU UUG AGG GGA ACG G TT) or Omi specific siRNA duplex (GGC AAG GGG AGU UUG UUG UTT) using Oligofectamine (Invitrogen-LifeTechnologies) as per manufacturer’s recommendations. 48 h after the transfection cells were...
either treated with TRAIL (100 ng/ml) for 6 hours or etoposide (100 µM) for 24 hours. Cells were collected for immunoblot analysis. A duplicate set of cells was stained with Annexin V and Propidium Iodide as per manufacturer’s recommendations (CLONTECH laboratories) to assay for cell death. Dead cells in each field (annexin V and/or PI positive) were counted using fluorescence microscopy. Total number of cells (live plus dead) in each field was counted by light microscopy. The percentage of cell death in each experiment was calculated from the values of dead cells divided on total cells. Data represents mean values ± standard deviation of three independent experiments.

In vitro protease assay—The protease activity of Omi was assayed with *in vitro* translated 35S-labeled XIAP, c-IAP1, or c-IAP2 as substrates. Bacterially expressed Omi/HtrA2 was purified on Talon affinity resins and then incubated with 35S-labeled proteins in buffer A (20 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 1mM EDTA and 1mM EGTA with protease inhibitors in a total 20 µl reaction. The cleavage products were analyzed by SDS-PAGE and visualized by autoradiography.

Endogenous Omi/HtrA2 was isolated by immunoprecipitation from 293T S100 extracts. The S100 extracts were incubated with Omi polyclonal antibody (made in the lab) for 1 h. After incubation, the Omi-antibody complexes were bound to protein G Sepharose and washed several times. The protein G Sepharose-bound complexes were incubated with 35S-labeled XIAP in buffer A for 1 h at 37° C in a total 20 µl reaction. The cleavage products were analyzed as outlined above.

Omi/HtrA2-induced cell death assay—The ability of Omi/HtrA2 variants to induce cell death in transfected cells was assayed as described (11). 293T cells were seeded in 12 well plates at a density of 1x10^6 cells/well and then transfected with 0.4 µg pEGFPN1 reporter
plasmid (CLONTECH laboratories) and 1.0 μg of empty vector or constructs encoding
Omi/HtrA2 variants using the LipofectAMINE™ method. After 36 h of transfection normal and
apoptotic GFP-expressing cells were counted using fluorescence microscopy. The percentage of
cell death in each experiment was calculated from the values of dead cells divided on total cells.
Data represents mean values ± standard deviation of three independent experiments.

**DEVD cleavage assay**—To assay caspase activity in 293 cytosolic S-100 extracts (50
mg), an equal amount of the extracts were incubated with 100 μM DEVD-AFC fluorogenic
substrate for 60 min at 37 °C. The amount of cleaved AFC was measured using 400 nm and 505
nm as the excitation and emission wavelengths, respectively. Results were expressed as DEVD
cleavage activity in relative fluorescence units. The data represents the average of three
independent experiments.
RESULTS

Suppression of Omi/HtrA2 by RNA interference suppresses cell death—Treatment of cancer cell lines with death stimuli causes the release of Omi/HtrA2 from the mitochondria into the cytoplasm (11-15). To evaluate the role of Omi/HtrA2 in the extrinsic (TRAIL) or intrinsic (etoposide) cell death pathways we used Omi/HtrA2-specific small interfering RNA (siRNA) oligonucleotides to reduce the protein level of Omi/HtrA2 in HeLa and MCF-7 cancer cells (Fig. 1A). Transfection of these cells with Omi/HtrA2-specific siRNA resulted in almost 40-50% reduction in the extent of TRAIL- or etoposide-induced cell death compared to untransfected or control siRNA-transfected cells (Fig. 1B and C). These results indicate that Omi/HtrA2 plays an important proapoptotic role in the extrinsic and intrinsic cell death pathways in cancer cell lines.

Mature Omi/HtrA2 can promote caspase activation and cell death—Recent results suggest that the serine protease activity of Omi/HtrA2 could be responsible for its potent killing activity (18). To determine whether Omi/HtrA2 protease activity can activate the caspase pathway, we measured caspase activity in 293 cells transfected with constructs encoding mature wildtype or active site mutant Omi/HtrA2 proteins. Expression of mature wildtype, but not the active site mutant Omi/HtrA2 resulted in significant caspase activation (Fig. 2A). This enhanced caspase activation was associated with increased cell death (Fig. 2B). Similar results were observed in MCF-7 and HeLa cell lines (ref (11) and data not shown). These results suggest that the ability of Omi/HtrA2 to promote caspase activation and cell death in transfected cells is largely dependent on its serine protease activity.

Omi/HtrA2 can degrade IAPs in vitro—Western blot analysis revealed that expression of mature wildtype Omi/HtrA2, but not an active site mutant protein results in significant reduction in the amount of XIAP in the transfected cells (Fig. 2C). These results suggest that degradation
of XIAP by Omi/HtrA2 could be responsible for this effect. To test this hypothesis we first
determined the physiological concentration of Omi/HtrA2 in 293 and HeLa cells by immunoblot
analysis with Omi/HtrA2 antibodies. The concentration of Omi/HtrA2 in 293 was found to be ~
150 nM and in HeLa cells ~ 60 nM. Next we assayed the activity of physiological amounts of
recombinant Omi/HtrA2 with $^{35}$S-labeled XIAP, cIAP-1 or cIAP-2 as substrates. As shown in
Fig. 3A, physiological amounts of Omi/HtrA2 were able to degrade these IAPs in a dose-
dependent manner. Consistent with these results, physiological amounts of endogenous
Omi/HtrA2 protein isolated by immunoprecipitation from 293 cells were also able to degrade
XIAP in a dose dependent manner (Fig. 3B). These results indicate that IAPs are physiological
substrates for Omi/HtrA2 protein.

Next we tested the effect of Omi/HtrA2 on the ability of IAPs to inhibit caspase
activation in S100 extracts stimulated with cytochrome c and dATP (Fig. 3C). Incubation of
XIAP with mature wildtype Omi resulted in a large dose dependent reduction in the inhibitory
activity of XIAP. Similar results were obtained with the active site mutant Omi/HtrA2-S306A.
In contrast, incubation of XIAP with protease active Omi/HtrA2 without its IAP binding motif
(ΔAVPS) did not cause significant reduction in the inhibitory activity of XIAP.

Western blot analysis showed that XIAP is degraded in the reaction mixtures containing
wildtype Omi and Omi-ΔAVPS, but not Omi/HtrA2-S306A. The IAP binding motif of Omi
seems to enhance its ability to cleave XIAP, because the Omi-ΔAVPS was less efficient in
cleaving XIAP. In addition, a distinct pattern of cleavage was observed with the wildtype
Omi/HtrA2 compared to Omi-ΔAVPS. Nevertheless, the cleavage products generated by Omi-
ΔAVPS were able to inhibit caspases indicating that cleavage alone might not be sufficient for
inactivation of XIAP. These interesting results suggest that the presence of the IAP binding
motif enhances the ability of Omi/HtrA2 to cleave and disrupt the association of the cleavage products of XIAP with caspases. In contrast, in the absence of the IAP binding motif, Omi/HtrA2 cleaves XIAP less efficiently at multiple sites and is not able to disrupt the association of the cleavage products with caspases.

IAP degradation during apoptosis is dependent on Omi/HtrA2 expression levels—To provide additional support for the role of Omi/HtrA2 in IAP-degradation we decreased the expression levels of Omi/HtrA2 in the mitochondria by transfection of MCF-7 cells with Omi-specific or non-specific siRNAs and then treated the cells with etoposide for different periods of time. Less IAP-degradation was observed in cells transfected with the Omi-specific siRNA compared to the cells transfected with the non-specific siRNA (Fig. 4). Taken together, these results indicate that Omi/HtrA2 plays a direct role in the degradation of IAPs during cell death.

DISCUSSION

IAP degradation is an important mechanism for caspase activation during developmental and perhaps p53-induced cell death in Drosophila (5,6,19). Drosophila DIAP-1 destruction is stimulated by binding of IAP-binding proteins such as Reaper, which is induced during development or DNA damage, to the BIR2 domain of DIAP1. This binding induces autoubiquitination of DIAP1, which targets it for degradation by the proteosomal pathways. So far, a similar mechanism of IAP destruction has not been identified in mammalian cells during cell death. However, recent results demonstrated that c-IAP1 is cleaved in human HeLa cells during p53-dependent apoptosis by a serine protease (20). Based on results showing
upregulation of Omi/HtrA2 mRNA by p53, it was suggested that Omi/HtrA2 could be responsible for the observed degradation of cIAP-1 during p53-dependent apoptosis (20).

Our biochemical and cellular data indicate that XIAP is also degraded in human cancer cell lines during cell death. Omi/HtrA2 appears to be directly responsible for degradation of XIAP, since increased level of Omi/HtrA2 induces more degradation (Fig. 2C) while decreased level has an opposite effect (Fig. 4). In addition, purified Omi/HtrA2 can directly degrade XIAP and other human IAPs in vitro (Fig. 3). The new results also indicate that the IAP binding motif of Omi/HtrA2 is important for efficient cleavage of IAPs at distinct sites, and for disruption of the association between caspases and the IAP fragments. In the absence of the IAP-binding motif, Omi/HtrA2 can still cleave IAPs with less efficiency but is unable to disrupt the association between caspases and the IAP fragments. Thus, the presence of the IAP-binding motif enhances the ability of Omi/HtrA2 to find and destroy IAPs after its release from the mitochondria into the cytosol during mammalian cell death.

Although the level of XIAP was reduced by the transfected wildtype Omi/HtrA2, there were no XIAP fragments detectable in the cellular extracts of the transfected cells (Fig. 2B). In contrast multiple fragments were detectable in vitro after incubation of Omi/HtrA2 with IAPs (Fig. 3). These interesting results suggest that in cells, the Omi/HtrA2-generated IAP fragments could be targeted for further degradation by the N-end rule degradation pathway. This is consistent with recent results, which demonstrated that caspase-cleavage of the Drosophila DIAP1 makes it unstable and targets it for degradation by the N-end rule degradation pathway (21). Thus Omi/HtrA2 could promote apoptosis in mammalian cells by two mechanisms. One mechanism relies on its IAP-binding motif to bind to IAPs and disrupts association of active caspases with IAPs and the other mechanism relies on its protease activity to cleave bound IAPs.
and targets them for further degradation by the proteosomal pathways. However, both mechanisms might be necessary for efficient killing by Omi/HtrA2. This could explain why the active site mutant Omi/HtrA2-S306A can disrupts IAP-caspase association and promote caspase activation in vitro (Fig. 3C) while it is not able to induce efficient cell death or caspase activation in transfected cells (Fig. 2).

Ironically, the similarity between Omi/HtrA2 and the bacterial survival chaperone-protease DegP/HtrA suggests that Omi/HtrA2 could have an important survival function in the mitochondria (16,17). Omi/HtrA2 may protect the mitochondria against cellular stresses by recognizing unfolded or misfolded proteins in the inter-membrane space and helps refold or degrade them. If this is the case, then loss of this protein could lead to accumulation of damaged proteins in the inter-membrane space of the mitochondria over a long period of time. This is likely to be deleterious to mitochondrial function and increase sensitivity of cells to stress-induced cell death. These possibilities are currently under investigation.

Note added in proof

While this paper was under review, Yang et al. reported that IAPs are substrates for Omi/HtrA2 (22). Their paper presents essentially the same results as ours.
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FIGURE LEGENDS

FIG. 1. Suppression of Omi/HtrA2 by RNA interference decreases cell death.  
A, Suppression of Omi/HtrA2 expression by Omi/HtrA2-Specific siRNA. HeLa (left panels) or MCF-7 (right panels) cells were transfected with Omi/HtrA2-specific (Omi lanes) or non-specific (NS lanes) small interfering RNA (siRNA) duplexes. 64 h after transfection cells were lysed and analyzed by western blotting with Omi/HtrA2-specific antibody (upper panels) or β-actin-specific antibody (lower panels).  
B and C, Suppression of cell death by Omi/HtrA2-Specific siRNA. HeLa or MCF-7 cells were transfected with siRNA duplexes as in A, and then treated with TRAIL (B) for 6 h or etoposide (C) for 24 h. The percentage of cell death in each case was estimated as described under “Experimental Procedures”.

FIG. 2. Ectopic expression of mature Omi/HtrA2 promotes caspase activation and cell death.  
A, DEVD-AFC cleaving activity in 293 cells transfected with Flag-Omi/HtrA2 plasmids. Cells were transfected with the indicated plasmids for 24 h. Cells were lysed and the cellular S-100 extracts were assayed with DEVD-AFC. RFU, relative fluorescence units.  
B, Analysis of cell death in 293 cells transfected with Flag-Omi/HtrA2 plasmids. Cells were transfected with the indicated plasmids for 36 h. The percentage of cell death in each case was estimated as described under “Experimental Procedures”.  
C, Analysis of XIAP in cells transfected with Flag-Omi/HtrA2 plasmids. The cellular extracts in (A) were analyzed by western blotting with Flag antibody (upper panel), XIAP antibody (middle panel) or GAPDH antibody (lower panel).

FIG. 3. IAPs are substrates for Omi/HtrA2.  
A, Degradation of IAPs by recombinant Omi/HtrA2 proteins. In vitro translated ^35S-labeled XIAP, c-IAP1 or c-IAP2 were incubated
with increasing physiological amounts of purified Omi/HtrA2 proteins (25, 75, 250 nM). The reaction products were analyzed by SDS-PAGE and autoradiography. B, Endogenous Omi/HtrA2 can cleave XIAP. Upper panel, endogenous Omi/HtrA2 was immunoprecipitated from 293 cellular extracts with Omi/HtrA2 antibody and different amounts of the immunoprecipitated Omi/HtrA2 (second-fourth lanes) or 293 immunoprecipitates with non-specific antibody (last lane) were incubated with $^{35}$S-labeled XIAP as substrate. The reaction products were analyzed by SDS-PAGE and autoradiography. Lower panel, western blot of the immunoprecipitates with Omi/HtrA2-specific antibody. The concentration of endogenous Omi/HtrA2 in the reaction mixtures is indicated at the bottom. C, Inhibition of XIAP-activity by Omi/HtrA2. Purified XIAP (10 nM) was preincubated with buffer or increasing amounts (50, 100, 250 nM) of wildtype Omi (WT), Omi-$\Delta$AVPS ($\Delta$AVPS) or Omi-S306A (S306A) for 10 min. The reaction mixtures were added to 293T S100 extracts and then stimulated with cytochrome c plus dATP. The reactions were carried out in the presence of DEVD-AMC as a substrate for 1 h. D, Immunoblot analysis of XIAP cleavage. The reaction mixtures in (C) were analyzed by SDS-PAGE and western blotted with XIAP antibody.

FIG 4. The level of Omi/HtrA2 protein in cells affects the extent of IAP degradation. MCF-7 cells were transfected with Omi/HtrA2-specific or non-specific (NS) siRNA duplexes as in Fig. 1A legend, and then treated with DMSO (24 h) or etoposide for the indicated periods of time. Cells were then lysed and analyzed by western blotting with XIAP-specific antibody or GAPDH antibody as an internal control. The intensities of the XIAP and GAPDH bands in each case were determined by densitometric scanning. The data represent the relative intensity of the
XIAP band to the GAPDH band as a percentage of the DMSO control in each experiment. DSU, densitometric scanning units.
Fig. 1

A

|     | Buffer | NS | Omi |
|-----|--------|----|-----|
| Omi |        |    |     |
| β-actin |    |    |     |

B

% cell death

Trail: - + - + - +

|        | Buffer | NS | Omi |
|--------|--------|----|-----|
| Omi |        |    |     |

C

% cell death

Etoposide: - + - + - +

|        | Buffer | NS | Omi |
|--------|--------|----|-----|
| Omi |        |    |     |
Fig. 2

A

![Bar graph showing DEVD cleavage (RFU) for Vector, WT, and S306A.]

B

![Bar graph showing % cell death for Vector, WT, and S306A.]

C

![Western blot images for Vector, WT, and S306A with bands for Omi, XIAP, and GAPDH.]
Fig. 3

A  Omi  Buffer

XIAP  c-IAP1  c-IAP2

B

Buffer  Omi  NS

nM  15  45  150  0

Cleavage products

C

% DEVD cleavage

120

100

80

60

40

20

0

XIAP  Buffer  WT  ΔAVPS  S306A

D

S306A  WT  ΔAVPS

Cleavage products
Fig. 4

- % Relative Intensity (DSU)

- DMSO
- 16h
- 20h
- 24h

- NS siRNA
- Omi siRNA
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