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Sanjeev Gupta

Rajesh Singh

Pinaki Datta

Zhi-Jia Zhang

Christopher Orr

See next page for additional authors

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The C-terminal Tail of Presenilin Regulates Omi/HtrA2 Protease Activity*

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Sanjeev Gupta‡§, Rajesh Singh‡§, Pinaki Datta‡, ZhiJia Zhang‡, Christopher Orr‡, Zhixian Lu‡, Garrett DuBois‡, Antonis S. Zervos¶, Miriam H. Meisler‖, Srinivasa M. Srinivasula‡, Teresa Fernandes-Alnemri‡, and Emad S. Alnemri‡ ‡‡**

From the ‡Center for Apoptosis Research and the Department of Microbiology and Immunology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, the ¶Biomolecular Science Center, University of Central Florida, Orlando, Florida 32826, and the ‖Department of Human Genetics, University of Michigan, Ann Arbor, Michigan 48109-0618

Presenilin mutations are responsible for most cases of autosomal dominant inherited forms of early onset Alzheimer disease. Presenilins play an important role in amyloid β-precursor processing, NOTCH receptor signaling, and apoptosis. However, the molecular mechanisms by which presenilins regulate apoptosis are not fully understood. Here, we report that presenilin-1 (PS1) regulates the proteolytic activity of the serine protease Omi/HtrA2 through direct interaction with its regulatory PDZ domain. We show that a peptide corresponding to the cytoplasmic C-terminal tail of PS1 dramatically increases the proteolytic activity of Omi/HtrA2 toward the inhibitor of apoptosis proteins and β-casein and induces cell death in an Omi/HtrA2-dependent manner. Consistent with these results, ectopic expression of full-length PS1, but not PS1 lacking the C-terminal PDZ binding motif, potentiated Omi/HtrA2-induced cell death. Our results suggest that the C terminus of PS1 is an activation peptide ligand for the PDZ domain of Omi/HtrA2 and may regulate the protease activity of Omi/HtrA2 after its release from the mitochondria during apoptosis. This mechanism of Omi/HtrA2 activation is similar to the mechanism of activation of the related bacterial DegS protease by the outer-membrane porins.

The presenilin proteins, presenilin-1 (PS1)[1] and presenilin-2 (PS2), have been implicated in the etiology of Alzheimer disease (AD) in humans (1–3). Mutations in the PS1 gene are responsible for about 60% of familial early-onset AD cases (4). Presenilins are highly conserved in evolution, being present also in *Caenorhabditis elegans* and *Drosophila melanogaster*, and are transcribed in many different cell types, both within the central nervous system and in peripheral tissues.

PS1 is a polytopic integral membrane protein composed of eight hydrophobic transmembrane (TM) segments, with N-terminal and C-terminal tails facing the cytoplasm. It is believed to be a catalytic subunit of a stable, high molecular mass γ-secretase complex, which contains also the three essential cofactors nicastrin, anterior pharynx defective 1, and presenilin enhancer 2 (5). In this complex the PS1 holoprotein (~44 kDa) undergoes constitutive endoproteolytic processing between TM6 and TM7 and, therefore, exists as a stable heterodimer composed of N-terminal fragment and C-terminal fragment (6). The presenilin-dependent γ-secretase activity is responsible for the proteolytic cleavage of the amyloid precursor protein, the NOTCH receptor protein, and several other substrates, including ErbB4, LRP, CD44, E-Cadherin, Nectin-1α, APLP-1, and Ire1α (7). PS1 interacts physically and functionally with proteins that are involved in a wide array of cellular functions. In particular, the C terminus of PS1 has been shown to interact with proteins such as PSAP, X11α, X11β, PAMP, and PARL but physiological function of these interactions is unknown (8–10).

Omi/HtrA2 is a serine protease that belongs to the high temperature requirement A (HtrA) protease family (11, 12). It is synthesized as a precursor protein and then targeted to the mitochondria where it is matured by the removal of N-terminal 133 residues. Recent studies have implicated Omi/HtrA2 in the process of apoptosis (13–16). The mature Omi/HtrA2 carries an IAP binding motif at its N terminus in addition to the serine protease domain. Following apoptotic stimuli, Omi/HtrA2 is released to the cytoplasm where it interacts with X-chromosome-linked inhibitor of apoptosis protein (XIAP) and relieves its inhibition of caspase-9 (13–16). In addition, Omi/HtrA2 can promote apoptosis in a caspase-independent mechanism through its ability to cleave IAPs in the cytoplasm (17–19).

In addition to its role in apoptosis we have recently shown that Omi/HtrA2 has a vital role in the mitochondria. The *mnd2* (motor neuron degeneration 2) mutant mice suffer from a neurodegenerative disease that causes muscle wasting and death by 6 weeks of age as a result of a point mutation in a noncatalytic serine residue (S276C) in the Omi/HtrA2 protein (20). The mutation does not affect the processing or localization of the mutant Omi/HtrA2 protein but renders it proteolytically inactive. The loss of Omi/HtrA2 protease activity increases the susceptibility of mitochondria to induction of the permeability

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§ Both authors contributed equally to this work.

** Sidney Kimmel Scholar.

‡‡ To whom correspondence should be addressed: Thomas Jefferson University, Kimmel Cancer Institute, Bluemle Life Sciences Bldg., Rm. 904, 233 S 10th St., Philadelphia, PA 19107. Tel.: 215-503-4632; Fax: 215-923-1098; E-mail: E_Alnemri@lac.jci.tju.edu.

[1] The abbreviations used are: PS1, -2, presenilins 1 and 2; AD, Alzheimer disease; TM, transmembrane; IAP, inhibitor of apoptosis protein; XIAP, X-chromosome-linked inhibitor of apoptosis protein; DMEM, Dulbecco's modified Eagle's medium; MEF, mouse embryonic fibroblast; GST, glutathione *S*-transferase; GFP, green fluorescent protein; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; DTBP, dimethyl-3,3-dithiobispropionimidate·2HCl; PI, propidium iodide; OMP, outer membrane porin; z-VAD-fmk, benzyloxycarbonyl-VAD-fluoromethyl ketone.

transition and increases the sensitivity of mouse embryonic fibroblasts to stress-induced cell death.

A defining feature of the HtrA protease family is the combination of a catalytic serine-protease domain with at least one PDZ domain (12, 21, 22). The PDZ domains are small globular domains that act as protein-protein recognition modules and are involved in many signaling cascades (12, 21, 22). They are named after the three proteins where they were originally found: postsynaptic density protein-95 (PSD-95) (23), *Drosophila* discs large tumor suppressor (Dlg) (24), and the tight junction protein (ZO-1) (25). The PDZ domains are defined by a unique sequence of 80–100 amino acids able to recognize specific C-terminal sequences in target proteins (26, 27). Recent structural and biochemical evidence suggested that the PDZ domain in the bacterial HtrA protease DegS modulates the activity of the protease domain upon binding to specific activation peptides derived from the C termini of the OMP proteins (28, 29). Here we report that peptides derived from the C termini of human Presenilins modulate the protease and apoptotic activities of Omi/HtrA2 by binding to its PDZ domain. Because mutations in Presenilins increase neuronal cell death, our findings suggest that activation of cytosolic Omi/HtrA2 by mutant Presenilins could be in part responsible for their increased apoptotic activity.

EXPERIMENTAL PROCEDURES

Cell Culture—Cells were cultured either in Dulbecco's modified Eagle's medium (DMEM) (HeLa and MEFs), DMEM/F-12 (293T cells), or RPMI 1640 (MCF-7 cells), supplemented with 10% fetal bovine serum, 200 $\mu g \cdot ml^{-1}$ penicillin, and 100 $\mu g \cdot ml^{-1}$ streptomycin sulfate. MEFs were isolated at E15 from mnd2/mnd2 embryos (20) and then immortalized by transfection with large T antigen cDNA. The human Omi/HtrA2-expressing mnd2-Omi/HtrA2 MEFs were generated by stable transfection of the immortalized mnd2 MEFs with a retroviral expression vector containing the full-length human Omi/HtrA2 cDNA (MSCV-hOmi). The mnd2-vector cells were generated from the immortalized mnd2 MEFs by stable transfection with the empty retroviral expression vector MSCV. HeLa cells expressing C-terminal FLAG-tagged full-length Omi/HtrA2 or only the PDZ domain of Omi/HtrA2 in the mitochondria were generated by stable transfection of HeLa Tet-Off™ (BD Biosciences) with the expression plasmid pTRE2-Omi/HtrA2 or pTRE2-Omi/HtrA2-MTS-PDZ, respectively.

Expression Constructs—Mammalian and bacterial expression constructs for WT and mutant Omi/HtrA2, β-casein, XIAP, c-IAP1, and c-IAP2 have been described previously (13, 17, 20). pTRE2-Omi/HtrA2 or pTRE2-Omi/HtrA2-MTS-PDZ were generated by inserting the cDNAs of full-length human Omi/HtrA2 or its mitochondrial targeting sequence fused in-frame to the PDZ domain, respectively, in the multiple cloning site of the expression plasmid pTRE2™ (BD Biosciences). DegS-ΔMA and RseA (residue 120 to end) were amplified by PCR using genomic DNA from *Escherichia coli* as a template and cloned in-frame into pET28a. The variants of GST having C-terminal tail of PS1 were generated by PCR using appropriate primers and cloned into pET28a. N-terminal GFP-tagged PS1 constructs were generated by PCR using appropriate primers and cloned into pEGFP vector. The nucleotide sequences of all constructs were confirmed by automated sequencing.

Peptide Synthesis—All peptides were synthesized by solid-phase techniques using a Symphony Multiplex synthesizer (Protein Technologies, Inc., Tucson, AZ) or a 9050 Pepsynthesizer Plus automated peptide synthesizer (Perseptive Biosystems, Cambridge, MA) with *N*-Fmoc/*tert*-butyl chemistry. For this procedure, Fmoc amino acids (4 equivalents) were activated with a mixture of 1-hydroxybenzo-triazole/2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (4 equivalents each) and diisopropylethylamine (8 equivalents) in dimethylformamide solvent. The Fmoc protecting group was removed with 20% piperidine in dimethylformamide. Deprotection of the amino acid side chains and cleavage of the peptide from the resin was performed by a 2.5-h incubation of the peptide in a mixture containing 95% trifluoroacetic acid, 2.5% water, and triisopropylsilane. For peptides containing cysteine and/or methionine, the deprotection mixture consisted of 94.5% trifluoroacetic acid, 2.5% water, 2.5% ethanedithiol, and 1% triisopropylsilane. Peptides were purified by reverse-phase high performance liquid chromatography (HPLC) on a C18 Vydac column using the 0.1% trifluoroacetic acid solvent system (aqueous buffer, 0.1% trifluoroacetic acid in HPLC grade H_2O-organic phase, 0.1% trifluoroacetic acid in 90% acetonitrile). The peptides were characterized by analytical HPLC and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. All the peptides were at least 95% pure, as determined by HPLC.

Transfections and Fluorescence Microscopy—Cells grown on 12-well plates were transfected with the required plasmids. The transfections were done using LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer's instructions. All the plasmids for transfection were prepared by using Qiagen columns. After 24–48 h of transfection, cells were observed using fluorescence microscope.

Co-immunoprecipitation Assays—HeLa Tet-Off™ cells stably expressing FLAG-Omi/HtrA2 and FLAG-Omi/HtrA2-PDZ under the control of the Tet-Off system were induced for 48 h, in DMEM medium without tetracycline. Cells were trypsinized, collected by centrifugation, and washed three times with ice-cold PBS. Cells were resuspended in PBS containing (1 mg/ml) dimethyl-3,3-dithiobispropionimidate·2HCl (DTBP) (protein cross-linker), diluted from freshly prepared stock solution (50 mg/ml), and incubated at 4 °C for 2 h. Subsequently cells were washed twice with PBS and resuspended in IP buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.5% Nonidet P-40, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Roche Applied Science)). Cells were lysed by passing 30 times through a 23.5-gauge needle and centrifuged at 14,000 rpm for 10 min. Cell lysates were pre-cleared with protein G-Sepharose and incubated with FLAG antibody (1:200) at 4 °C for 4 h. The immunocomplexes were precipitated and analyzed by Western blotting with PS1 antibody (Ab14) (kind gift from Dr. Sam Gandy, Thomas Jefferson University, Philadelphia, PA). In some experiments, 293T cells were co-transfected with constructs encoding GFP or C-terminal GFP-tagged full-length PS1, and FLAG-tagged mature Omi/HtrA2. 36 h after transfection, the cells were incubated with the cross-linker DTBP and then lysed in IP buffer as above, and the lysates were immunoprecipitated with an anti-GFP antibody. The immunoprecipitates were fractionated by SDS-PAGE and Western blotted with a FLAG antibody or an Omi/HtrA2-specific antibody. In other experiments, endogenous PS1 was immunoprecipitated with the PS1-Ab14 antibody from 293T cells after incubation with the cross-linker DTBP, and the immunoprecipitates were Western blotted with an Omi/HtrA2-specific antibody.

Cell Death Assays—Cells were seeded in 24-well plates at a density of 2×10^4 cells per well and treated with PTD or PTD-PS1-C15 peptides followed by staining with propidium iodide (PI). Cells showing PI staining, loss of cell volume, loss of refractility, and membrane blebbing were scored as apoptotic. At least 200 cells were counted in each well. The data represent mean ± S.D. from at least three independent experiments. Cell death induced by transient transfection of the GFP-tagged PS1 constructs was determined as above.

RESULTS

Omi/HtrA2 Interacts with the C-terminal Tail of PS1—The 3- to 4-residue PDZ binding motif located at the extreme C terminus in bacterial outer membrane porins (OMP) is an important regulator of the enzymatic activity of the bacterial HtrA protease DegS (28, 29). Because Omi/HtrA2 shares extensive sequence and structural homology with DegS (28, 29), we reasoned that its enzymatic activity might be regulated by a similar mechanism. Computer searches for mammalian membrane proteins with PDZ binding motifs similar to those present in the OMP proteins identified PS1 and PS2, as likely regulators for the serine protease activity of Omi/HtrA2. To test this hypothesis, we examined the ability of the last 15 residues of the cytoplasmic tail of PS1, which contains a consensus PDZ binding motif (FYI-COOH), to interact with Omi/HtrA2 by *in vitro* GST-pull-down assays. For this purpose we generated variants of GST having residues from the cytoplasmic tail of PS1 fused to the C terminus of GST (Fig. 1*A*). As shown in Fig. 1*B*, 15 residues of the C terminus of PS1 fused to GST (GST-PS1-C15) were able to precipitate endogenous Omi/HtrA2 from 293T cell extracts. However, no interaction was seen when the last 3 residues of PS1 were deleted (GST-PS1-ΔC3), or when only the last 3 residues of PS1 were used (GST-PS1-C3). These results indicate that the C-terminal 15 residue tail of PS1 interacts with Omi/HtrA2 and that the PDZ binding motif and residues adjacent to it are required for this interaction.

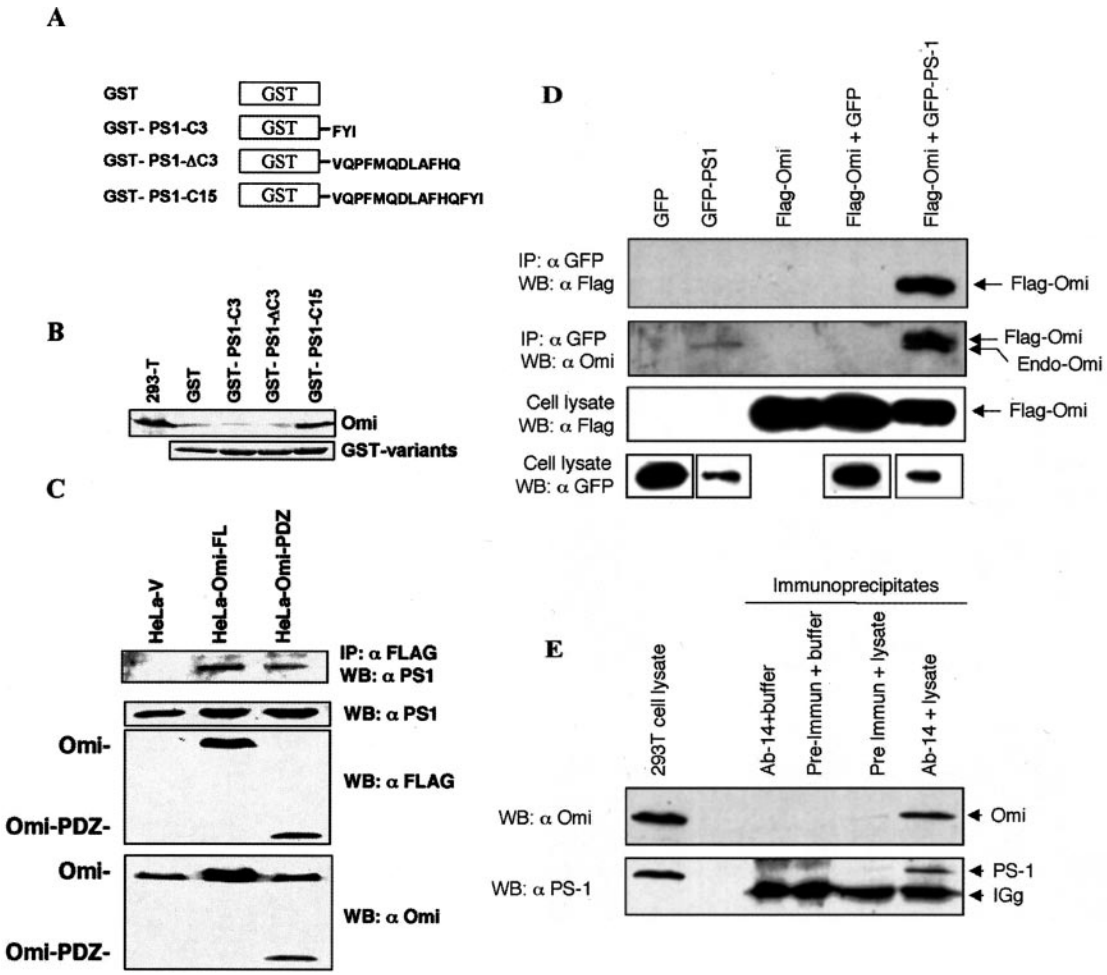

FIG. 1. **Interaction of Omi/HtrA2 with the C-terminal tail of PS1.** *A*, schematic representation of various GST-PS1 fusion proteins having PS1 residues fused at the C terminus of GST. *B*, *in vitro* interaction of Omi/HtrA2 with GST-PS1-C15 fusion protein. Extracts from 293T cells were incubated with GST, GST-PS1-C3, GST-PS1-ΔC3, or GST-PS1-C15 fusion protein immobilized on glutathione-Sepharose beads. The beads were washed, and the bound proteins were eluted with SDS sample buffer and Western blotted with Omi/HtrA2-specific antibody. *First lane*, total extracts from human 293T cells. *C*, *in vivo* interaction of Omi/HtrA2 and PS1. FLAG-tagged Omi/HtrA2 and Omi/HtrA2-PDZ domains were immunoprecipitated from HeLa cells with anti-FLAG antibody after cross-linking the cells with 1 mg/ml DTBP. *Upper panel*, Western blot analysis of immunoprecipitates with PS1 antibody. *Lower panel*, expression of PS1, FLAG-tagged proteins, and Omi/HtrA2 proteins in these cells. Note that the expression of the Omi/HtrA2-PDZ domain is comparable to the expression of endogenous Omi/HtrA2 in HeLa cells. *D*, 293T cells were transfected with the indicated constructs, and their lysates were then immunoprecipitated with anti-GFP antibody and Western blotted with anti-FLAG (*first panel from top*) or anti-Omi/HtrA2 antibody (*second panel from top*). Note that the FLAG-tagged Omi/HtrA2 migrates slower than the endogenous Omi/HtrA2 (*Endo-Omi*). The total cell lysates were Western blotted with anti-FLAG and anti-GFP antibodies as indicated. *E*, lysates from 293T cells or control buffer were immunoprecipitated with the PS1-Ab-14 antibody or pre-immune serum as indicated. The immunoprecipitates were fractionated and Western blotted with anti-Omi/HtrA2 antibody (*upper panel*) or PS1-Ab-14 antibody (*lower panel*). The *first lane* shows total 293 cell lysates.

To further investigate the interaction between Omi/HtrA2 and PS1, *in vivo*, we performed co-immunoprecipitation assays. For this purpose, we used HeLa cells stably expressing FLAG-tagged Omi/HtrA2 and Omi/HtrA2-PDZ domains (Fig. 1*C*). Endogenous PS1 was immunoprecipitated with the FLAG antibody from both the full-length Omi/HtrA2- and Omi/HtrA2-PDZ-expressing cells, but not the vector control (Fig. 1*C*). We also co-transfected 293T cells with constructs encoding N-terminal GFP-tagged full-length PS1 and FLAG-tagged mature Omi/HtrA2. As predicted, the GFP-PS1 fusion protein was also able to bind the ectopically expressed FLAG-tagged Omi/HtrA2 (Fig. 1*D*, *fifth lane*) and the endogenous Omi/HtrA2 (Fig. 1*D*, *second lane*). Furthermore, we immunoprecipitated endogenous PS1 from 293T cells with a PS1-specific antibody and probed the immunoprecipitates with an Omi/HtrA2-specific antibody (Fig. 1*E*). Endogenous Omi/HtrA2 was immunoprecipitated with the PS1 antibody (*fifth lane*) but not with the non-specific antibody (*fourth lane*), indicating that endogenous Omi/HtrA2 interacts specifically with endogenous PS1. Combined, these results demonstrate that PS1 does indeed interact with Omi/HtrA2 via the PDZ domain.

C-terminal Presenilin Peptides Regulate Omi/HtrA2 Protease Activity—Recent structural information suggests that the active sites of Omi/HtrA2 and the bacterial homolog DegS are present in a nonfunctional state when their PDZ domains are not bound to the activation peptides (28). Binding of the activation peptides to the PDZ domain induces a conformational change in the flexible L3 loop, which triggers reorientation of the activation domain yielding a functional active site, thereby promoting degradation of the substrates. To test whether the C terminus of PS1 is an activation peptide for Omi/HtrA2, we assayed the activity of purified mature Omi/HtrA2 with ^{35}S-β-casein and IAPs as substrates, in the presence or absence of a peptide corresponding to the last 15 residues of the C terminus of PS1 (PS-C15). As predicted the PS-C15 peptide drastically increased the protease activity of Omi/HtrA2 toward these

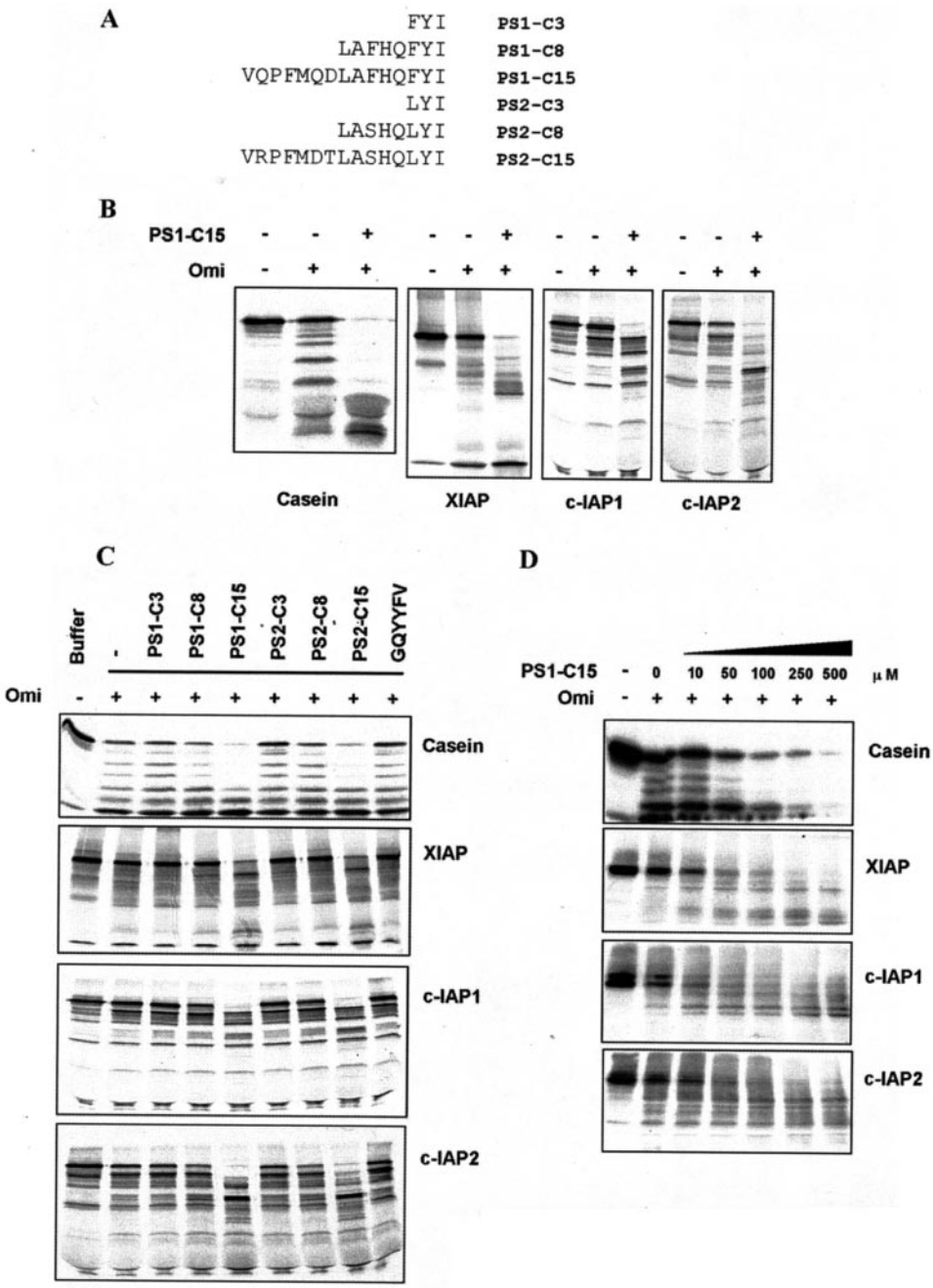

FIG. 2. **Activation of Omi/HtrA2 protease activity by the C-terminal peptides of presenilins.** *A*, sequences of the peptides designed from the C termini of PS1 and PS2. *B–D*, *in vitro* translated ^{35}S-labeled β-casein, XIAP, c-IAP1, or c-IAP2 (2 μl) was incubated with purified Omi/HtrA2 (5 μM) protein in the presence (100 μM) of the indicated peptides (*B* and *C*) or the indicated increasing concentrations of the PS1-C15 peptide (*D*) for 30 min (for β-casein) or 1 h (for IAPs) at 37 °C in a reaction volume of 30 μl. The *first lane* in *each panel* is the buffer control. The reaction products were analyzed by SDS-PAGE and autoradiography.

substrates, indicating that binding of this peptide to the PDZ domain could indeed modulate the protease activity of Omi/HtrA2 (Fig. 2*B*).

The PDZ domains usually recognize 3–4 residue motifs that occur at the C terminus of target proteins, although in some rare cases specificity of recognition extends beyond 4 residues. To determine the optimal number of amino acid residues required for recognition of Omi/HtrA2 PDZ domain, we used peptides corresponding to the last 3, 8, and 15 residues of PS1 and its homolog PS2 (Fig. 2*A*). In addition, we also used GQYYFV-COOH peptide, recently reported to bind Omi/HtrA2 PDZ domain and increase protease activity (30). As shown in Fig. 2*C*, the 15-residue peptides patterned after PS1 and PS2 significantly increased the protease activity of Omi/HtrA2 against ^{35}S-β-casein, whereas the 3-residue or 8-residue peptides had no or slight effect, respectively (Fig. 2*C*). Surprisingly, the previously described GQYYFV-COOH peptide showed no effect on Omi/HtrA2 protease activity under these conditions. Essentially similar results were obtained with XIAP, c-IAP1, and c-IAP2 as substrates. The PS1-C15 peptide had no enzymatic activity when assayed without Omi/HtrA2 and had no effect on the activity of an Omi/HtrA2 mutant without PDZ domain (data not shown). Combined, the above data indicate that the PDZ domain regulates Omi/HtrA2 protease activity and that the 3-residue PDZ binding motif in PS1 or PS2 is not sufficient for this effect but adjacent residues are required to relieve the inhibition of the PDZ domain. Because there was no significant difference between

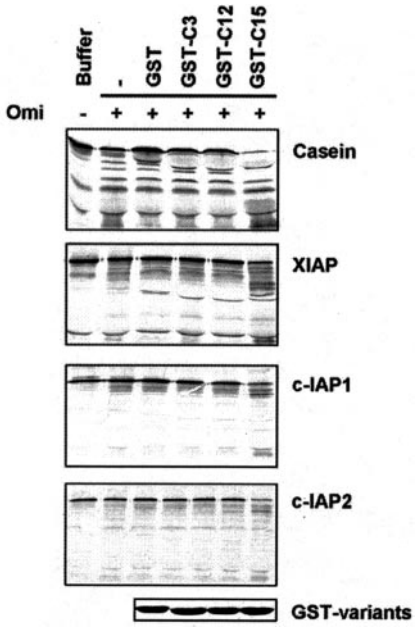

FIG. 3. **Effect of PS1 C-terminal peptides fused to the C terminus of GST on Omi/HtrA2 protease activity.** *In vitro* translated ^{35}S-labeled β-casein, XIAP, c-IAP1, and c-IAP2 (2 μl) were incubated with purified Omi/HtrA2 (5 μM) protein in the absence (−) or presence of GST (15 μM) or the indicated GST-PS1 fusion proteins (15 μM) for 30 min (for β-casein) or 1 h (for IAPs) at 37 °C in a reaction volume of 30 μl. The reaction products were analyzed by SDS-PAGE and autoradiography. The indicated purified GST or GST-PS1 fusion proteins were resolved by SDS-PAGE and stained by Coomassie Blue.

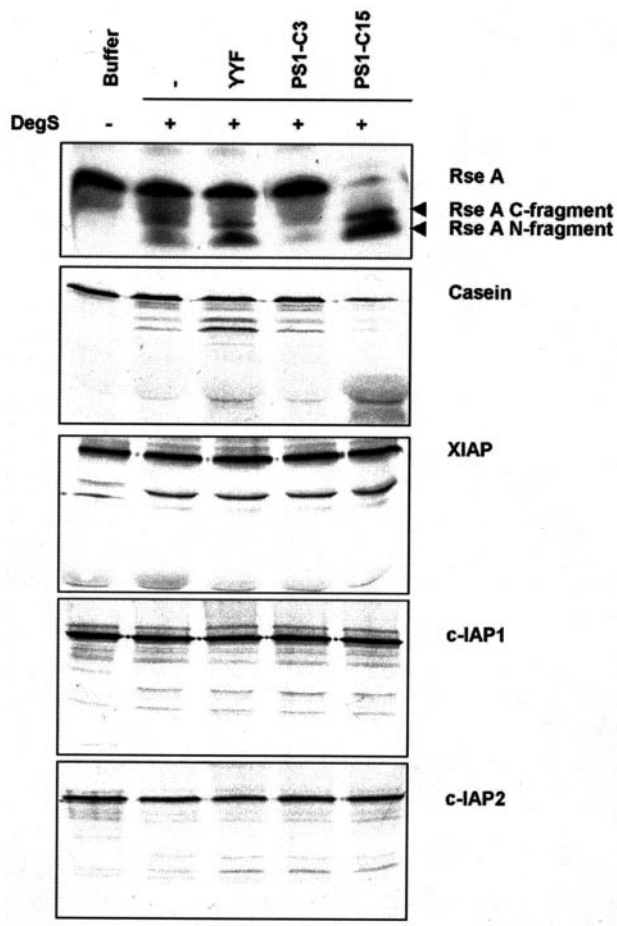

FIG. 4. **Effect of C-terminal PS1 peptides on protease activity of DegS.** *In vitro* translated ^{35}S-labeled RseA, β-casein, XIAP, c-IAP1, and c-IAP2 (2 μl) were incubated with purified DegS protein (5 μM) in the presence of indicated peptides (100 μM) for 16h at 37 °C in a reaction volume of 30 μl. The reaction products were analyzed by SDS-PAGE and autoradiography.

the protease-promoting activity of PS1-C15 and PS2-C15 peptides, we decided to use the PS1-C15 peptide for further studies.

To determine the effective concentration of the PS1 peptide, we incubated Omi/HtrA2 together with substrates and increasing concentrations of this peptide. PS1 peptide caused a dose-dependent increase in Omi/HtrA2 protease activity toward β-casein, XIAP, c-IAP1, and c-IAP2 substrates (Fig. 2*C*). PS1 peptide showed a marked protease stimulatory activity at 10 μM concentration. Similar concentrations of peptides were used recently to demonstrate activation of DegS by OMP peptides (28, 29).

To further investigate the activity of the last 15 residues of PS1 in a context of a globular protein, we fused this peptide or shorter variants of this peptide to the C terminus of GST (Fig. 1*B*). Next we tested the effect of purified GST and the GST-PS1 fusion variants on protease activity of Omi/HtrA2. As shown in Fig. 3, GST protein having the last 15 amino acid residues (GST-PS1-C15) of PS1 at its C terminus markedly enhanced the protease activity of Omi/HtrA2. However, GST or the GST variant with only the last 3 residues of PS1 (GST-PS1-C3) had no such effect on protease activity of Omi/HtrA2. No effect was also seen with the GST-PS1-ΔC3 variant, in which the 3-residue PDZ binding motif at the extreme C terminus of the PS1 peptide is deleted (Fig. 3). Combined, these results indicate that the last 3 amino acids in the PS1 peptide are important for interaction with the PDZ domain of Omi/HtrA2, but adjacent residues are also required. Furthermore, the presence of the PS1 peptide in the context of a globular protein such as GST does not affect their activity toward Omi/HtrA2.

C-terminal Peptide of PS1 Activates DegS Protease Activity—DegS is a bacterial trimeric serine protease related to Omi/HtrA2. Binding of tripeptides derived from the C termini of OMP proteins to the PDZ domain of DegS has been shown to activate DegS protease activity (28, 29). This indicates that both Omi/HtrA2 and DegS are activated by a similar mechanism; binding of peptide ligands to the PDZ domain relieves the inhibitory effect, resulting in activation of the protease domain. If this is the case then the 15-residue PS1 peptide might also activate DegS. To test this hypothesis we compared the activation of DegS protease activity by the OMP C-terminal tripeptide YYF-COOH and the PS1 C-terminal 15- or 3-residue peptides. Surprisingly, the 15-residue PS1 peptide activated DegS protease activity much more efficiently than the YYF-COOH peptide, whereas 3-residue PS1 peptide had no effect (Fig. 4). Similar results were obtained using RseA, a physiological substrate of DegS and the nonphysiological substrate β-casein. However, DegS was unable to cleave IAPs under similar conditions (Fig. 4) indicating that IAPs are specific substrates for Omi/HtrA2. These results also suggest that the peptide recognition site in the PDZ domains of DegS and Omi/HtrA2 are evolutionarily conserved, a conclusion that is supported by recent structural data (28). Nevertheless, the two proteases differ in their responsiveness to tripeptides; whereas DegS could be activated by tripeptides (29), neither the OMP (YYF-COOH) nor the PS1 (FYI-COOH) C-terminal tripeptides were able to activate Omi/HtrA2 under these conditions (Fig. 2*C* and data not shown).

Cell-permeable Peptide Having C-terminal Residues of PS1 Induces Omi/HtrA2-dependent Cell Death—Next we decided to examine the effect of Omi/HtrA2 activation by PS1-C15 peptide

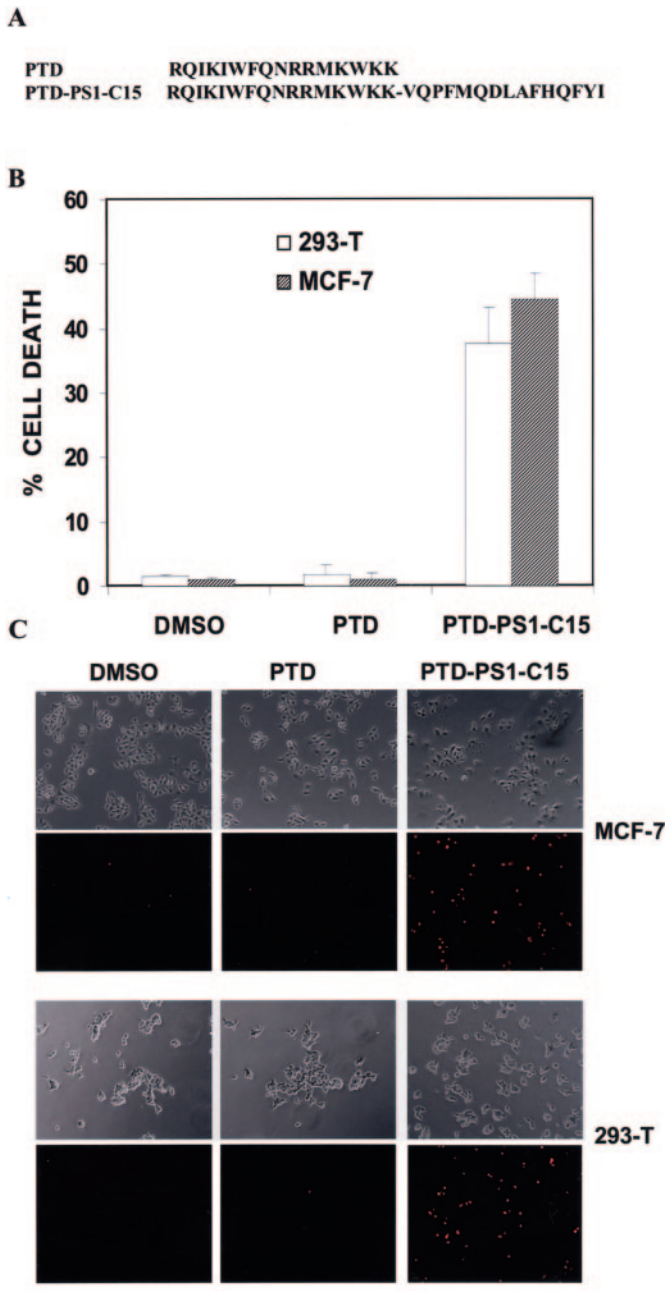

FIG. 5. **Effect of PS1 peptide fused to protein transduction domain of antennapedia on cell viability.** *A*, sequence of protein transduction domain of antennapedia (*PTD*) and PS1 peptide fused to PTD of antennapedia (*PTD-PS1-C15*). *B*, 293T and MCF-7 cells were treated with vehicle (Me_2SO (*DMSO*)), PTD, or PTD-PS1-C15 peptides at 50 μM and 25 μM, respectively, and then stained with propidium iodide. The percentage of cell death was determined as described under "Experimental Procedures." *C*, representative fields of Me_2SO-treated (*DMSO*), PTD-treated (*PTD*), and PTD-PS1-C15-treated (*PTD-PS1-C15*) cells. Cells were visualized by fluorescence microscopy (×10).

on the viability of mammalian cells. For this purpose we synthesized 15-residue peptide corresponding to PS1 C terminus fused to the C terminus of the protein transduction domain of antennapedia (PTD-PS1-C15) and control peptide having only the protein transduction domain of antennapedia (PTD) (Fig. 5*A*). The PTD allows the hybrid peptides to cross the cell membrane. Once inside the cell they could enter the mitochondria because of their small size, because the mitochondrial outer membrane is permeable to molecules of ~6 kDa or less (31). First, we checked the activation of Omi/HtrA2 protease activity by PTD-PS1-C15 and PTD *in vitro*. The PTD-PS-C15 peptide activated Omi/HtrA2 protease activity, whereas the PTD peptide did not show any activation under similar conditions (data not shown). When 293T or MCF-7 cells were treated with the PTD or the PTD-PS1-C15, the PTD-PS1-C15 peptide induced substantial cell death in both 293T and MCF-7 cells as depicted by the increase in propidium iodide (PI)-positive cells (Fig. 5, *B* and *C*). However, PTD-treated cells showed no significant difference from control untreated cells (Fig. 5, *B* and *C*). In addition treatment of 293T and MCF-7 cells with 15-residue PS1 peptide lacking protein transduction domain had no effect on cell viability (data not shown).

In the *mnd2* mouse the protease activity of Omi/HtrA2 is abolished by a missense mutation, which changes a conserved serine at position 276 to cysteine (20). To determine the role of Omi/HtrA2 in PTD-PS1-C15 peptide-induced cell death, we generated isogenic cell lines differing only in the Omi/HtrA2-function from mnd2 mouse embryonic fibroblasts (MEFs). For this purpose we immortalized mnd2 MEFs, which lack Omi/HtrA2 activity, and then stably transfected the immortalized mnd2 MEFs with an empty vector control or a vector expressing human Omi/HtrA2. The expression of Omi/HtrA2 was confirmed both by degradation of ^{35}S-labeled β-casein by immunoprecipitated Omi/HtrA2 from the mnd2-Omi/HtrA2 cells but not the mnd2-vector cells (Fig. 6*A*) and by Western blot analysis (Fig. 6*B*). mnd2-vector and mnd2-Omi/HtrA2 cells were treated with PTD (50 μM) and PTD-PS1-C15 (50 μM) and subsequently stained with PI. The proportion of PI-positive cells was significantly higher in mnd2-Omi/HtrA2 cells after treatment with the PTD-PS1-C15 peptide compared with untreated control cells or cells treated with the PTD peptide (Fig. 6*C*). However, there was only a slight increase in the number of PI-stained cells in the mnd2-vector cells after treatment with the PTD-PS peptide (Fig. 6*C*). The PTD-PS1-C15 peptide induced ~50% cell death in mnd2-Omi/HtrA2 cells and ~15% cell death in mnd2-vector cells (Fig. 6*D*). Cell death induced by the PTD-PS1-C15 peptide was not inhibited by the pan-caspase inhibitor z-VAD-fmk in both vector and Omi/HtrA2-expressing cells. This difference in cell death was not due to compromised cell death machinery in mnd2-vector cells, because the percentage of cell death in the mnd2-vector cells was similar to that in the mnd2-Omi/HtrA2 cells upon treatment with tunicamycin or 5-fluorouracil (data not shown).

Earlier we have shown that deletion of the PDZ domain partially restores protease activity to the S276C mutant Omi/HtrA2. Thus, we decided to examine whether the 15-residue PS1 peptide can also restore protease activity to the S276C mutant Omi/HtrA2. As shown in Fig. 6*E*, the 15-residue PS1 peptide partially restored the protease activity to the S276C mutant Omi/HtrA2 (*fifth lane*), but had no effect on the activity of the S276C Omi/HtrA2-ΔPDZ mutant, which lacks the PDZ domain (*seventh lane*). These data provide further proof that the PS1 peptide modulates the activity of Omi/HtrA2 by impacting its PDZ domain. Nevertheless, the activation of the protease activity of the S276C mutant Omi/HtrA2 by the PS1 peptide was significantly less than that of the wild type Omi/HtrA2 (*third lane*). This difference in potentiation of protease activity correlates well with the difference in cell death between mnd2-vector and mnd2-Omi/HtrA2 cells after treatment with the PTD-PS1-C15 peptide (Fig. 6, *C* and *D*). These results suggest that constitutive activation of the Omi/HtrA2 protease in the mitochondria by the PTD-PS1-C15 peptide leads to cell death.

PS1 Augments Omi/HtrA2-induced Cell Death—During apoptosis Omi/HtrA2 is released from mitochondria to the cyto-

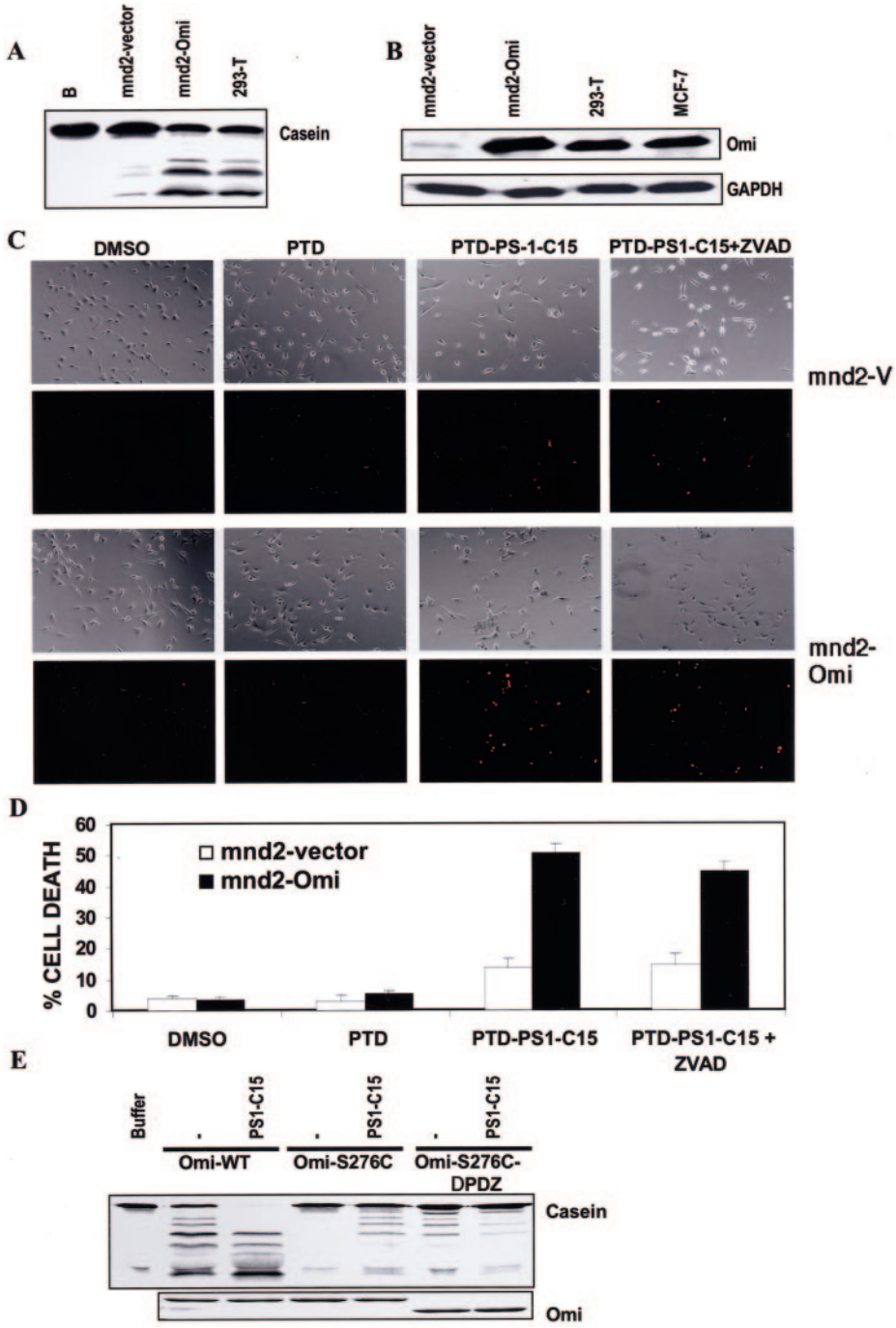

FIG. 6. **Role of Omi/HtrA2 in PS1 peptide-induced cell death.** *A*, Omi/HtrA2 protein was immunoprecipitated from mnd2-vector, mnd2-Omi/HtrA2, and 293T cells and assayed with *in vitro* translated ^{35}S-labeled β-casein. The reaction products were analyzed by SDS-PAGE and autoradiography. *B*, Western blots of whole cell lysates from mnd2-vector, mnd2-Omi/HtrA2, 293T, and MCF-7 cells with Omi/HtrA2 and GAPDH antibodies. The Omi/HtrA2 antibody was raised against human Omi/HtrA2 but can also detect mouse Omi/HtrA2 to a lesser extent. *C*, mnd2-vector and mnd2-Omi/HtrA2 cells were treated with vehicle (Me_2SO (*DMSO*)), PTD, or PTD-PS1-C15 (50 μM) peptides in the absence or presence of the pan-caspase inhibitor z-VAD-fmk (50 μM). Cells were visualized by fluorescence microscopy (×10). Representative fields of control Me_2SO-treated (*DMSO*), PTD-treated (*PTD*), PTD-PS1-C15-treated (*PTD-PS1-C15*) and PTD-PS1-C15 plus zVAD-fmk-treated (*PTD-PS1-C15+z-VAD-fmk*) cells. *D*, cells were treated as in *C*, and then the percentage of cell death was determined as described under "Experimental Procedures." *E*, *in vitro* translated ^{35}S-labeled casein was incubated with equal amounts (5 μM) of purified wild type Omi/HtrA2 (*Omi/HtrA2-WT*), S276C mutant Omi/HtrA2 (*Omi/HtrA2-S276C*), or S276C mutant Omi/HtrA2 ΔPDZ (*Omi/HtrA2-S276C ΔPDZ*) protein in the absence or presence of the 15-residue PS1 peptide PS1-C15 (100 μM) for 30 min at 37 °C. The reaction products were analyzed by SDS-PAGE and autoradiography. The indicated purified Omi/HtrA2 proteins were resolved by SDS-PAGE and stained by Coomassie Blue.

plasm where it binds to inhibitor of apoptosis proteins (IAPs) and cleave IAPs in the cytoplasm (17, 18). To determine the effect of ectopic expression of PS1 on Omi/HtrA2-induced cell death, we co-expressed full-length PS1 or PS1 lacking the last three C-terminal residues together with mature wild-type or a catalytically inactive (S306A) Omi/HtrA2 in MCF-7 cells. As shown in Fig. 7*A*, expression of mature wild-type Omi/HtrA2 in MCF-7 cells induced significant apoptosis, which was further enhanced by co-expression of full-length PS1, but not PS1 lacking the PDZ binding motif. The catalytically inactive mutant Omi/HtrA2 did not show any significant cell death in the presence or absence of ectopic PS1 (Fig. 7*A*). Consistent with these results we also found that ectopic expression of wild-type Omi induces less cell death in PS1-deficient or PS1- and PS2-deficient MEFs compared with wild-type MEFs (Fig. 7*B*). These results indicate that PS1 can increase Omi/HtrA2-mediated cell death by enhancing its protease activity in the cytosol. In addition, the ability of PS1 to enhance Omi/HtrA2-induced cell death was dependent on its PDZ binding motif.

Expression of Mutant PS1 Induces Omi/HtrA2-dependent Cell Death—Neuronal apoptosis has been implicated in Alzheimer disease and mutations in PS may predispose neurons to apoptotic cell death. It has been previously demonstrated that expression of the Alzheimer mutant PS1 (M146V and L286V) sensitizes PC12 cells to amyloid-β and trophic factor withdrawal induced cell death (32). We evaluated the effect of mutant PS1 (M146V and L286V missense mutations) on cell viability. First, we compared the apoptotic activity of full-length wild-type and M146V mutant PS1 with or without the PDZ binding motif. As shown in Fig. 8*A*, expression of the full-length M146V mutant PS1 induced significantly more cell

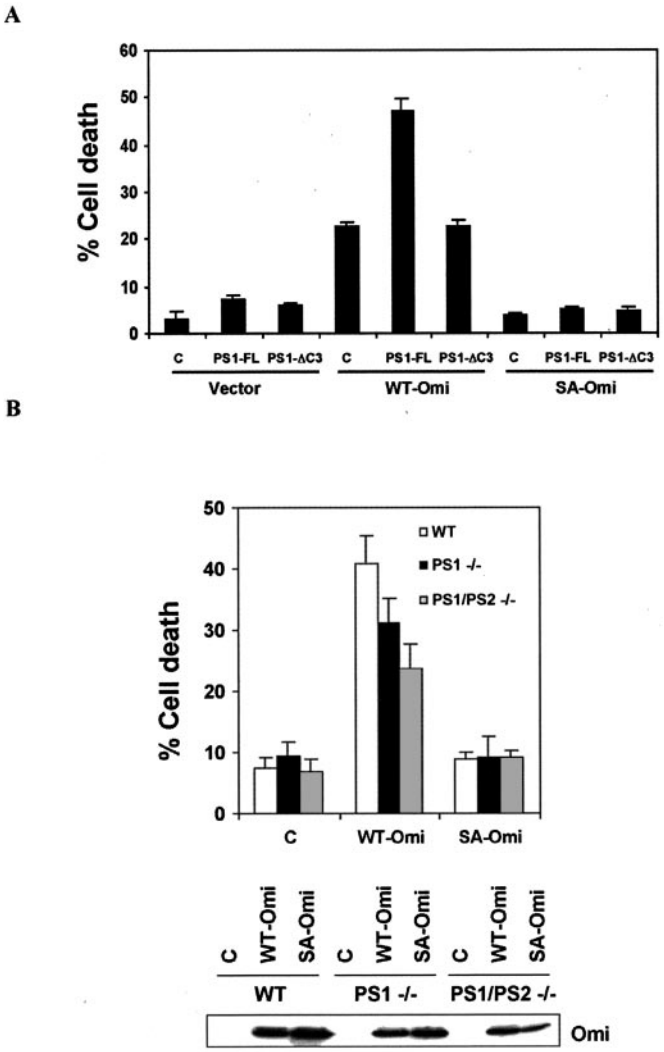

FIG. 7. **Presenilins augments Omi/HtrA2-induced cell death.** *A*, MCF-7 cells were transiently transfected with vector control, wild-type mature Omi/HtrA2, or S306A mature Omi/HtrA2 constructs together with GFP (*C*), or N-terminal GFP-tagged wild-type PS1-FL (*PS1-FL*) or wild-type PS1-ΔC3 (*PS1-ΔC3*) constructs as indicated. After 24 h of transfection the percentage of cell death was determined as described under "Experimental Procedures." *B*, wild-type (*WT*), PS1-knockout (*PS1* −/−), or PS1 and PS2 double-knockout (*PS1/PS2* −/−) MEFs were transfected with vector control, wild-type mature Omi/HtrA2, or S306A mature Omi/HtrA2 constructs together with GFP. After 16 h of transfection, the percentage of cell death (*upper panel*) was determined as described under "Experimental Procedures." The expression of the transfected Omi/HtrA2 proteins is shown in the *lower panel*.

death than the wild-type PS1. However, very little difference was observed between the wild-type and M146V mutant PS1 constructs without the PDZ binding motif (Fig. 8*A*). These results indicate that the PDZ binding motif of PS1 contributes to its apoptotic activity.

Next we determined the role of Omi/HtrA2 in the Alzheimer mutant PS1-induced cell death. For this purpose, mnd2-Omi/HtrA2 and mnd2-vector cells were transfected with wild-type and mutant (M146V and L286V) PS1 with or without the PDZ binding motif. Expression of mutant PS1 (M146V and L286V) induced substantially more cell death in mnd2-Omi/HtrA2 cells as compared with mnd2-vector cells (Fig. 8*B*). However, there was little difference in cell death induced by mutant PS1 (M146V and L286V) without the PDZ binding motif, in mnd2-Omi/HtrA2 and mnd2-vector cells (Fig. 8*B*). These results suggest that mutant PS1 induces cell death in Omi/HtrA2-dependent and -independent fashions. However, the PDZ binding motif of PS1 appears to be required for Omi/HtrA2-dependent cell death.

DISCUSSION

Omi/HtrA2 is a member of the HtrA serine protease family, which contains a central trypsin-like protease domain followed by one or two C-terminal regulatory PDZ domains (21). The functional unit of Omi/HtrA2 is a homotrimer, which is formed by three monomers of Omi/HtrA2 packed symmetrically to create a funnel-like structure with the protease domains forming the core of the molecule and the PDZ domains protruding outwards (33). The active sites of the protease are located on the concave side of the funnel. Recent structural data (28) indicate that the PDZ domain of Omi/HtrA2 might function similarly as in DegS, to regulate the catalytic activity of the protease domain. In the unbound state, the PDZ domain is tethered by multiple interactions to the protease domain trapping the flexible L3 loop and the so-called "activation domain," which is formed by loops L1, L2, and LD in an inactive conformation. Upon binding of the PDZ domain to specific activation peptides in target proteins, it releases the flexible L3 loop, which in turn triggers reorientation of the activation domain resulting in increased protease activity. So far, there are no known physiological activation peptides for the PDZ domain of Omi/HtrA2. In this study we demonstrated that the C terminus of Presenilin contains a PDZ binding motif, which functions as a physiological activation peptide for the PDZ domain of Omi/HtrA2. Our results suggest that the C-terminal PDZ binding motif of PS1 interacts with the PDZ domain of Omi/HtrA2 and relieves its inhibition of the protease domain resulting in increased protease activity toward IAPs and β-casein (Fig. 2). These results demonstrate that the PDZ binding motif of PS1 functions similarly as in the OMP proteins to modulate the protease activity of Omi/HtrA2.

The first identified and the most important function of PDZ domains is the recognition of specific C-terminal PDZ binding motifs found in target proteins, most often in the cytoplasmic tails of transmembrane receptors and channels (34, 35). Our results demonstrated that the conserved PDZ binding motif (FYI-COOH) in the C-terminal tail of PS1 is critical for binding to Omi/HtrA2. Substituting the C-terminal isoleucine with glycine or deleting this motif abolished binding to Omi/HtrA2 and the effect on Omi/HtrA2 protease activity (Figs. 1 and 3, and data not shown). However, the effect on Omi/HtrA2 activity requires more than the canonical $\phi X\phi$-COOH PDZ binding motif. Our data demonstrated that the PS1 FYI-COOH peptide is not sufficient for activation of Omi/HtrA2, suggesting that specific residues as far back as −15 appear to be involved in the interaction between the PDZ domain of Omi/HtrA2 and the C terminus of presenilins (Fig. 2*C*). The involvement of residues upstream of the C-terminal tripeptide in PDZ recognition has also been reported for interactions between CRIPT postsynaptic protein and the third PDZ domain of PSD-95/SAP90 (36), and between muscle sodium channels and the syntrophin PDZ domain (37, 38).

Recently, Martins *et al.* (30) described a synthetic six-residue optimal Omi/HtrA2 PDZ domain-binding peptide (GQYYFV-COOH), which could increase Omi/HtrA2 activity toward a synthetic octapeptide substrate. However, in our hands the same GQYYFV-COOH hexapeptide was not effective with β-casein or IAPs as substrates (Fig. 2*C*). This suggest that binding of short peptides such as the GQYYFV-COOH to the PDZ domain of Omi/HtrA2 does not trigger complete conformational changes in the L3 loop and the adjacent activation domain to allow binding of large protein substrates to the active site pocket. Longer peptides, such as the 15-residue PS1 peptide described here, might be required to trigger complete reorien-

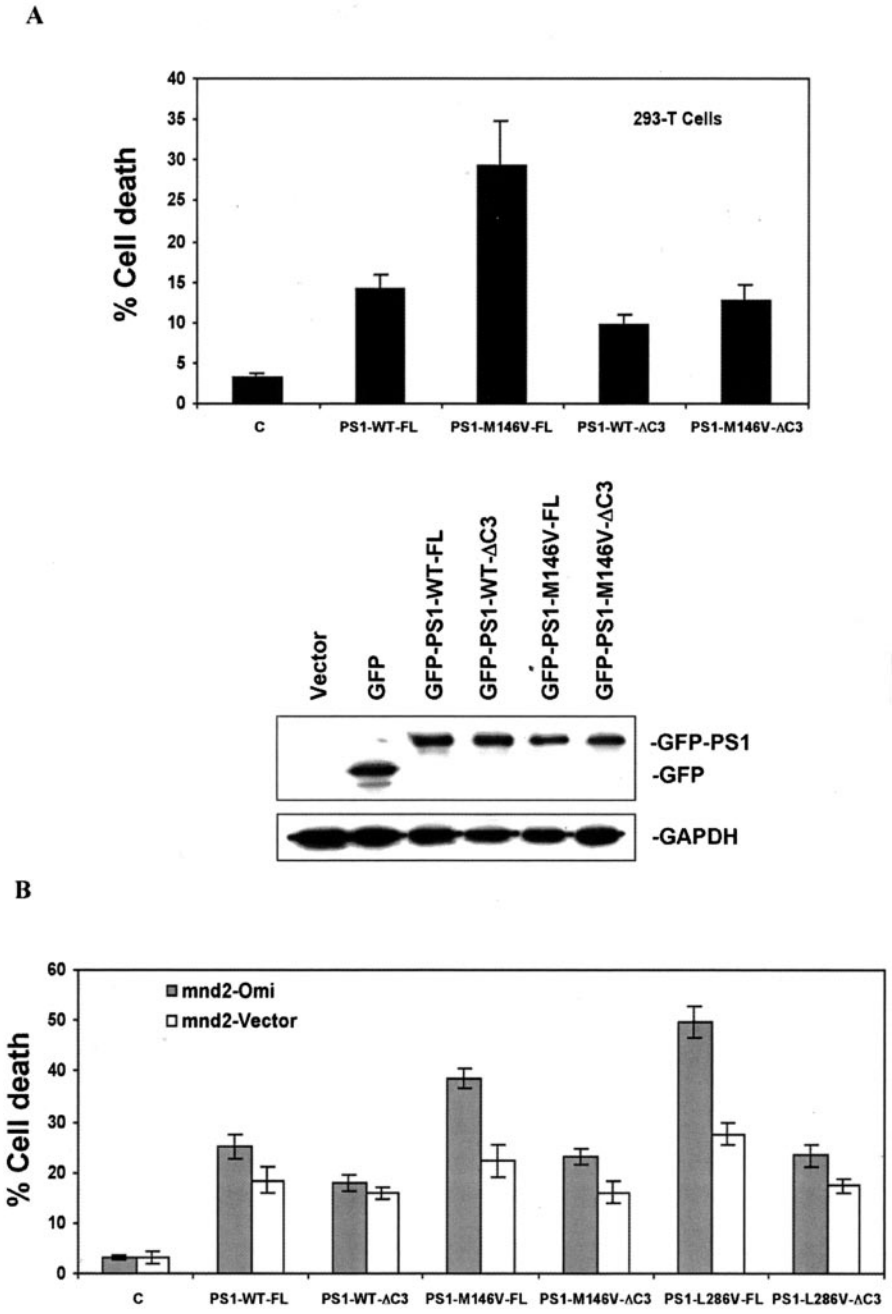

FIG. 8. **Mutant PS1 induces Omi/HtrA2-dependent cell death.** *A*, 293T cells were transiently transfected with GFP (*C*), N-terminal GFP-tagged wild-type PS1-FL (*PS1-WT-FL*), mutant M146V PS1-FL (*PS1-M146V-FL*), or their corresponding ΔC3 (lacking the PDZ binding motif) constructs as indicated. After 48 h of transfection the percentage of cell death (*upper panel*) was determined as described under "Experimental Procedures." The expression of the transfected proteins is shown in the *lower panel*. *B*, mnd2-Omi/HtrA2 and mnd2-vector cells were transiently transfected with the indicated constructs as in *A*. After 48 h of transfection the percentage of cell death was determined as described under "Experimental Procedures."

tation of the L3 loop and the adjacent activation domain and maintain a functional active site, which allows access or binding of large protein substrates to the active site pocket. It is also possible that the length of the peptide is not the determining factor in triggering Omi/HtrA2 activation but that specific residues N-terminal to the 3- to 4-residue PDZ binding motif in the 15-residue PS1 peptide might be required to trigger complete conformational changes.

The structure of Omi/HtrA2 is similar to that of the bacterial HtrA protease DegS, which has been shown to protect against environmental stresses by acting as a sensor for unfolding stresses (29). In *E. coli*, stress-induced accumulation of misfolded and unfolding outer membrane porins (OMPs) in the periplasm is believed to expose their C-terminal PDZ binding motifs, which bind the PDZ domain of DegS (28, 29). This binding relieves the inhibitory interactions between the PDZ and protease domains of DegS resulting in activation of its proteolytic activity. The activated DegS cleaves and destroys the transcription factor σ^E-inhibitory protein RseA. The free σ^E then induces the σ^E-dependent transcription of stress-responsive genes.

In mammalian cells, exposure of mitochondria to stress could also lead to accumulation of misfolded and unfolded proteins in the intermembrane space, which might be sensed by Omi/HtrA2, thereby triggering an Omi/HtrA2-specific stress response pathway. Consistent with this hypothesis, Omi/HtrA2 was found to be essential for survival (20). The loss of Omi/HtrA2 protease activity in mice is responsible for the increased susceptibility of *mnd2* mitochondria to induction of the permeability transition and mitochondrial dysfunction (20), perhaps due to a defect in sensing and responding to mitochondrial folding stresses. Considering this important role of Omi/HtrA2 in maintenance of healthy mitochondria, its activity is probably regulated by a mechanism similar to the mechanism that regulates DegS activity in bacteria.

PS1, primarily located in the endoplasmic reticulum and cell membranes, has also been recently found by subcellular fractionation and immuno-electron microscopy in rat mitochondrial membranes and proposed to be part of the mitochondrial megapore complex (39). We also found PS1 in the mitochondrial membranes of human cells (data not shown). The presence of PS1 in the mitochondrial membranes raises an interesting possibility that PS1 might have a similar role in the mitochondria analogous to the role of OMP proteins in bacteria. During mitochondrial stress unfolded PS1 might accumulate in the mitochondria and trigger activation of Omi/HtrA2 via its PDZ binding motif. The activated Omi/HtrA2 in turn recognizes and degrades specific mitochondrial substrates. The degradation of these substrates might be required to induce stress-responsive genes in the mitochondria to prevent accumulation of damaged or misfolded proteins. However, more analysis is required to determine the topology of PS1 in the mitochondrial membranes and whether mitochondrial stress induces accumulation of PS1 in the mitochondria.

Presenilins are expressed in neurons throughout the brain and appear to be present in both degenerating and nondegenerating neurons in AD brain. It is interesting that the mutations in presenilins identified in patients with familial AD are missense mutations or in-frame deletions that do not truncate the protein (40). The mechanisms by which these mutations promote neuron degeneration in AD are not fully understood, but it is possible that they may cause misfolding or significant conformational changes in Presenilins, which increase cellular stress. Consistent with this hypothesis, Presenilin mutations appear to increase neuronal cell death by increasing oxidative stress and accumulation and oligomerization of amyloid β (32). These changes are thought to play a central role in the pathogenesis of this disease by probably directly leading to mitochondrial dysfunction (41). Indeed, mitochondrial impairment, including mitochondrial membrane depolarization, has been documented in brain tissues from AD patients and in fibroblasts from patients harboring PS1 mutations (42). These mitochondrial alterations could therefore lead to release of Omi/HtrA2 together with other inducers of cell death into the cytoplasm where it binds to the C-terminal tail of the mutant PS1 and becomes active. Activation of Omi/HtrA2 might in turn contribute to cytoplasmic activation of apoptosis by direct degradation of IAPs and other unknown cellular substrates. Indeed, our results indicate that full-length AD mutant PS1 proteins induce more cell death in Om/HtrA2-containing cells than in Omi/HtrA2-deficient cells. Thus, mutant presenilins could act as both the inducers and enhancers of cell death in AD.

Another potential mechanism that might contribute to neuronal cell death would be conformational changes in the mutant presenilin protein that leads to accumulation of mutant PS1 in the mitochondria and increased interaction of mutant PS1 with the PDZ domain of Omi/HtrA2. The increased interaction between mutant presenilins and Omi/HtrA2 might contribute to mitochondrial dysfunction by inducing prolonged activation (overactivation) of Omi/HtrA2. Indeed, overactivation of Omi/HtrA2 protease activity using a cell-permeable peptide having C terminus residues of PS1 induced potent Omi/HtrA2-dependent cell death (Figs. 5 and 6). This corroborates the fact that all our attempts to obtain stable clones of constitutively active Omi/HtrA2 (Omi/HtrA2 ΔPDZ) have failed.[2] Therefore, based on our recent observations that inactivation of Omi/HtrA2 in the mitochondria causes increased cell death in mnd2 mice (20) and our present observations that overactivation of Omi/HtrA2 in the mitochondria also causes cell death, we suggest that the activity of Omi/HtrA2 is critically regulated and either loss of activity or excess activity causes mitochondrial dysfunction leading to neurodegeneration.

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[2] S. Gupta, R. Singh, P. Datta, Z. Zhang, C. Orr, Z. Lu, G. DuBois, A. S. Zervos, M. H. Meisler, S. M. Srinivasula, T. Fernandes-Alnemri, and E. S. Alnemri, unpublished observations.

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The C-terminal Tail of Presenilin Regulates Omi/HtrA2 Protease Activity

Sanjeev Gupta, Rajesh Singh, Pinaki Datta, ZhiJia Zhang, Christopher Orr, Zhixian Lu, Garrett DuBois, Antonis S. Zervos, Miriam H. Meisler, Srinivasa M. Srinivasula, Teresa Fernandes-Alnemri and Emad S. Alnemri

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