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Regulation of HAX-1 Anti-apoptotic Protein by Omi/HtrA2 Protease during Cell Death*

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Omi/HtrA2 is a nuclear-encoded mitochondrial serine protease that has a pro-apoptotic function in mammalian cells. Upon induction of apoptosis, Omi translocates to the cytoplasm and participates in caspase-dependent apoptosis by binding and degrading inhibitor of apoptosis proteins. Omi can also initiate caspase-independent apoptosis in a process that relies entirely on its ability to function as an active protease. To investigate the mechanism of Omi-induced apoptosis, we set out to isolate novel substrates that are cleaved by this protease. We identified HS1-associated protein X-1 (HAX-1), a mitochondrial anti-apoptotic protein, as a specific Omi interactor that is cleaved by Omi both *in vitro* and *in vivo*. HAX-1 degradation follows Omi activation in cells treated with various apoptotic stimuli. Using a specific inhibitor of Omi, HAX-1 degradation is prevented and cell death is reduced. Cleavage of HAX-1 was not observed in a cell line derived from motor neuron degeneration 2 mice that carry a mutated form of Omi that affects its proteolytic activity. Degradation of HAX-1 is an early event in the apoptotic process and occurs while Omi is still confined in the mitochondria. Our results suggest that Omi has a unique pro-apoptotic function in mitochondria that involves removal of the HAX-1 anti-apoptotic protein. This function is distinct from its ability to activate caspase-dependent apoptosis in the cytoplasm by degrading inhibitor of apoptosis proteins.

Omi/HtrA2 is a mitochondrial serine protease that is released to the cytoplasm upon induction of apoptosis (1–4). In the cytoplasm, Omi binds and cleaves IAPs[1] leading to activation of caspase-dependent apoptosis (5, 6). Omi can also induce caspase-independent apoptosis through an as yet unknown mechanism that requires its proteolytic activity (7, 8). In addition to its pro-apoptotic function, Omi has another unique role in maintaining mitochondrial homeostasis, but the details of this mechanism are still unclear (9). The serine protease activity of Omi is necessary and essential for its normal function whether it acts as a pro-apoptotic protein in the cytoplasm or as a potential chaperone in the mitochondria (9). The proteolytic activity of Omi has been associated with autoprocessing to form the mature protein as well as cleavage of IAPs to activate caspase-dependent apoptosis (5, 6, 8). To understand the mechanism of Omi's function, it will be necessary to identify new substrates for this protease. These substrates might be mitochondrial or cytoplasmic proteins, and their degradation and removal by Omi could be part of the apoptotic process. In this report, we used the yeast two-hybrid system to isolate and characterize new Omi-interacting proteins. One of these interactors isolated from this screen was the HS1-associated protein X-1 (HAX-1) anti-apoptotic protein (10). HAX-1 interacted with Omi both *in vitro* and *in vivo*. Furthermore, HAX-1 was degraded and removed by Omi when cells were treated with various apoptotic stimuli. Using a specific inhibitor of the proteolytic activity of Omi, we could block HAX-1 degradation and protect cells from apoptosis. We also used a cell line derived from motor neuron degeneration 2 (*mnd2*) mice that carry a mutated form of Omi affecting its proteolytic activity (9). There was little degradation of HAX-1 in these cells upon induction of apoptosis. When functional Omi was reconstituted in the *mnd2* cells, degradation of HAX-1 was also restored. Our present study identified the HAX-1 protein as a new substrate for Omi. Cleavage of HAX-1 occurs in mitochondria before Omi translocates to the cytoplasm. This suggests HAX-1 degradation precedes cleavage of IAPs that takes place in the cytoplasm. HAX-1 degradation occurs in response to various apoptotic stimuli and requires the presence of a proteolytically active Omi. Our results define a new mechanism by which Omi activates apoptosis from inside the mitochondria by removing the HAX-1 anti-apoptotic protein. Furthermore, we show a very significant inverse correlation between the level of HAX-1 protein and the degree of cell death. This suggests that the HAX-1 protein (and its regulation by Omi protease) plays a central role in mammalian cell death.

MATERIALS AND METHODS

Yeast Two-hybrid Screen—EGY48 (*MATa trp1 ura3 his3 LEU2*::*pLex Aop6-LEU2*) was used as the host yeast strain for all two-hybrid interaction experiments (11). This yeast strain has both an integrated LEU reporter gene with upstream LexA operators as well as a pSH18–34 (LexAop-lacZ) 2 μ plasmid that directs the synthesis of β-galactosidase (12).

Positive protein-protein interactions between the bait and prey were initially detected by the ability of the yeast to grow on galactose-Ura-Trp-His-Leu-selective yeast plates. They were further tested on Ura-His-Trp-X-gal plates. Plasmids were rescued from positive clones and

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[1] The abbreviations used are: IAP, inhibitor of apoptosis protein; HAX-1, HS1-associated protein X-1; EGFP, enhanced green fluorescent protein; TMRM, tetramethylrhodamine methyl ester; mnd2, motor neuron degeneration 2; MSCV, murine stem cell virus.

introduced into KC8 *Escherichia coli* by electroporation. DNA sequence was determined by sequencing both DNA strands with a commercially available kit (CEQ DTCS-Quick Start kit (Beckman Coulter)) using a CEQ 2000 DNA analysis system (Beckman Coulter). The presence and stability of the recombinant proteins in yeast cells was monitored by Western blot analysis with either LexA-antibodies (for baits) or HA-antibodies (for preys).

Degradation Assay—The ability of His-Omi$_{134-458}$ to cleave HAX-1 *in vitro* was investigated. For this the full-length cDNA for HAX-1 protein was cloned in the pET-28 vector (Novagen) containing the T7 promoter and used in an *in vitro* transcription-translation system (Promega) in the presence of [^{35}S]methionine and [^{35}S]cysteine (Amersham Biosciences). Bacterially expressed recombinant His-Omi$_{134-458}$ was purified on nickel-nitrilotriacetic acid affinity resin as described (13). His-Omi$_{134-458}$ (0.5 μg) was incubated with ^{35}S-labeled HAX-1 in 20 μl of reaction volume in assay buffer (20 mM Na_2HPO_4 (pH 8), 10% glycerol, 200 mM NaCl). After 2 or 6 h of incubation at 37 °C the reactions were stopped through the addition of SDS-sample buffer. Reaction products were analyzed by SDS-PAGE followed by autoradiography.

Cell Culture—HEK293 cells were grown using Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Hyclone), 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). HK-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 15 mM Hepes, 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, and 5 μg/ml apotransferrin (Sigma). *mnd2*-derived cell lines stably transfected with MSCV vector or MSCV-Omi (14) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin and 0.5 μg/ml of puromycin (14).

In Vivo Interaction between Omi and HAX-1 Proteins—HEK293 cells were transfected with either the pEGFP-N1 vector (Clontech) or EGFP-OmiS/A plasmid using LipofectAMINE 2000 reagent (Invitrogen). EGFP-OmiS/A encodes the full-length Omi protein that carries a mutation that replaces the serine in position 306 with an alanine (S → A) fused to EGFP protein. This mutation has been shown previously to inactivate the proteolytic activity of Omi (6, 8). Twenty-four h after transfection, cell lysates were prepared using radioimmune precipitation assay buffer (1× phosphate-buffered saline, 1% Nonidet P-40, 0.1% SDS) containing protease-inhibitor mixture (Roche Applied Science). After a low spin centrifugation, the supernatant of the cell lysates was precleared by mixing them with protein G-agarose beads (Roche Applied Science) for 1 h followed by a GFP-polyclonal antibody (Invitrogen) for 2 h at 4 °C. Protein G-agarose beads were then added and allowed to bind for an additional 4 h. Immunoprecipitates were collected by brief centrifugation, washed extensively with Nonidet P-40 buffer, and resolved by SDS-PAGE. They were then electrotransferred onto a polyvinylidene difluoride membrane and treated with a mouse monoclonal HAX-1 antibody (BD Biosciences) followed by a secondary horseradish peroxidase-conjugated antibody.

We also investigated the potential interaction between endogenous Omi and HAX-1 proteins. For this, HEK293 cells were grown in 100-mm dishes and subconfluent cultures were lysed using radioimmune precipitation assay buffer. Approximately 400 μg of total protein cell lysate was precleared using Protein G-agarose (Roche Applied Science) for 2 h followed by incubation with Omi rabbit polyclonal antiserum overnight at 4 °C. A control (preimmune) antiserum was also used. Protein G-agarose beads were collected by centrifugation, washed four times with 1 ml of radioimmune precipitation assay buffer, and then resuspended in 50 μl of SDS sample buffer. Proteins were subjected to SDS-PAGE and Western blot analysis using HAX-1 monoclonal antibodies.

Western Blot Analysis—After various pro-apoptotic treatments, cells were lysed using a Triton X-100 based lysis buffer (1% Triton X-100, 10% glycerol, 150 mM NaCl, 20 mM Tris (pH 7.5), 2 mM EDTA) in the presence of a protease inhibitor mix (Roche Applied Science). Approximately 20 μg of whole cell extracts were resuspended in SDS sample buffer and boiled for 3 min. The samples were resolved on SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes (Pall Corporation) using a semi-dry cell transfer blot (Bio-Rad). 2% nonfat dry milk in TBST buffer (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.1% Tween 20) was used to block nonspecific binding of the membrane. The membrane was incubated with the indicated primary antibodies: HAX-1 monoclonal antibody at 1:300 dilution (BD Biosciences), Omi rabbit polyclonal antibody at 1:5000 dilution (prepared in our lab), XIAP monoclonal antibody (BD, Transduction Laboratories) at 1:500 dilution, and β-actin monoclonal antibody (Sigma) at 1:5,000 dilution followed by a secondary peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibody (Jackson ImmunoResearch) at 1:5,000 dilution; the immunocomplex was then visualized by ECL (Pierce).

Cell Death Assays—HK-2 cells were grown in six-well plates in the appropriate medium until they reached 80% confluence; they were then treated with ucf-101 (50 or 70 μM) for 20 min followed by cisplatin (50 μM) or H_2O_2 (2 mM) treatment for 14 h. HEK293 cells were treated with cisplatin (50 μM) or H_2O_2 (1 mM) for 14 h. *mnd2*-derived cell lines were treated with cisplatin (5 μM), H_2O_2 (100 μM), or etoposide (100 μg/ml) for 14 h. Cells were detached using 1× trypsin-EDTA (Invitrogen), washed twice with ice-cold phosphate-buffered saline, and resuspended in 1× binding buffer (BD Biosciences) according to BD Biosciences protocol. Cell death was estimated by staining with Annexin V (apoptotic cell) and 7-aminoactinomycin D (necrotic cells) (15–17). Samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences).

Subcellular Fractionation—HEK293 cells were grown in 100-mm dishes to 80% confluence. After treatment with cisplatin (50 μM) or H_2O_2 (1 mM) for 14 h, cell fractionation was performed using the ApoAlert cell fractionation kit (Clontech) according to the instructions supplied by the manufacturer. Cells were homogenized with a tissue grinder in a fractionation buffer mix containing protease inhibitors and dithiothreitol. The homogenate was then centrifuged at 700 × *g*. The pellet representing the crude nuclear fraction was discarded. The supernatant was collected and centrifuged further at 10,000 × *g*. Both the resultant crude pellet mitochondrial fraction and the crude supernatant cytosolic fraction were collected. The purity of the mitochondrial fractions was monitored by assaying the activity of succinate dehydrogenase and cytochrome *c* oxidase (18). The majority of the activity of these two enzymes was associated with the mitochondrial fraction. The fractions were resolved by SDS-PAGE and Western blot analysis using the following antibodies: Omi (1:5,000), HAX-1 (1:300), XIAP (1:500), and cytochrome *c* (1:500).

Transient Expression of HAX-1 and Mitochondrial Membrane Potential ($\Delta\psi_m$)—HEK293 cells were plated in 12-well plates and transfected with either the EGFP-C1 vector or EGFP-HAX-1 plasmid using LipofectAMINE 2000 (Invitrogen). Eighteen h after transfection, cells were treated with various concentrations of cisplatin (30, 50, and 70 μM) for 14 h. The percentage of apoptotic cells was estimated by staining with Annexin V. The change in the mitochondrial membrane potential was monitored by staining the cells with tetramethylrhodamine methyl ester (TMRM) (Sigma) using the method described in Ref. 19 with some modifications. Briefly, each sample (1×10^6 cells) was divided in three test tubes: (*a*) control GFP, (*b*) TMRM, and (*c*) phycoerythrin-conjugated Annexin V, and incubated either at 37 °C ((*b*)) or at room temperature ((*a*) and (c)) for 15 min in 1× binding buffer (BD Biosciences). All samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences).

Statistical Analysis—All quantitative data are expressed as mean ± S.D. Differences among groups were analyzed by one-way analysis of variance followed by Tukey's post-hoc test. A value of $p < 0.05$ was considered significant.

RESULTS

Isolation of Omi Interactors—We used the yeast two-hybrid system to screen three different cDNA libraries constructed in the pJG4–5 vector (11, 12). We used a HeLa cDNA library (11) as well as cDNA libraries prepared from primary human melanocytes or keratinocytes.[2] We selected these cDNA libraries to screen as many diverse proteins as possible, including any potential tissue-specific interactors. Furthermore, cDNAs encoding pro-apoptotic proteins might be underrepresented or absent in cDNA libraries prepared from cell lines. The bait used was the mature, proteolytically active form of the Omi protein (amino acids 134–458) cloned in the pGilda (Clontech) bait vector. Initially, we used the PL202 (11) vector and found that constitutive high expression of the LexA-Omi$_{134-458}$ was detrimental to yeast. Therefore, we chose to use the pGilda vector, where expression of the bait protein is under the control of the GAL1 inducible promoter that allows expression of the LexA-bait fusion protein only when yeast are grown in galactose medium. Expression and stability of the LexA-Omi$_{134-458}$ was verified by Western blot analysis using LexA antibodies

[2] A. S. Zervos, unpublished data.

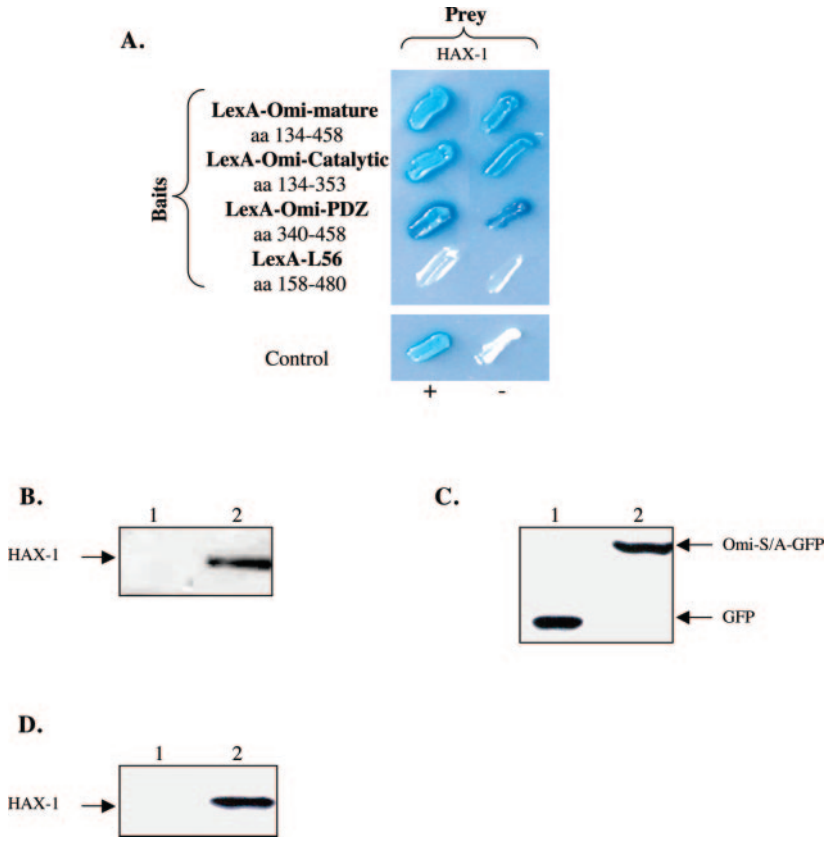

FIG. 1. **Interaction of HAX-1 with Omi serine protease.** *A*, interaction and specificity of HAX-1 with different domains of Omi protein. Yeast colonies were transformed with plasmids encoding the indicated baits and full-length HAX-1 as prey (11); *blue color* results from a positive protein-protein interaction. L56/HtrA1 is a mammalian homolog of Omi (23). *B*, association of HAX-1 with Omi in mammalian cells. Cells were transfected with either EGFP or EGFP-OmiS/A. Twenty-four h later, cell lysates were prepared as described under "Materials and Methods." GFP antibodies were used to immunoprecipitate proteins that were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane, and the presence of HAX-1 was detected using specific antibodies. *Lane 1* shows an absence of HAX-1 in cells transfected with empty EGFP vector; *lane 2* shows that HAX-1 has co-precipitated with EGFP-Omi-S/A. *C*, the same blot was also incubated with anti-GFP antibodies to show expression and stability of the GFP-fusion proteins. *D*, co-immunoprecipitation of endogenous Omi and HAX-1 proteins. HEK293 cells were lysates, and Omi antibodies were used to precipitate endogenous protein. Immunoprecipitated complexes were analyzed as described in *B*. HAX-1 co-precipitated with Omi (*lane 2*) but not when pre-immune serum was used (*lane 1*).

(results not shown). Several interacting proteins were identified in this screen. A detailed description of these interactors and their properties will be presented elsewhere.[3] One of the Omi interactors isolated from both the melanocyte and keratinocyte cDNA libraries was the full-length HAX-1 protein. Fig. 1 shows that HAX-1 interacts specifically and strongly with Omi in yeast. The interaction is mediated through the PDZ domain of Omi as well as its catalytic domain.

The specificity of HAX-1 interaction with Omi in yeast was tested using L56/HtrA1, a mammalian homolog of Omi that has 68% amino acid sequence similarity. No interaction between HAX-1 and L56/HtrA1 was observed in this yeast two-hybrid assay (Fig. 1*A*).

Interaction of Omi with HAX-1 in Mammalian Cells—To investigate if Omi interacts with HAX-1 *in vivo*, HEK293 cells were transfected with a construct encoding a proteolytically inactive form of Omi (Omi-S/A, serine 306 was replaced with alanine) fused to the GFP protein. GFP antibodies were used to precipitate GFP-Omi-S/A; the presence of any HAX-1 protein in the precipitated complex was monitored by Western blot analysis using HAX-1 antibodies. Fig. 1*B* shows that HAX-1 is in a complex with GFP-Omi-S/A in HEK293 cells. Furthermore, interaction between endogenous Omi and HAX-1 proteins was also investigated in HEK293 cells. Polyclonal Omi antiserum or control pre-immune serum was used to precipitate endogenous Omi and any associated proteins. The presence of HAX-1 in this immunoprecipitated complex was detected by Western blot using HAX-1 antibodies. Fig. 1*D* shows endogenous Omi and HAX-1 proteins interact in HEK293 cells.

Omi Can Degrade HAX-1 Protein in Vitro—To test the ability of Omi to cleave the HAX-1 protein *in vitro*, ^{35}S-labeled HAX-1 protein was made using a TNT-Quick coupled transcription/translation system (Promega). Bacterially made His-$Omi_{134-458}$ is an active serine protease and has been described previously (13). Fig. 2 is an autoradiograph of an SDS-PAGE gel showing specific cleavage of ^{35}S-labeled HAX-1 by His-$Omi_{134-458}$ *in vitro*.

HAX-1 Protein Level Decreases during Cell Death—To investigate whether the level of HAX-1 protein is regulated in mammalian cells during cell death, we treated HK-2 cells with various concentrations of cisplatin or H_2O_2. Fig. 3 shows the protein level of HAX-1 proportionally decreases as the concentration of cisplatin is raised from 30 to 70 μM. HAX-1 protein also decreased in cells treated with increasing concentration of H_2O_2.

ucf-101 Inhibitor Prevents HAX-1 Degradation—We investigated whether the Omi protease is specifically responsible for the degradation of HAX-1 observed in HK-2 cells during apoptosis. To accomplish this, HK-2 cells were treated with cisplatin and the percentage of cell death was monitored by flow cytometry. Cell extracts were also prepared from the same cell populations, and the levels of HAX-1 or Omi proteins were monitored by Western blot analysis. HK-2 cells treated with 50 μM cisplatin for 14 h resulted in 94% cell death of the population (Fig. 4). The cell death in these cells coincided with a dramatic reduction in the level of HAX-1 protein. When the same experiment was performed in the presence of ucf-101, a specific inhibitor of the proteolytic activity of Omi, the percentage of apoptotic cells decreased to 66%. HAX-1 degradation was also inhibited in the presence of ucf-101. At the higher concentration of 70 μM ucf-101, cisplatin-induced apoptosis of HK-2 cells was further reduced to 48%. At this higher concentration, ucf-101 also had a more pronounced effect in blocking HAX-1 degradation. The level of Omi protein in HK-2 cells did not show any significant variation under any of these conditions.

HAX-1 Is Not Degraded in mnd2 Cells That Carry an Inactive Omi Protease—To further validate that Omi, and not some other protease, is responsible for HAX-1 degradation during apoptosis, we used cell lines derived from *mnd2* mice (9, 20).

[3] M. M. Soundarapandian, G. A. Kyriazis, L. Cilenti, and A. S. Zervos, unpublished data.

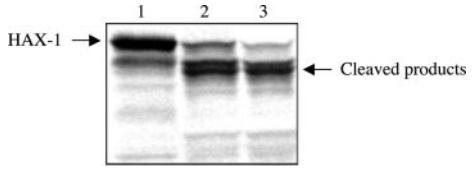

FIG. 2. **HAX-1 can be cleaved by Omi protease *in vitro*.** *In vitro* translated ^{35}S-labeled HAX-1 protein was incubated at 37 °C without (*lane 1*) or with 500 ng of recombinant His-Omi$_{134-458}$ for 2 h (*lane 2*) and 6 h (*lane 3*). The reactions were resolved on SDS-PAGE, and the gel was transferred on a PDVF membrane and exposed on X-OMAT-AR film.

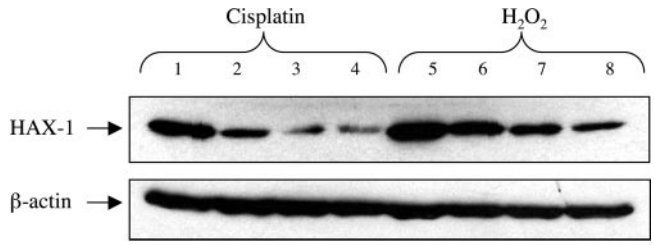

FIG. 3. **The level of HAX-1 protein is regulated by cisplatin or H_2O_2 treatment.** Total cell lysates were prepared from HK-2 cells after induction of apoptosis using different concentrations of cisplatin or H_2O_2 for 14 h. *Lane 1* shows lysates from control cells treated with vehicle (*N*,*N*-dimethylformamide). *Lanes 2*, *3*, and *4* show cell extracts from HK-2 treated with 30, 50, and 70 μM cisplatin, respectively. *Lane 5* shows cell lysates without treatment; *lanes 6*, *7*, and *8* contain lysates obtained after 1, 2, and 4 mM of H_2O_2 treatment. In the *lower panel*, β-actin antibody was used to verify that equal amounts of protein were present in each lane.

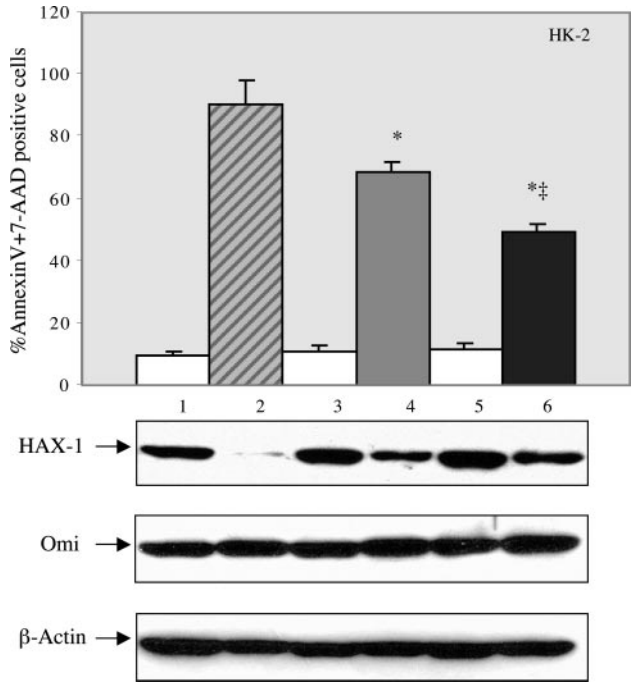

FIG. 4. **ucf-101 protects HK-2 cells from cisplatin-induced apoptosis and inhibits HAX-1 degradation.** HK-2 cells were treated with 50 or 70 μM of ucf-101, and apoptosis was induced with 50 μM cisplatin for 14 h. Cell death was monitored using Annexin V (apoptosis) and 7-aminoactinomycin D (necrosis) staining and analyzed by flow cytometry (15, 16). Extracts were prepared from the same cell population and analyzed by SDS-PAGE and Western blot using HAX-1 and Omi antibodies. This corresponds with increased apoptosis in the cell population. When HK-2 cells were treated with ucf-101 followed by cisplatin, the inhibitor substantially protected HAX-1 proteins and the percentage of apoptotic cells was significantly reduced (*lanes 4* and *6*). *Lane 1* shows cell lysates obtained from untreated cells, *lane 2* shows lysates after 50 μM of cisplatin, *lane 3* shows lysates after treatment with 50 μM of ucf-101, *lane 4* shows lysates after treatment with 50 μM of ucf-101 followed by 50 μM of cisplatin, *lane 5* shows cell lysates after treatment with 70 μM of ucf-101, and *lane 6* shows lysates after treatment with 70 μM of ucf-101 followed by 50 μM of cisplatin. Results shown are means ± S.D. of four independent experiments. *, $p < 0.05$ *versus* cisplatin 50 μM (*lane 2*); ‡, $p < 0.05$ *versus* ucf-101+cisplatin (*lane 4*).

These mice carry a single mutation in the Omi gene that changes the amino acid serine in position 276 to a cysteine; this amino acid substitution abolishes the proteolytic activity of Omi (9). *mnd2* mouse embryonic fibroblasts were immortalized and then stably transfected with an empty vector (*mnd2*-MSCV) or a vector expressing human Omi (*mnd2*-MSCV-Omi) (14). Apoptosis was induced in these two cell lines using various stimuli, and the percentage of cell death was estimated. HAX-1 and Omi protein levels were also monitored by Western blot analysis. The three different apoptotic stimuli employed induced cell death in *mnd2*-MSCV-Omi cells to various degrees. Under these conditions, H_2O_2 induced more cell death than etoposide or cisplatin. The level of HAX-1 protein was dramatically decreased during apoptosis in these cells, and there was an inverse correlation between the level of HAX-1 protein and the percentage of apoptotic cells (Fig. 5).

When the same experiment was performed with the *mnd2*-MSCV parental cell line that carries the mutated Omi protein, the results were very different. The percentage of apoptotic cells after treatment was significantly lower when compared with *mnd2*-MSCV-Omi cells. Furthermore, no significant HAX-1 degradation was observed in these cells. The level of Omi protein was much higher in the *mnd2*-MSCV-Omi cells than in the parental cell line (*mnd2*-MSCV) presumably caused by multiple copies of Omi gene introduced during transfection (Fig. 5).

Omi-induced HAX-1 Degradation Occurs in the Mitochondria—HAX-1 protein is reported to be present in the mitochondria as well as in the cytoplasm (10, 21). We investigated where in the cell Omi-induced degradation of HAX-1 occurs. Fig. 6 shows that the HAX-1 protein is present in the mitochondria. During induction of apoptosis with cisplatin or H_2O_2, HAX-1 remained in the mitochondria, and its protein level dramatically decreased. XIAP, on the other hand, is found in the cytoplasm and there was a decrease in its protein level after induction of apoptosis. The protein level of Omi in the cytoplasm increased after the pro-apoptotic treatment because of its release from the mitochondria (1, 2). Some Omi protein remained associated with the mitochondrial fraction even after induction of apoptosis. Cytochrome *c* was also released from the mitochondria to the cytoplasm in a similar manner as the Omi protein (Fig. 6). The small amount of XIAP protein detected in the mitochondrial fractions as well as the cytochrome *c* and Omi proteins seen in the control cell lysates are probably caused by cross-contamination between the mitochondrial and cytosolic fractions.

HAX-1 Overexpression Has an Anti-apoptotic Effect and Regulates Mitochondrial Membrane Potential—Previous studies clearly demonstrated that HAX-1 has an anti-apoptotic function, but the mechanism of its action is not known (10, 22). We tested the anti-apoptotic function of HAX-1 in our system and any effect HAX-1 overexpression might have on the mitochondrial membrane potential. HEK293 cells were transfected with EGFP-C1 vector or EGFP-HAX-1, and apoptosis was induced using different concentration of cisplatin. Fourteen h after treatment, the percentage of apoptotic cells was estimated by Annexin V staining, and mitochondrial membrane potential was monitored using TMRM followed by FACS analysis. Fig. 7*B* shows that the percentage of apoptotic cells (Annexin V positive) increased when the concentration of cisplatin was raised from 50 μM to 70 μM (42% to 67% annexin V positive). Furthermore, cells transfected with EGFP-HAX-1 were signif-

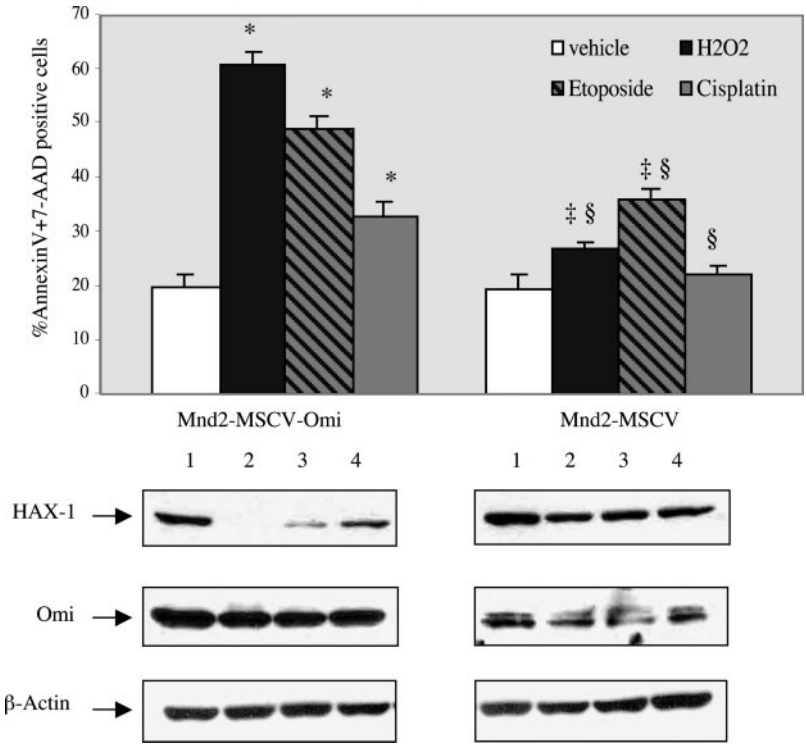

FIG. 5. **HAX-1 degradation is absent in *mnd2* cells.** Mouse embryonic fibroblasts from *mnd2* mice were transformed and then stably transfected with MSCV vector or MSCV-Omi (14). Cells were grown in six-well plates, and cell death was induced with H_2O_2, etoposide, or cisplatin. Cells were collected, and cell death was monitored by flow cytometry using Annexin V and 7-aminoactinomycin D, or cell lysates were prepared and processed for Western blot analysis using HAX-1 or Omi antibodies. β-actin antibody was used to verify equal amounts of protein were loaded in each lane. Results shown are means ± S.D. of four independent experiments. *, $p < 0.05$ *versus* vehicle *mnd2*-MSCV-Omi; ‡, $p < 0.05$ *versus* vehicle *mnd2*-MSCV; §, $p < 0.05$ *versus* their correspondent treatment in *mnd2*-MSCV-Omi.

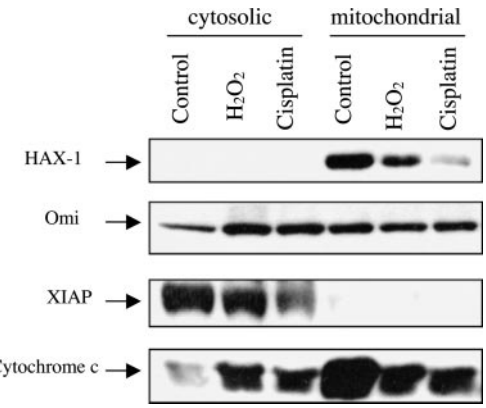

FIG. 6. **HAX-1 is cleaved in the mitochondria during induction of cell death.** HEK293 cells were treated with H_2O_2 or cisplatin; the control lane represents untreated cells. After treatment, cells were fractionated into cytosolic and mitochondrial fractions. The fractions were resolved by SDS-PAGE and analyzed by Western blot using HAX-1, Omi, XIAP, or cytochrome *c* antibodies.

icantly more resistant to cisplatin-induced apoptosis than cells transfected with the empty vector pEGFP-C1 (Fig. 7*B*). Loss of mitochondrial membrane potential ($\Delta\Psi_m$) in cells overexpressing HAX-1 and treated with cisplatin was also significantly different from the control cells. This result was more pronounced in cells treated with 50 μM cisplatin. In these cells loss of mitochondrial membrane potential occurred in 45% of cells transfected with pEGFP-C1. In contrast, there was only 16% loss of mitochondrial potential in cells transfected with EGFP-HAX-1 (Fig. 7, *A* and *C*).

DISCUSSION

Omi is a serine protease with homology to bacterial *HtrA* chaperones (23–26). It is made as a precursor protein that translocates to the mitochondria where it is processed to the mature form by proteolytic cleavage of a 133 amino acid amino-terminal peptide (1, 2, 4). This cleavage exposes an AVPS sequence motif at the new amino terminus; this motif is found to be present in all known IAP-binding proteins (2, 4). Upon induction of apoptosis, Omi translocates to the cytoplasm where it binds to the baculovirus IAP repeat domain of IAPs via its AVPS sequence motif (2, 4). This follows a specific cleavage and degradation of IAPs by Omi. Removal of IAPs initiates caspase-dependent apoptosis. Omi is also able to induce caspase-independent apoptosis via a poorly understood mechanism that relies entirely on its ability to function as a protease (4).

While confined in the mitochondria, Omi might also have a distinct function maintaining the mitochondrial homeostasis (9, 27). This was suggested by the identification of a single mutation in the Omi gene as the cause of the *mnd2* phenotype in mice (9, 20). This mutation inactivates the proteolytic activity of Omi without affecting its protein level or subcellular localization. The phenotype of the *mnd2* homozygous mice is muscle wasting, neurodegenerative disease, and death by 6 weeks of age (9). This suggests that the primary function of Omi in mammalian cells might be a chaperone-like activity in the mitochondria (9). Other known apoptotic proteins, such as cytochrome *c* and endonuclease G, are also found to have mundane functions in healthy cells while playing different and distinct roles in apoptosis (28). The proteolytic activity of Omi is necessary and essential for its pro-apoptotic function as well as its "chaperone-like" activity in the mitochondria (27). This suggests that cleavage of specific substrates is involved in each process. The only known substrates of Omi are the IAPs (5, 6, 8) but this helps only to explain how Omi activates caspase-dependent apoptosis. Therefore, identification of new substrates for Omi is necessary to fully understand its normal function in mammalian cells. In this report, we used the yeast two-hybrid system to isolate cDNAs encoding proteins that interact with Omi with the assumption that some of them might also be substrates for this protease. Several clones were isolated, and a detailed description of this work will be presented elsewhere.[3] One of the clones isolated multiple times from both the human melanocyte and human keratinocyte cDNA library encoded for HAX-1 (10). HAX-1 is a 35-kDa protein that has sequence similarity to Nip3 protein and shares homology to the BH1 and BH2 domains from the Bcl-2 family of proteins (10). HAX-1 was originally isolated as an interactor of HS1 (10) and later shown

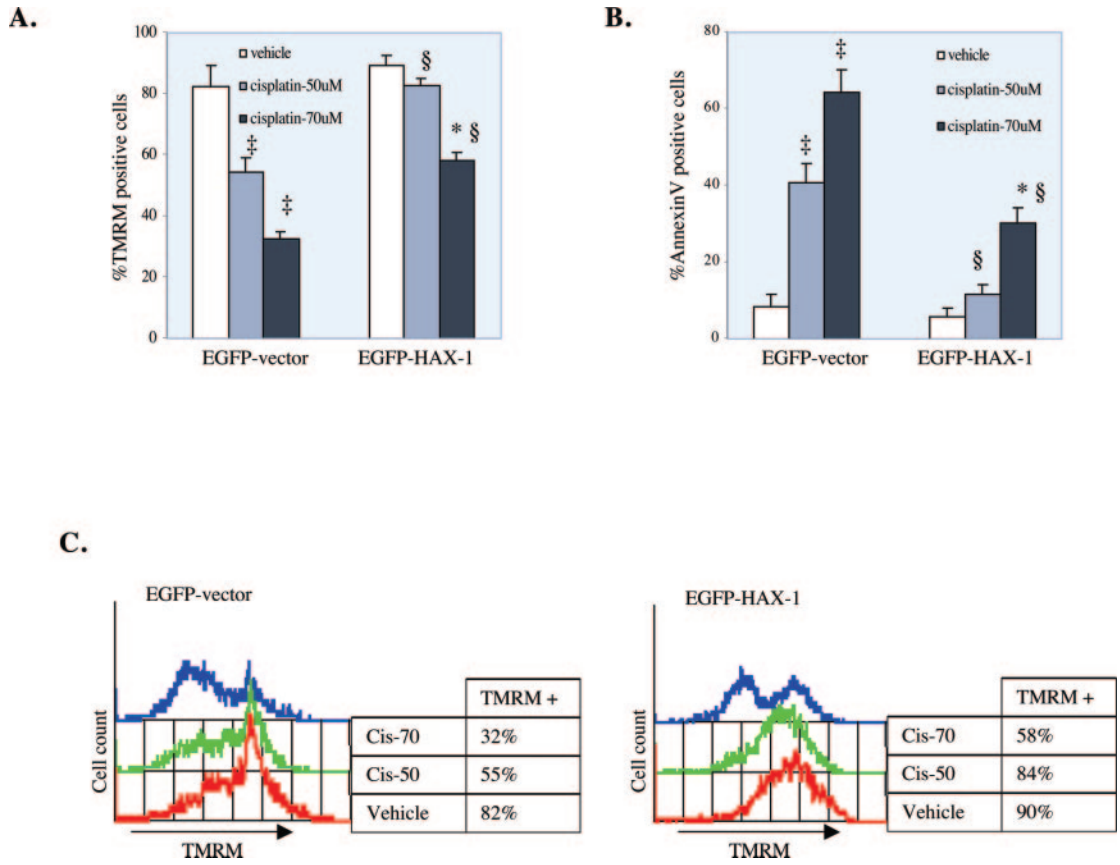

FIG. 7. **HAX-1 overexpression has an anti-apoptotic effect and regulates mitochondrial membrane potential.** *A*, HEK293 cells were transfected with empty EGFP vector or EGFP-HAX-1 as described under "Material and Methods." Apoptosis was induced using 50 and 70 μM of cisplatin for 14 h. Cells were stained with either TMRM (*A*) or Annexin V (*B*), incubated at 37 °C for 15 min, and analyzed using a FACSCalibur flow cytometer (BD Biosciences). Data are means ± S.D. of four independent experiments. ‡, $p < 0.05$ *versus* vehicle EGFP-vector; *, $p < 0.05$ *versus* vehicle EGFP-HAX-1; §, $p < 0.05$ *versus* their correspondent treatment in EGFP-vector. *C*, histogram showing results from a representative experiment that measures changes in the mitochondrial membrane potential ($\Delta\Psi_m$) in HEK293 cells over-expressing HAX-1 and treated with cisplatin. HEK293 cells were transfected with EGFP-vector (*left panel*) or EGFP-HAX-1 (*right panel*) and treated with two different concentrations of cisplatin, 50 μM (*green*) or 70 μM (*blue*). Control denotes cells treated with vehicle alone (*red*). The percentage of TMRM-positive cells in the transfected population of cells is indicated.

to also interact with PKD2 (21), EBNA-LP (29, 30), and K15-Kaposi's sarcoma proteins (22). Down-regulation of HAX-1 using antisense RNA has been shown to induce apoptosis in HaCaT cells (31). We found that HAX-1 protein could specifically interact with Omi in yeast; this interaction involved both the PDZ-domain and the catalytic domain of Omi. When HAX-1 was tested in the same system against L56/HtrA1, a mammalian homolog of Omi with extensive similarity, no interaction was observed. Co-precipitation experiments showed HAX-1 and Omi also associate in mammalian cells. Omi was also able to cleave HAX-1 *in vitro*. Because HAX-1 has been reported to be an anti-apoptotic protein (10, 22), we investigated if it is removed during apoptosis and whether Omi could be the protease responsible for its degradation. When apoptosis was induced in HK-2 cells using cisplatin or H_2O_2, there was a dramatic decrease in the level of HAX-1 protein.

To show that HAX-1 degradation is part of the apoptotic process and any involvement Omi may have, we used the ucf-101 inhibitor. ucf-101 is a specific inhibitor of the proteolytic activity of Omi and has been described previously (13). When HK-2 cells were treated with cisplatin in the presence of ucf-101, the percentage of apoptotic cells decreased and the inhibitor significantly blocked HAX-1 degradation. This effect was more pronounced when a higher concentration of the inhibitor was used.

To confirm the specificity of the inhibitor in this system and exclude the possibility that another protease rather than Omi is involved in HAX-1 cleavage, we used cell lines derived from *mnd2* mice (9). The parent cell line (*mnd2*-MSCV) derived from mouse embryo fibroblasts has no detectable Omi proteolytic activity (9). The same cell line has been transfected with wild type human Omi cDNA (*mnd2*-MSCV-Omi) and expresses high levels of active Omi protein (14). We found that in *mnd2*-MSCV cells, when induced to undergo apoptosis with various stimuli, the number of apoptotic cells was very low. Furthermore, no detectable cleavage of HAX-1 was observed. This is in contrast with the *mnd2*-MSCV-Omi cells where apoptosis was robust, and HAX-1 levels were inversely proportional to the degree of apoptosis. This experiment clearly shows that Omi is solely responsible for HAX-1 cleavage, which is essential for apoptosis under the conditions used in these experiments. HAX-1 subcellular localization depends on cell type (21, 30) and has been reported to be present in the mitochondria, cytoplasm, or plasma membrane (10, 21, 22, 30). We performed subcellular fractionation to investigate where HAX-1 cleavage by Omi takes place. We found that, in HEK293 cells, HAX-1 was predominantly present in the mitochondria, and this localization did not change in response to apoptotic stimuli. This suggests that Omi can initiate apoptosis in the mitochondria by cleaving HAX-1 protein. This is in accord with a recent study that shows Omi can induce apoptosis in human neutrophils treated with TNF-α without being released from the mitochondria (7). Although several studies clearly define HAX-1 as an anti-apoptotic protein, the mechanism of its function is unknown.

HAX-1 has sequence similarity to Bcl-2 family of proteins (10, 22). When we overexpressed HAX-1 protein into HEK293 cells, the cells became significantly more resistant to cisplatin-induced apoptosis than cells transfected with vector alone. HAX-1-overexpressing cells were also more resistant to loss of mitochondrial membrane potential ($\Delta\Psi_m$) than control cells following cisplatin treatment. This suggests that HAX-1 anti-apoptotic function might be mediated through its ability to block $\Delta\Psi_m$ collapse during induction of apoptosis. Therefore, cleavage of HAX-1 by Omi in mitochondria would allow the normal depolarization of the mitochondrial membrane leading to the release of pro-apoptotic proteins to the cytoplasm. The fact that both Omi and HAX-1 are localized in mitochondria suggests the existence of yet another control mechanism that prevents HAX-1 degradation by Omi under normal conditions, but allows it upon induction of apoptosis. This could be achieved through "modulator" proteins that bind to Omi after induction of apoptosis and regulate its proteolytic activity. We have reported previously that the proteolytic activity of Omi is regulated and dramatically increased in the kidneys of mice that have undergone ischemia/reperfusion (23). The presence of modulators of the proteolytic activity of Omi was recently confirmed by the isolation of presenilin as a specific Omi interactor that regulates its proteolytic activity (14). HAX-1 is not the only anti-apoptotic protein that is cleaved and inactivated by Omi during cell death. Ped/pea-15 has recently been identified as a specific Omi interactor that is also degraded upon induction of apoptosis (32). Ped/pea-15 is a cytoplasmic protein, whereas HAX-1 is in the mitochondria. Therefore, cleavage of HAX-1 by Omi might be an early event and defines a potential new pro-apoptotic pathway initiated in the mitochondria. The strict inverse correlation we observed between the protein level of HAX-1 and the degree of cell death suggest that this protein and its regulation by Omi play a central role in mammalian cell death.

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Regulation of HAX-1 Anti-apoptotic Protein by Omi/HtrA2 Protease during Cell Death

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