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## Omi/HtrA2 Promotes Cell Death by Binding and Degrading the Anti-apoptotic Protein ped/pea-15\*

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ped/pea-15 is a ubiquitously expressed 15-kDa protein featuring a broad anti-apoptotic function. In a yeast two-hybrid screen, the pro-apoptotic Omi/HtrA2 mitochondrial serine protease was identified as a specific interactor of the ped/pea-15 death effector domain. Omi/HtrA2 also bound recombinant ped/pea-15 *in vitro* and co-precipitated with ped/pea-15 in 293 and HeLa cell extracts. In these cells, the binding of Omi/HtrA2 to ped/pea-15 was induced by UVC exposure and followed the mitochondrial release of Omi/HtrA2 into the cytoplasm. Upon UVC exposure, cellular ped/pea-15 protein expression levels decreased. This effect was prevented by the ucf-101 specific inhibitor of the Omi/HtrA2 proteolytic activity, in a dose-dependent fashion. *In vitro* incubation of ped/pea-15 with Omi/HtrA2 resulted in ped/pea-15 degradation. In intact cells, the inhibitory action of ped/pea-15 on UVC-induced apoptosis progressively declined at increasing Omi/HtrA2 expression. This further effect of Omi/HtrA2 was also inhibited by ucf-101. In addition, ped/pea-15 expression blocked Omi/HtrA2 co-precipitation with the caspase inhibitor protein XIAP and caspase 3 activation. Thus, in part, apoptosis following Omi/HtrA2 mitochondrial release is mediated by reduction in ped/pea-15 cellular levels. The ability of Omi/HtrA2 to relieve XIAP inhibition on caspases is modulated by the relative levels of Omi/HtrA2 and ped/pea-15.

Apoptosis is mediated by proteolytic activation of caspases. Activated caspases catalytically degrade important intracellular substrates and induce cell death (1, 2). The initial proteolytic cleavage of caspases may be induced by the extrinsic cell-surface pathway through the activation of the tumor necrosis family of receptors, and/or by the intrinsic route through the release of

different apoptotic proteins from the mitochondria to the cytoplasm (3, 4). Because of its destructive nature, caspase activity must be tightly regulated inside the cells. However, the identity of the molecules involved in restraining apoptosis and their mechanism of action have been only partially elucidated.

ped/pea-15 is a ubiquitously expressed cytosolic protein exerting a broad anti-apoptotic action (5–10). First, by virtue of its death effector domain (DED),<sup>1</sup> ped/pea-15 binds other DED-containing proteins, preventing formation of the death-inducing signaling complex and inhibiting activation of the caspase cascade (8). Indeed ped/pea-15 blocks apoptotic responses initiated by Fas ligand, tumor necrosis factor- $\alpha$ , and tumor necrosis factor-related apoptosis-inducing ligand (7, 8, 10). Second, ped/pea-15 inhibits p38 and JNK activation by stress-inducing agents at a very upstream step in the stress-activated protein kinase activation cascade (9). Thus ped/pea-15 exerts its anti-apoptotic function by acting at multiple steps in the processes leading to caspase activation. Whether ped/pea-15 also affects apoptotic mechanisms triggered upon release of mitochondrial proteins is unknown at the present.

The Omi/HtrA2 serine protease is an antagonist of the inhibitor of apoptosis proteins (IAPs) identified in mammals (11, 12). Omi/HtrA2 is a nuclear-encoded mitochondrial protein. Cellular stresses, such as UV exposure, induces cleavage of the Omi/HtrA2 mitochondrial localization sequence thereby generating a mature active molecule featuring a new apoptogenic NH<sub>2</sub> terminus, termed the IAP-binding motif (13). This motif consists of a short stretch of hydrophobic amino acids. Hence, Omi/HtrA2 is released into the cytosol and competitively binds to the BIR domain of IAPs through the IAP-binding motif (14–16). This event leads to the release and reactivation of the BIR-bound caspases. Thus, Omi/HtrA2 binding displaces IAPs from caspases releasing the suppressive effect on caspase activity. Furthermore, Omi/HtrA2 can also trigger apoptosis in a caspase-independent pathway, which entirely depends on its serine protease function (17–20). However, neither the mechanism nor the significance of this function is clearly understood.

In this article, we show that, upon release into the cytoplasm of 293 cells, Omi/HtrA2 binds to and degrades ped/pea-15, thereby removing its anti-apoptotic action and triggering apoptosis. Thus, in addition to controlling the extrinsic pathway, ped/pea-15 is also embedded into the intrinsic pathway inducing caspase activation. We show that the relative levels of

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<sup>1</sup> The abbreviations used are: DED, death effector domain; JNK, c-Jun NH<sub>2</sub>-terminal kinase; IAP, inhibitor of apoptosis proteins; GST, glutathione S-transferase.

ped/pea-15 and Omi/HtrA2 play an important role in committing the cells to undergo apoptosis or survival.

#### EXPERIMENTAL PROCEDURES

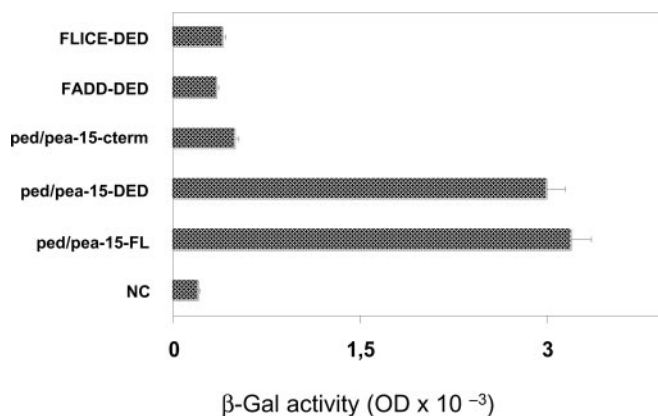
**Materials**—Media, sera, and antibiotics for cell culture and the LipofectAMINE reagent were from Invitrogen. PED polyclonal antibodies have been previously described (6). Rabbit Omi/HtrA2 antisera were raised by PRIMM (Milan, Italy), using the OVA-conjugated peptide NH<sub>2</sub>-HRGEKKNSSSGISGSQRRY-COOH. Mouse monoclonal antibodies toward XIAP were purchased from BD Biosciences and caspase 3 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The pcDNAIII vector encoding Omi/HtrA2 cDNA was a generous gift of Dr. I. Iaccarino (Imperial Cancer Research Fund, London, UK), and was reported by Martins *et al.* (20). The pGBKT7 yeast expression plasmids encoding FADD and FLICE have been previously reported (21). The yeast strain AH109, the human HeLa cDNA library in the pGADT7 vector, and the Matchmaker Gal4 two-hybrid system 3 were from Clontech (Palo Alto, CA). Generation of pGBKT7 yeast expression plasmids encoding the ped/pea-15 FL or DED cDNAs or the ped/pea-15-(80–130) COOH terminus cDNA were obtained by amplifying ped/pea-15, respectively, with the following sets of primers. ped/pea-15 5' NdeI (5'-GGGAATTCATATGGTTGAGTACGGG-3') and 3'ped/pea-15 BamHI (5'-CGCGGATCCTCAGGCCTTCTTCGCTGG GGGACC-3'); ped/pea-15 5' NdeI (5'-GGGAATTCATATGGTTGAGTAC GGG-3') and ped/pea-15 3' EcoRI (5'-CCGGAATTCCTCAAACCACCATAGTGAGTAGGTC-3'); ped/pea-15 5' EcoRI (5'-CCGGAATTCGACTACAGAA CCCGTGTGCTG-3') and ped/pea-15 3' BamHI (5'-CGCGGATCCTCAGGCCTTCTTCGCTGGGGGACC-3'). SDS-PAGE reagents were purchased from Bio-Rad. Radiochemicals, Western blotting, and ECL reagents were from Amersham Biosciences. All other chemicals were from Sigma.

**Cell Culture and Transfection, Cell Death, and Caspase 3 Cleavage Assays**—The 293 kidney embryonic cells stably expressing ped/pea-15 cDNA have been previously described (9). The 293 and HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 IU/ml streptomycin, and 2% L-glutamine in a humidified CO<sub>2</sub> incubator. Transient transfection experiments with Omi/HtrA2 and ped/pea-15 cDNAs were performed using the LipofectAMINE method according to the manufacturer's instructions (Invitrogen). Briefly, the cells were cultured in 60-mm dishes up to 80% confluence and incubated for 24 h with the indicated amounts of cDNA and 15  $\mu$ l of LipofectAMINE in serum-free Dulbecco's modified Eagle's medium. An equal volume of Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum was added for 5 h. The medium was then replaced with Dulbecco's modified Eagle's medium supplemented with 10% serum and the cells were further incubated for 24 h before the assay.

For detecting apoptosis, the cells were exposed to UVC (100 J/m<sup>2</sup>), as indicated in the description of the individual experiments. Apoptosis was then estimated using the Apoptosis ELISA Plus kit (Roche Diagnostics), according to the manufacturer's instructions. For caspase 3 cleavage, subconfluent cells were exposed to UVC as above. The cells were lysed in 1% Triton X-100, 150 mM NaCl, 10% glycerol, 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 16,000  $\times$  g for 15 min at 4  $^{\circ}$ C, supernatants were subjected to Western blotting with caspase 3 antibodies.

**Transformation of Yeast Strains and  $\beta$ -Galactosidase Assay**—Plasmid DNA transformations were performed using an high efficiency lithium acetate procedure (22). Cotransformants were propagated on Trp<sup>-</sup>, Leu<sup>-</sup> plates, and potential interacting clones selected in Trp<sup>-</sup>, Leu<sup>-</sup>, His<sup>-</sup>, Ade<sup>-</sup> media. After 4 days of incubation at 30  $^{\circ}$ C, positive clones were further tested for  $\beta$ -galactosidase activity by liquid culture assays using the substrate *o*-nitrophenyl- $\beta$ -D-galactopyranoside as described by Miller *et al.* (23). Clones of interest were analyzed by DNA sequencing and BLAST analysis.

**Cell Subfractionation and Western Blot Analysis**—Subcellular fractions were obtained as described in Refs. 24 and 25. Briefly, cells were lysed in ice-cold 10 mM HEPES, pH 7.4, 5 mM MgCl<sub>2</sub>, 40 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin. Lysed cells were centrifuged at 200  $\times$  g to pellet nuclei. Supernatants were centrifuged at 10,000  $\times$  g to pellet the heavy membrane fraction containing mitochondria, and the resulting liquid phase was further centrifuged at 150,000  $\times$  g to pellet plasma membranes. The last supernatant represented the cytosolic fraction (24). Mitochondria were further purified by resuspending heavy membrane pellets in 250 mM mannitol, 0.5 mM EGTA, 5 mM HEPES, pH 7.4, 0.1% bovine serum albumin, and layering on 30% Percoll, 225 mM mannitol, 1 mM EGTA, 25 mM HEPES, pH 7.4, 0.1% bovine serum albumin. After centrifugation at 95,000  $\times$  g, mitochon-



**FIG. 1. Analysis of Omi/HtrA2 interaction with ped/pea-15 fragments.** Measurement of the  $\beta$ -galactosidase activity in transformed yeasts is shown. The yeast reporter strain AH109 was co-transformed with the pGADT7 plasmid encoding Omi/HtrA2 full-length cDNA in combination with the pGBKT7 plasmid encoding the FL, DED, or COOH terminus regions of ped/pea-15, or the DED of FADD or FLICE. Transformants were isolated on selective plates. Activation of the LacZ reporter gene was monitored by measuring  $\beta$ -galactosidase activity in cell lysates using *o*-nitrophenyl- $\beta$ -D-galactopyranoside as substrate. The activities are expressed in Miller's units (23) and are the average  $\pm$  S.D. of values obtained with samples prepared from five independent transformants.

dria were recovered from the lower phase (26). Mitochondria were then centrifuged, washed, and resuspended in 50 mM potassium phosphate, pH 7.4, and used in the experiments described below. Purity of the mitochondrial fraction was assessed by assaying succinate dehydrogenase and cytochrome *c* oxidase activities (27, 28). Results showed that >99% activity of these enzymes associated with the mitochondrial fractions and <1% of the total activities with the other fractions.

For Western blot assays, the cells were solubilized in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 4 mM EDTA, 10 mM Na<sub>4</sub>PO<sub>7</sub>, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 100 mM NaF, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 100  $\mu$ g/ml aprotinin, 1 mM leupeptin) for 30 min at 4  $^{\circ}$ C. Lysates were centrifuged at 5,000  $\times$  g for 15 min and solubilized proteins were separated by SDS-PAGE and transferred on 0.45- $\mu$ m Immobilon-P membranes (Millipore, Bedford, MA). Upon incubation with primary and secondary antibodies, immunoreactive bands were detected by ECL according to the manufacturer's instructions.

**In Vitro Interaction of Omi/HtrA2 and ped/pea-15 and Protease Assay**—To analyze ped/pea-15 interaction with Omi/HtrA2, a ped/pea-15-glutathione *S*-transferase (GST) fusion protein was generated as described in Ref. 29. Cell lysates (500  $\mu$ g of protein) were incubated in the presence of Sepharose-bound GST-ped/pea-15 (2  $\mu$ g) for 2 h at 4  $^{\circ}$ C. The beads were washed four times with TnT buffer (0.5% Nonidet P-40, 25 mM Tris, pH 7.5, 30 mM MgCl<sub>2</sub>, 40 mM NaCl, 1 mM dithiothreitol) and then resuspended in Laemmli buffer followed by boiling for 4 min and centrifugation at 25,000  $\times$  g for 3 min. Supernatants were separated by SDS-PAGE followed by blotting with Omi/HtrA2 antibodies.

Omi/HtrA2 protease activity was assayed by incubating 2  $\mu$ g of recombinant his-Omi/HtrA2-(134–458) protein with 10  $\mu$ g of recombinant ped/pea-15 in 20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA for 1 h at 37  $^{\circ}$ C. The reactions were stopped with SDS-sample buffer and samples were boiled for 5 min. The reaction products were analyzed by SDS-PAGE followed by Western blot analysis using ped/pea-15 antibodies. Alternatively, the reaction products were resolved by SDS-PAGE followed by Coomassie Blue staining.

#### RESULTS

**Isolation and Identification of Omi/HtrA2 as a Novel ped/pea-15 Interacting Protein**—To search for proteins that specifically interact with ped/pea-15, a yeast two-hybrid system was established using the full-length (FL) ped/pea-15 gene (pGBKT7-ped/pea-15) as bait to screen a human HeLa library (Clontech). Upon HIS3 selection, 45  $\beta$ -galactosidase positive cDNA clones were detected. Based upon sequence analysis and BLAST searching, 6 of these clones were shown to match the Omi/HtrA2 serine protease (11, 12, 30).



We also sought to define the region responsible for the interaction of Omi/HtrA2 with ped/pea-15. We generated two further pGBKT7 plasmids encoding either the DED (amino acids 1–80), or the COOH terminus region of ped/pea-15 (amino acids 80–130). We then compared the ability of the pGADT7-Omi/HtrA2 to interact with the full-length, DED, and the COOH terminus regions of ped/pea-15 in the yeast system. Measurement of  $\beta$ -galactosidase activity revealed that Omi/HtrA2 interacted with both the FL and ped/pea-15 DED fusion proteins (Fig. 1). However, no Omi/HtrA2 interaction was detected when the ped/pea-15 COOH terminus was used as bait. No interaction was also detected when the DEDs of the FADD or FLICE proteins were used, suggesting that Omi/HtrA2 specifically interacts with the ped/pea-15 death effector domain.

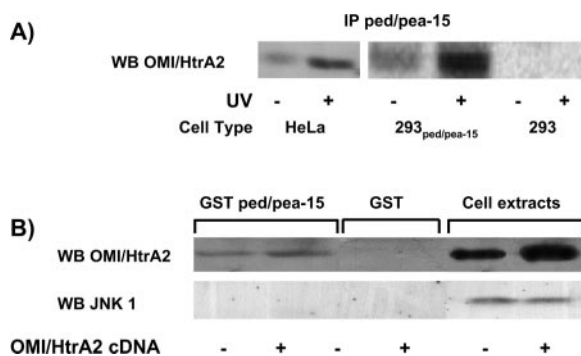
**Omi/HtrA2 Interacts with ped/pea-15 in Vitro as Well as in Mammalian Cells**—Upon cell exposure to UV and other stress-inducing agents, Omi/HtrA2 is released from mitochondria (18, 30) to the cytoplasm, where ped/pea-15 is largely localized (5, 31). To verify the interaction of Omi/HtrA2 with ped/pea-15 in mammalian cells, we used HeLa cells expressing endogenous ped/pea-15 and 293 cells stably transfected with ped/pea-15

cDNA (293<sub>PED</sub>). As shown in Fig. 2A, precipitation of lysates from the two cell types with ped/pea-15 antibodies followed by blotting with Omi/HtrA2 antibodies revealed little ped/pea-15 co-precipitation with Omi/HtrA2. UV exposure of the cells, however, induced a >20-fold increase in the co-precipitation, suggesting that UV-induced cleavage of Omi/HtrA2 is necessary to enable interaction with ped/pea-15 as well as with IAPs. Indeed, in control experiments, UV treatment caused the appearance of the 37,000 mature Omi/HtrA2 in the total cell lysates and simultaneous disappearance of the 49,000 precursor (data not shown). Whether in the absence or presence of UV irradiation no co-precipitation occurred in untransfected 293 cells, as these cells do not express detectable levels of endogenous ped/pea-15 (Fig. 2A).

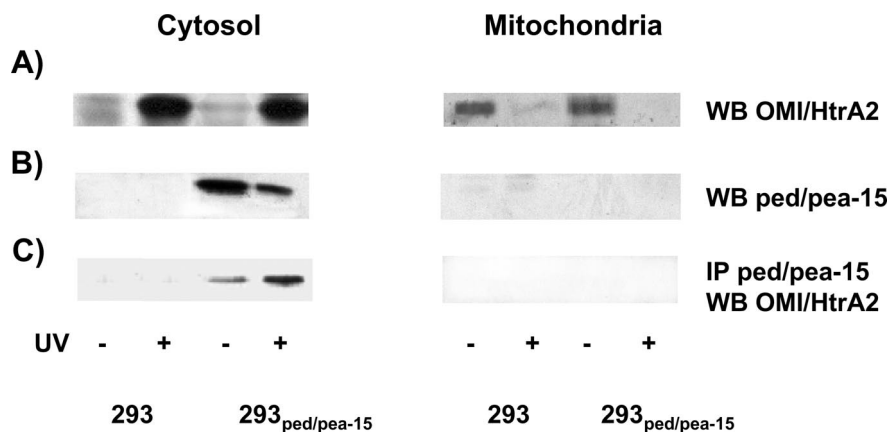
Based on pull-down assays, GST-fused recombinant ped/pea-15 bound to the endogenous Omi/HtrA2 in 293 cell extracts (Fig. 2B). Omi/HtrA2 interaction with ped/pea-15 was >2-fold more evident in lysates from 293 cells that have been transiently transfected with the Omi/HtrA2 cDNA and overexpress Omi/HtrA2 by 2.5-fold. No pull-down was detectable using GST alone. No pull-down was detectable blotting with JNK rather than with Omi/HtrA2 antibodies either, indicating that Omi/HtrA2 specifically binds ped/pea-15 *in vitro* as well as in intact mammalian cells.

**Omi/HtrA2 Mitochondrial Release Is Accompanied by Reduced ped/pea-15 Cellular Levels**—To further investigate the biological significance of Omi/HtrA2 interaction with ped/pea-15, we performed subcellular fractionation experiments in UV-exposed and unexposed cells. In the absence of UV treatment, Omi/HtrA2 was mainly mitochondrial both in the 293<sub>PED</sub> and the ped/pea-15 untransfected cells (Fig. 3, A and B). In both cell types, UV exposure led to an almost complete disappearance of mitochondrial Omi/HtrA2 and a parallel appearance of Omi/HtrA2 in the cytoplasmic fraction. At variance, ped/pea-15 was only detected in the cytoplasm of the 293<sub>PED</sub> cells, whether UV-treated or not. Indeed, co-precipitation of Omi/HtrA2 with ped/pea-15 only occurred in the cytoplasmic fraction of the 293<sub>PED</sub> cells and only upon UV-induced mitochondrial release (Fig. 3C). Importantly, upon UV exposure, ped/pea-15 cellular levels declined in parallel with the release of Omi/HtrA2 into the cytoplasm (Fig. 3B), raising the possibility that Omi/HtrA2 may reduce stability of ped/pea-15.

To test this hypothesis, we transiently overexpressed Omi/HtrA2 in 293<sub>PED</sub> as well as in HeLa cells (expressing only endogenous ped/pea-15). Then, upon UV light exposure, we compared ped/pea-15 levels in the transfected *versus* the un-



**FIG. 2. Interaction of Omi/HtrA2 with ped/pea-15 in HeLa and 293 cells and *in vitro*.** A, HeLa cells expressing endogenous ped/pea-15 and 293 cells, either expressing transfected ped/pea-15 (293<sub>ped/pea-15</sub>) or not, were exposed to UVC (100 J/m<sup>2</sup>) and then solubilized as described under “Experimental Procedures.” Lysates were immunoprecipitated (IP) with ped/pea-15 antibodies followed by blotting with Omi/HtrA2 antibodies. B, upon transient transfection with Omi/HtrA2 cDNA, 293 cells were solubilized and lysates incubated for 2 h at 4 °C with Sepharose-GST-bound recombinant ped/pea-15 or GST alone, as indicated. Pulled down proteins and total cell extracts were then blotted with Omi/HtrA2 or JNK antibodies as described under “Experimental Procedures.” Blots were revealed by ECL and autoradiography. The autoradiographs shown are representative of four (A) and three (B) independent experiments. WB, Western blot.



**FIG. 3. Subcellular localization of Omi/HtrA2 and ped/pea-15.** 293 cells stably overexpressing ped/pea-15 and control 293 cells were exposed to UVC (100 J/m<sup>2</sup>) and cytosolic and mitochondrial fractions obtained as described under “Experimental Procedures.” 80  $\mu$ g of proteins from each fraction were blotted with Omi/HtrA2 (A) or ped/pea-15 (B) antibodies or, precipitated with ped/pea-15 antibodies followed by blotting with Omi/HtrA2 antibodies (C). Blots were revealed by ECL and autoradiography. The autoradiographs shown are representative of four (A and B) and three (C) independent experiments. IP, immunoprecipitated; WB, Western blot.

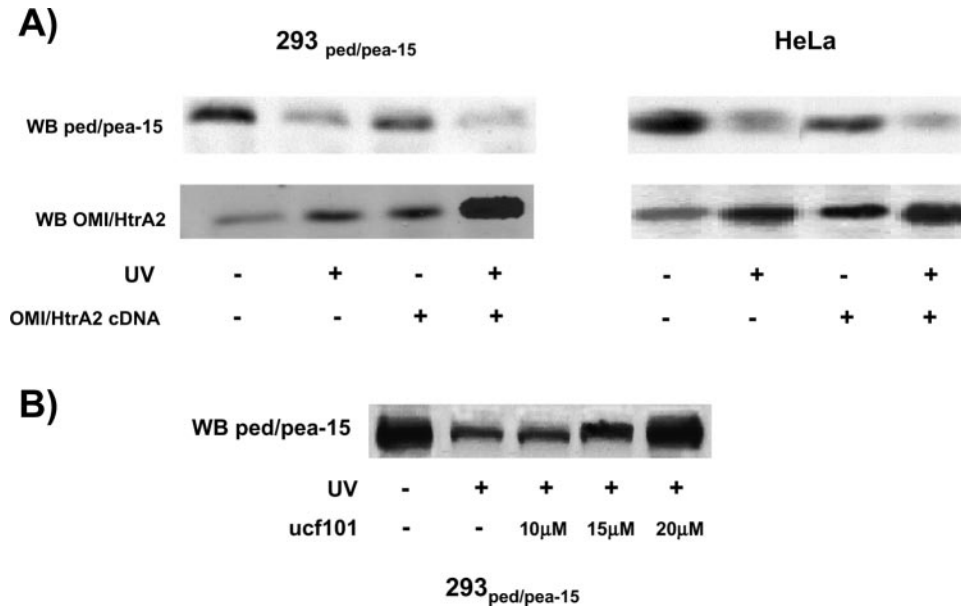


FIG. 4. **Effect of Omi/HtrA2 overexpression on ped/pea-15 levels.** *A*, 293 cells stably expressing ped/pea-15 and HeLa cells were transiently transfected with Omi/HtrA2 cDNA. The cells were then exposed to UVC (100 J/m<sup>2</sup>), solubilized, and then blotted with ped/pea-15 antibodies. For control, aliquots of the lysates were also blotted with Omi/HtrA2 antibodies. Blots were revealed by ECL and autoradiography. The autoradiographs shown are representative of four independent experiments. *B*, the 293<sub>ped/pea-15</sub> cells were transiently transfected with Omi/HtrA2 and exposed to UVC (100 J/m<sup>2</sup>) in the absence or presence of the indicated concentrations of ucf-101. The cells were then solubilized and blotted with ped/pea-15 antibodies. Blots were revealed by ECL and autoradiography. The autoradiograph shown is representative of three independent experiments. *WB*, Western blot.

transfected cells. In both the 293<sub>PED</sub> and HeLa cells, overexpression of Omi/HtrA2 (20-fold above the endogenous levels) caused only a slight 10% reduction in ped/pea-15 levels (Fig. 4A;  $p < 0.01$ ). However, upon UV irradiation and Omi/HtrA2 release into the cytoplasm, the cells overexpressing Omi/HtrA2 exhibited a 2-fold larger reduction in ped/pea-15 levels compared with those expressing only the endogenous Omi/HtrA2. Importantly, this decrease in ped/pea-15 levels was inhibited in a dose-dependent fashion by the specific Omi/HtrA2 protease inhibitor ucf-101 (Fig. 4B) (32), indicating that the UV effect is largely dependent on Omi/HtrA2 protease activity.

We have therefore tested the hypothesis that ped/pea-15 may serve as a direct Omi/HtrA2 substrate. We incubated recombinant ped/pea-15 (rped/pea-15) with active recombinant Omi/HtrA2-(134–458). After a 2-h incubation, we compared the amount of intact ped/pea-15 remaining in the absence or presence of Omi/HtrA2. Based on Western blot analysis and Coomassie Blue staining (A), ped/pea-15 protein level was decreased by 60% in the presence of Omi/HtrA2 (Fig. 5). GST was not affected by Omi/HtrA2, however (B). As shown in panel A, Omi/HtrA2-induced disappearance of ped/pea-15 was prevented by ucf-101, indicating specific cleavage of ped/pea-15 by the Omi/HtrA2 protease *in vitro*.

**Omi/HtrA2 Reduces ped/pea-15 Anti-apoptotic Function—** We also sought to examine the functional consequences of Omi/HtrA2 mitochondrial release on ped/pea-15 function. To this end, we analyzed UV-induced apoptosis in 293<sub>PED</sub> cells upon transient transfection of increasing amounts of Omi/HtrA2 cDNA. As shown in Fig. 6, the expression of ped/pea-15 in 293 cells was accompanied by a 90% reduction in UV apoptosis. However, the anti-apoptotic function of ped/pea-15 was reduced by Omi/HtrA2 transfection. This reduction paralleled the increase in the Omi/HtrA2 expression level in the cells and was prevented by ucf-101 treatment, suggesting that, in part, apoptosis following Omi/HtrA2 mitochondrial release is mediated by reduction in ped/pea-15 cellular levels. Indeed, in HeLa cells, increased levels of transfected Omi/HtrA2 caused increased degradation of endogenous ped/pea-15, which was in-

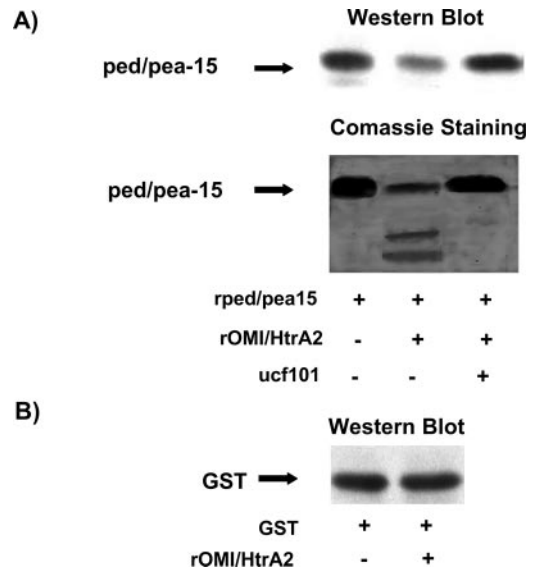
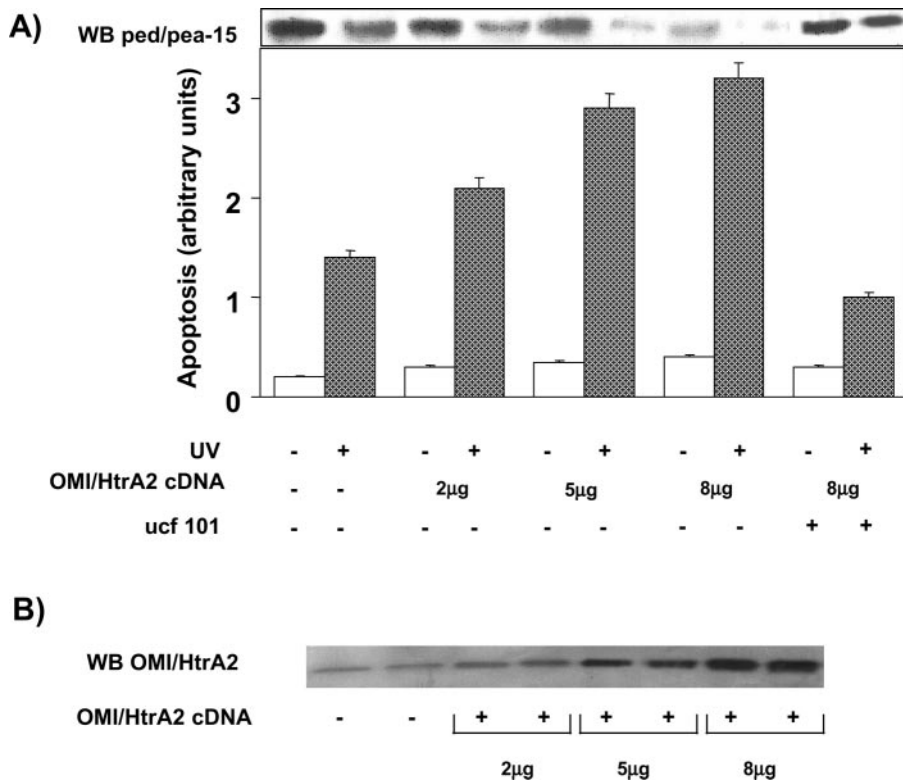
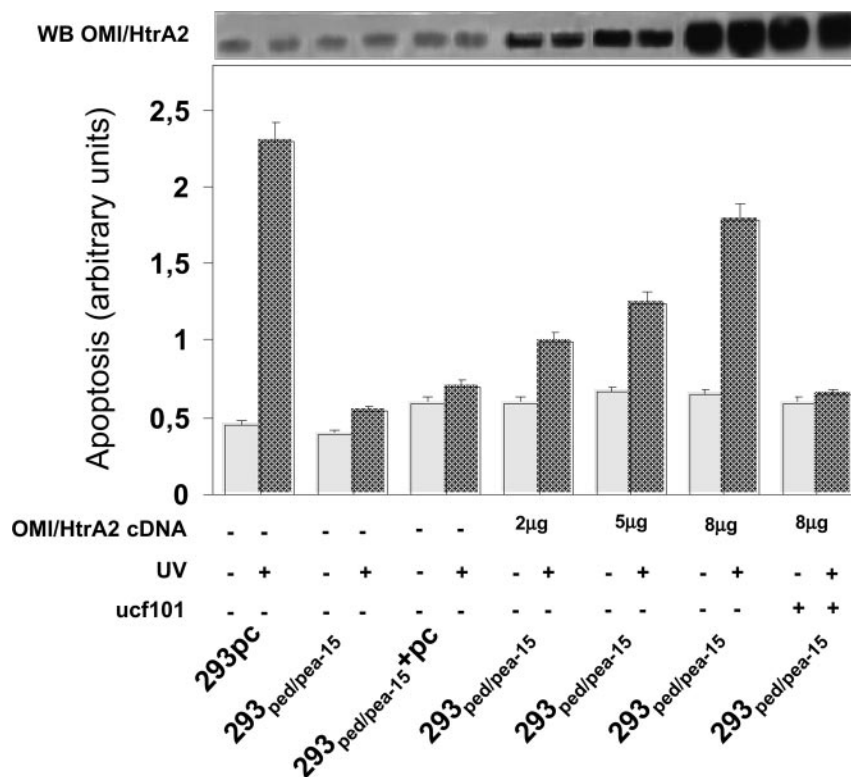


FIG. 5. **Effect of Omi/HtrA2 on ped/pea-15 *in vitro*.** *A*, recombinant ped/pea-15 (rped/pea-15; 10 mg) was incubated with the recombinant Omi/HtrA2-(134–458) active fragment (rOmi/HtrA2-(134–458); 2 μg) for 60 min at 37 °C. The reaction was stopped with Laemmli buffer at 100 °C and proteins were separated by SDS-PAGE followed by blotting with ped/pea-15 antibodies or Coomassie Blue staining. For control, GST was also incubated with rOmi/HtrA2-(134–458) as above, followed by blotting with GST antibodies (B). Blots were revealed by ECL and autoradiography. The images shown are representative of four (A) and three (B) independent experiments.

hibited by ucf-101 (Fig. 7A, top). As in the 293<sub>PED</sub> cells, Omi/HtrA2 transfection in HeLa cells was accompanied by augmented UV-induced apoptosis (Fig. 7A, bottom), as a function of the expression level of Omi/HtrA2 achieved (Fig. 7B).

If reducing the ped/pea-15 cytoplasmic level is relevant to Omi/HtrA2 apoptosis in UV-exposed cells, one would also predict that increasing cellular levels of ped/pea-15 results in limited Omi/HtrA2 function. To test this hypothesis, we fo-

**FIG. 6. Effect of Omi/HtrA2 on ped/pea-15 function.** 293 cells stably expressing either the control vector (*pc*) or ped/pea-15 were transiently transfected with 8  $\mu$ g of pcDNAIII ( $293_{ped/pea-15}^{+pc}$ ) or the indicated amounts of Omi/HtrA2 cDNA and further incubated with 20  $\mu$ M ucf-101 for 60 min, as indicated. The cells were then exposed to UVC (100 J/m<sup>2</sup>) and apoptosis was quantitated by the ELISA Plus detection kit as described under "Experimental Procedures." For control, aliquots of the samples were solubilized, blotted with Omi/HtrA2 antibodies, and revealed by ECL and autoradiography (*top panel*). Bars represent the mean  $\pm$  S.D. of duplicate determinations in four independent experiments. The blot in the *top panel* represents duplicate controls for Omi/HtrA2 expression in UVC-treated cells and are representative of three independent experiments. WB, Western blot.



**FIG. 7. Modulation of the anti-apoptotic function of endogenous ped/pea-15 by Omi/HtrA2 in HeLa cells.** A, HeLa cells were transiently transfected with the indicated amounts of Omi/HtrA2 cDNA and further incubated with 20  $\mu$ M ucf-101 for 60 min, as indicated. The cells were then exposed to UVC (100 J/m<sup>2</sup>) and apoptosis was quantitated by the ELISA Plus detection kit as described under "Experimental Procedures." Aliquots of the samples were solubilized, blotted with ped/pea-15 antibodies (*top panel*) or, for control, with Omi/HtrA2 antibodies (*B*) and revealed by ECL and autoradiography (*top panel*). Bars represent the mean  $\pm$  S.D. of duplicate determinations in three independent experiments. WB, Western blot.

cused on UV-triggered apoptosis in 293 cells expressing increasing amounts of ped/pea-15. In these cells, apoptosis progressively declined with increasing ped/pea-15 expression levels (Fig. 8). Simultaneously, in 293<sub>PED</sub> as well as in HeLa cells, ped/pea-15 overexpression inhibited Omi/HtrA2 co-precipitation with the caspase inhibitor protein XIAP (Fig. 9, A and B). In HeLa cells, the effect of ped/pea-15 was accompanied by an almost complete inhibition of UV-induced activation of caspase 3 (Fig. 9C). Caspase 3 cleavage was well evident in

cells expressing only the endogenous ped/pea-15, however. Similar results were obtained in 293 cells (data not shown). Thus, in the cell, the ability of Omi/HtrA2 to release the XIAP brake on caspase activation may be modulated by the relative concentrations of Omi/HtrA2 and ped/pea-15.

#### DISCUSSION

Omi/HtrA2 is a nuclear-encoded mitochondrial serine protease serving as a sensor of unfolding stresses in the mitochon-



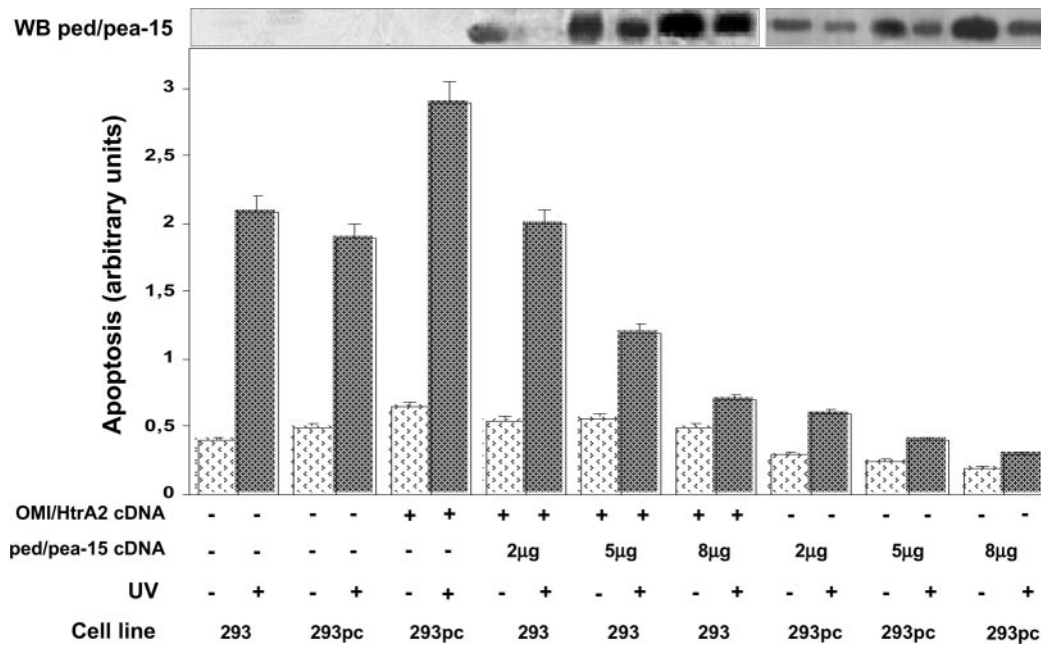


FIG. 8. **Effect of ped/pea-15 on Omi/HtrA2 function.** 293 cells were transiently transfected with either the control plasmid (293pc) or Omi/HtrA2 cDNA. Co-transfection with the indicated amounts of ped/pea-15 cDNA was also achieved. The cells were then exposed to UVC (100 J/m<sup>2</sup>) and apoptosis was quantitated by the ELISA Plus detection kit as described under "Experimental Procedures." For control, aliquots of the samples were solubilized, blotted with ped/pea-15 antibodies, and revealed by ECL and autoradiography (top panel). Bars represent the mean  $\pm$  S.D. of duplicate determinations in four independent experiments. The blots shown are representative of those obtained in the four experiments. WB, Western blot.

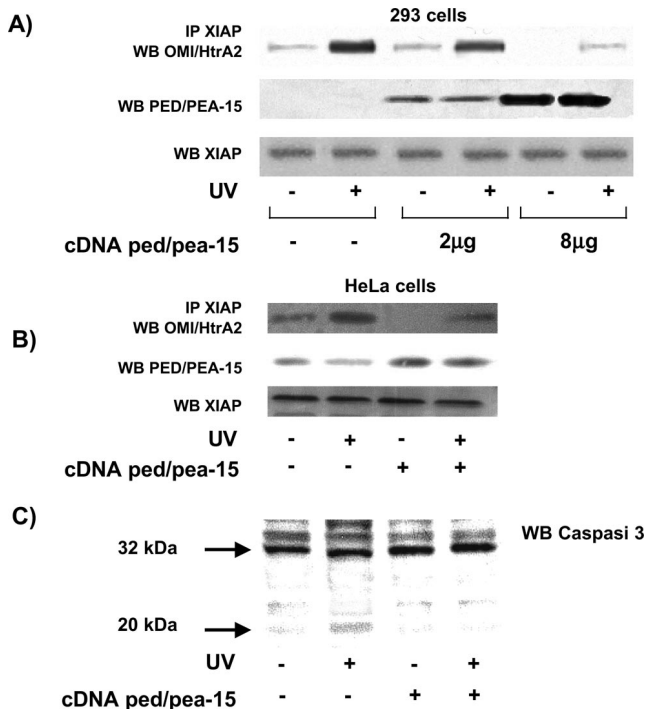


FIG. 9. **Effect of ped/pea-15 on Omi/HtrA2 co-precipitation with XIAP and caspase 3 activation.** A, 293 cells were transiently transfected with the indicated amounts of ped/pea-15 cDNA and then exposed to UVC (100 J/m<sup>2</sup>). The cells were solubilized and precipitated with XIAP antibodies, followed by blotting with Omi/HtrA2 antibodies. For control, aliquots of the cell lysates were also blotted with ped/pea-15 or XIAP antibodies, as indicated. B and C, HeLa cells were transfected with ped/pea-15 cDNA or with a control plasmid. Aliquots of the lysates obtained from unexposed or UVC-exposed cells were either precipitated with XIAP antibodies, followed by blotting with Omi/HtrA2 antibodies (B, top panel) or directly blotted with ped/pea-15 (B, middle panel), XIAP (B, bottom panel), and caspase 3 antibodies (C). Blots were revealed by ECL and autoradiography. The autoradiographs shown are representative of three independent experiments. WB, Western blot.

dria. In the mutant *mnd2* mice, loss of the Omi/HtrA2 protease activity leads to mitochondrial disintegration, neuronal degeneration, and death (33). It has been shown that, once in the cytoplasm, Omi/HtrA2 promotes caspase activation and apoptosis by binding to the BIR3 domain of the mammalian caspase inhibitor XIAP (13, 30).

Omi/HtrA2 binding displaces XIAP from caspases, inhibiting the suppressive effect on caspase activity (13–16). There is also evidence that interaction with IAPs is not the only mechanism involved in Omi/HtrA2-induced cell death, as the serine protease activity of Omi/HtrA2, when localized in the cytosol, is sufficient to cause caspase-independent cell death (17). This evidence led to the concept that protease activity plays an important role in regulation of apoptosis by Omi/HtrA2 (32). Indeed, Srinivasula *et al.* (34) have shown that IAPs are substrates for Omi/HtrA2 and their degradation may contribute to caspase activation by Omi/HtrA2. However, whether other substrates also play a role in Omi/HtrA2-induced cell death is unknown.

In the present work, we have identified the serine protease Omi/HtrA2 as a novel ped/pea-15 interactor, by yeast two-hybrid screening. Furthermore, we show that the anti-apoptotic protein ped/pea-15 co-precipitates with Omi/HtrA2 in mammalian cell extracts and specifically binds Omi/HtrA2 in pull-down assays. Thus, Omi/HtrA2 may also bind ped/pea-15 in mammalian cells. At variance with ERK, another ped/pea-15-binding protein (35), Omi/HtrA2 interaction requires the DED of ped/pea-15. Indeed, a truncated protein, lacking the entire DED, does not interact with Omi/HtrA2 in yeast. Furthermore, the DED of ped/pea-15 but not that of other DED-containing proteins, is sufficient for the interaction to occur, indicating specificity of Omi/HtrA2 for the DED of ped/pea-15.

Interestingly, the mitochondrial release of Omi/HtrA2 in response to UV exposure was accompanied by a significant decrease in cellular ped/pea-15 levels. This effect was amplified upon overexpression of Omi/HtrA2 cDNA and might have been produced either by Omi/HtrA2 decreasing ped/pea-15 synthesis



and/or by increasing its degradation. The latter appeared a more likely possibility based upon the information that Omi/HtrA2 features serine-protease activity (11, 18–20). Consistently, we have shown that, (i) ped/pea-15 is a substrate of Omi/HtrA2 serine protease *in vitro*; and (ii) the reduction in ped/pea-15 levels accompanying UV-induced release of Omi/HtrA2 from mitochondria is blocked by a specific inhibitor of its serine protease activity. Thus, the evidence indicates that, upon being released into the cytoplasm, Omi/HtrA2 binds to and degrades ped/pea-15.

Degradation of ped/pea-15 by Omi/HtrA2 was accompanied by reduction of the ped/pea-15 apoptosis-inhibiting function. Indeed, the protection from UV-induced apoptosis accomplished by ped/pea-15 in both HeLa and 293 cells was progressively abolished by increasing the expression levels of Omi/HtrA2 in the cell, and this effect was blocked by inhibition of the Omi/HtrA2 serine protease. We suggest therefore that, in part, the caspase-independent cell death induced by cytoplasmic release of Omi/HtrA2 is mediated by ped/pea-15 degradation. The relevance of ped/pea-15 to Omi/HtrA2 apoptosis was further underlined by the finding that, in 293 cells, the ability of Omi/HtrA2 to bind XIAP and induce cleavage and activation of caspase 3 progressively declined in close parallel with increasing ped/pea-15 cellular levels and with increasing protection from UV-induced apoptosis. Thus, the relative concentrations of Omi/HtrA2 and ped/pea-15 in the 293 cell cytoplasm play an important role in committing the cells to apoptosis.

Compartmentalization appears to be a major mechanism through which control of this balance is accomplished in the 293 cells. Indeed, in the present work, we show that Omi/HtrA2 interaction with ped/pea-15 only occurs in the cytoplasm and upon UV-induced release of Omi/HtrA2 from the mitochondria. Apoptotic signals alter Omi/HtrA2 compartmentalization enabling its protease activity toward different cytoplasmic substrates whose cleavage fosters activation of the apoptotic machinery of the cell.

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#### REFERENCES

- Cryns, V., and Yuan, J. (1998) *Genes Dev.* **12**, 1551–1570
- Thornberry, N. A., and Lazebnik, Y. (1998) *Science* **281**, 1312–1316
- Nagata, S. (1997) *Cell* **88**, 355–365
- Wang, X. (2001) *Genes Dev.* **15**, 2922–2933
- Danziger, N., Yokoyama, M., Jay, T., Cordier, J., Glowinski, J., and Chneiweiss, H. (1995) *J. Neurochem.* **64**, 1016–1025
- Condorelli, G., Vigliotta, G., Iavarone, C., Caruso, M., Tocchetti, C. G., Androzzi, F., Cafieri, A., Tecce, M. F., Formisano, P., Beguinot, L., and Beguinot, F. (1998) *EMBO J.* **17**, 3858–3866
- Kitsberg, D., Formstecher, E., Fauquet, M., Kubes, M., Cordier, J., Canton, B., Pan, G., Rolli, M., Glowinski, J., and Chneiweiss, H. (1999) *J. Neurosci.* **19**, 8244–8251
- Condorelli, G., Vigliotta, G., Cafieri, A., Trencia, A., Andalo, P., Oriente, F., Miele, C., Caruso, M., Formisano, P., and Beguinot, F. (1999) *Oncogene* **18**, 4409–4415
- Condorelli, G., Trencia, A., Vigliotta, G., Perfetti, A., Goglia, U., Cassese, A., Musti, A. M., Miele, C., Santopietro, S., Formisano, P., and Beguinot, F. (2002) *J. Biol. Chem.* **277**, 11013–11018
- Hao, C., Beguinot, F., Condorelli, G., Trencia, A., Van Meir, E. G., Yong, V. W., Parney, I. F., Roa, W. H., and Petruk, K. C. (2001) *Cancer Res.* **61**, 1162–1170
- Gray, C. W., Ward, R. V., Karran, E., Turconi, S., Rowles, A., Vigliangi, D., Southan, C., Barton, A., Fantom, K. G., West, A., Savopoulos, J., Hassan, N. J., Clinkenbeard, H., Hanning, C., Amegadzie, B., Davis, J. B., Dingwall, C., Livi, G. P., and Creasy, C. L. (2000) *Eur. J. Biochem.* **267**, 5699–5710
- Faccio, L., Fusco, C., Chen, A., Martinotti, S., Bonventre, J. V., and Zervos, A. S. (2000) *J. Biol. Chem.* **275**, 2581–2588
- Yang, Q. H., Church-Hajduk, R., Ren, J., Newton, M. L., and Du, C. (2003) *Genes Dev.* **17**, 487–496
- Liu, Z., Sun, C., Olejniczak, E. T., Meadows, R. P., Betz, S. F., Oost, T., Herrmann, J., Wu, J. C., and Fesik, S. W. (2000) *Nature* **408**, 1004–1008
- Wu, G., Chai, J., Suber, T. L., Wu, J. W., Du, C., Wang, X., and Shi, Y. (2000) *Nature* **408**, 1008–1012
- Srinivasula, S. M., Hegde, R., Saleh, A., Datta, P., Shiozaki, E., Chai, J., Lee, R. A., Robbins, P. D., Fernandes-Alnemri, T., Shi, Y., and Alnemri, E. S. (2001) *Nature* **410**, 112–116
- Li, W., Srinivasula, S. M., Chai, J., Li, P., Wu, J. W., Zhang, Z., Alnemri, E. S., and Shi, Y. (2002) *Nat. Struct. Biol.* **9**, 436–441
- Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., and Takahashi, R. (2001) *Mol. Cell* **8**, 613–621
- Hegde, R., Srinivasula, S. M., Zhang, Z., Wassell, R., Mukattash, R., Cilenti, L., DuBois, G., Lazebnik, Y., Zervos, A. S., Fernandes-Alnemri, T., and Alnemri, E. S. (2002) *J. Biol. Chem.* **277**, 432–438
- Martins, L. M., Iaccarino, I., Tenev, T., Gschmeissner, S., Totty, N. F., Lemoine, N. R., Savopoulos, J., Gray, C. W., Creasy, C. L., Dingwall, C., and Downward, J. (2002) *J. Biol. Chem.* **277**, 439–444
- Guilet, C., and Vito, P. (2000) *J. Cell Biol.* **148**, 1131–1140
- Gietz, D., St. Jean, A., Woods, R. A., and Schiestl, R. H. (1992) *Nucleic Acids Res.* **20**, 1425
- Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Ruvolo, P. P., Deng, X., Carr, B. K., and May, W. S. (1998) *J. Biol. Chem.* **273**, 25436–25442
- Wang, H. G., Rapp, U. R., and Reed, J. C. (1996) *Cell* **87**, 629–638
- Hovius, R., Lambrechts, H., Nicolay, K., and de Kruijff, B. (1990) *Biochim. Biophys. Acta.* **1021**, 217–226
- Gateau, O., Morelis, R., and Louisot, P. (1978) *Eur. J. Biochem.* **88**, 613–622
- Pennington, R. (1961) *Biochem. J.* **80**, 649–654
- Trencia, A., Perfetti, A., Cassese, A., Vigliotta, G., Miele, C., Oriente, F., Santopietro, S., Giacco, F., Condorelli, G., Formisano, P., and Beguinot, F. (2003) *Mol. Cell. Biol.* **23**, 4511–4521
- van Loo, G., van Gurp, M., Depuydt, B., Srinivasula, S. M., Rodriguez, I., Alnemri, E. S., Gevaert, K., Vandekerckhove, J., Declercq, W., and Vandenaebroeck, P. (2002) *Cell Death Differ.* **9**, 20–26
- Condorelli, G., Vigliotta, G., Trencia, A., Maitan, M. A., Caruso, M., Miele, C., Oriente, F., Santopietro, S., Formisano, P., and Beguinot, F. (2001) *Diabetes* **50**, 1244–1252
- Cilenti, L., Lee, Y., Hess, S., Srinivasula, S., Park, K. M., Junqueira, D., Davis, H., Bonventre, J. V., Alnemri, E. S., and Zervos, A. S. (2003) *J. Biol. Chem.* **278**, 1489–1494
- Jones, J. M., Datta, P., Srinivasula, S. M., Ji, W., Gupta, S., Zhang, Z., Davies, E., Hajnoczky, G., Saunders, T. L., Van Keuren, M. L., Fernandes-Alnemri, T., Meisler, M. H., and Alnemri, E. S. (2003) *Nature* **425**, 721–727
- Srinivasula, S. M., Gupta, S., Datta, P., Zhang, Z., Hegde, R., Cheong, N., Fernandes-Alnemri, T., and Alnemri, E. S. (2003) *J. Biol. Chem.* **278**, 31469–31472
- Formstecher, E., Ramos, J. W., Fauquet, M., Calderwood, D. A., Hsieh, J. C., Canton, B., Nguyen, X. T., Barnier, J. V., Camonis, J., Ginsberg, M. H., and Chneiweiss, H. (2001) *Dev. Cell* **1**, 239–250

**Omi/HtrA2 Promotes Cell Death by Binding and Degrading the Anti-apoptotic Protein ped/pea-15**

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