β-Amyloid Precursor Protein Is a Direct Cleavage Target of HtrA2 Serine Protease

IMPLICATIONS FOR THE PHYSIOLOGICAL FUNCTION OF HtrA2 IN THE MITOCHONDRIA

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Hyo-Jin Park,† Sang-Soo Kim‡, Young-Mo Seong‡§, Kyung-Hee Kim‡§, Hui Gwan Goo‡, Eun Jin Yoon‡, Do Sik Min†, Seongman Kang†§, and Hyangshuk Rhim‡§¶

From the †School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, the §Research Institute of Molecular Genetics, ¶Department of Biomedical Sciences, College of Medicine, the Catholic University of Korea, Seoul 137-701, and the ‡Department of Molecular Biology, College of Natural Science, Pusan National University, Busan 609-735, South Korea

The processing and metabolism of amyloid precursor protein (APP) is a major interest in Alzheimer disease (AD) research, because not only amyloid β (Aβ) peptide, but also cellular or mitochondrial APP are intimately involved in cellular dysfunction and AD pathogenesis. Here we demonstrate that APP is directly and efficiently cleaved by the HtrA2 serine protease in vitro and in vivo. Using several APP mutants and N-terminal amino acid sequencing, we identified that the HtrA2-mediated APP cleavage product is the C161 fragment encompassing amino acids 535–695 of APP695. The immunofluorescence and subcellular fractionation studies indicate that APP is partly colocalized with HtrA2 in the mitochondria where HtrA2 can cleave APP under normal conditions. The HtrA2-cleaved C161 fragment was detected in the cytosolic fraction; therefore, we postulate that the C161 fragment is released into the cytosol after cleavage of APP by HtrA2. Interestingly, the level of C161 was remarkably decreased in motor neuron degeneration (mnd2) mice in which the serine protease activity of HtrA2 was severely reduced. These results show that the protease activity of HtrA2 is essential for the production of C161 and that processing of APP into C161 is a natural event occurring under normal physiological conditions. Our study suggests that the direct cleavage of mitochondrial APP by HtrA2 may prevent mitochondrial dysfunction caused by accumulation of APP and that the regulation of HtrA2 protease activity may be a therapeutic target in AD.

Alzheimer disease (AD) is the most common neurodegenerative disorder characterized by accelerated neuronal cell death in the cerebral cortex and the hippocampus (1). Neuronal loss in AD is accompanied by the progressive accumulation of amyloid β (Aβ) peptide and the presence of neurofibrillary tangles made up of abnormally phosphorylated Tau protein (1, 2). The Aβ peptide is generated by endoproteolytic processing of a large type 1 transmembrane protein, the amyloid precursor protein (APP). Numerous recent studies have demonstrated that in the amyloidogenic pathway, aspartic proteases called β- and γ-secretase sequentially cleave APP at the N and C termini of the Aβ peptide, respectively, leading to the formation of amyloidogenic or neurotoxic Aβ in the brains of AD patients (3). In addition, APP can also undergo non-amyloidogenic processing by α-secretase to generate the 83-residue C-terminal APP fragment (C83) and an α- and γ-secretase cleaved APP fragment, p3 (Fig. 1A) (4). Recently, APP-like proteins (APLP1 and APLP2), which share similar domain structures with APP, were also shown to be processed by secretases (5).

Several lines of evidence indicate that other proteases are also involved in the proteolytic processing and degradation of APP. The cysteine protease calpain and caspasas such as caspase-3, -6, -7, and -8 that play an important role for executing cell death in apoptosis can cleave APP, resulting in elevated toxic Aβ or C31 fragments that are associated with AD pathology (6–9). Moreover, the APP cleavage by a trypsin-like serine protease has been reported in chicken sympathetic neurons (10). Also, HtrA1, a member of the HtrA serine proteases family, appears to be involved in proteolytic cleavage of Aβ and C99 (11). Although, research on the secretases and other APP processing proteases has made rapid progress, the exact mechanism and biological meaning of the APP processing and degradation remain elusive.

Besides the primary role of APP processing into Aβ42 in AD, a number of in vivo and in vitro studies suggest that APP is intimately involved in AD pathogenesis (12–15). More recently, it has been reported that APP can accumulate in the mitochondria as a transmembrane-arrested form, causing mitochondrial dysfunction with impaired energy metabolism in cell culture systems as well as in the APP-transgenic mice, an animal model for AD (16). The molecular mechanism of mitochondrial dysfunction caused by APP accumulation remains unclear, and the molecules that physiologically regulate the functional metabolic balance of mitochondrial APP have not yet been identified.

HtrA2 (also known as Omi) is a serine protease that localizes to the mitochondrial intermembrane space (17, 18). Upon apoptosis induction by cellular stresses such as UV exposure,
β-Amyloid Precursor Protein Is a Direct Cleavage Target

TRAIL, staurosporine, or etoposide, the processed, mature HtrA2 is released from the mitochondria into the cytoplasm and promotes cell death via two different mechanisms: a caspase-independent manner through its protease activity as well as in a caspase-dependent manner by antagonizing XIAP-mediated caspase-3 (18–22). Moreover, recent studies using motor neuron degeneration 2 (mnd2) and HtrA2 knock-out mice suggest that HtrA2 may play a crucial role in regulating mitochondria biogenesis through its serine protease activity in the mitochondria (23, 24). Furthermore, we have previously shown that HtrA2 interacts with APP through the Aβ region (25). Therefore, the mitochondrial localization and binding capability of both HtrA2 and APP increases the possibility that HtrA2 serine protease may regulate the physiological or pathological role of APP. Here we demonstrate that the mitochondrial serine protease HtrA2 cleaves APP in vitro and in vivo. The functional relation between HtrA2 and APP was confirmed by the marked reduction of C161 in mnd mice. Taken together, our data suggests a possible role for HtrA2 in the cleavage or clearance of APP in the mitochondria.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies (Ab)—All chemicals were purchased from Sigma unless otherwise stated. Rabbit polyclonal anti-HtrA2 Ab was described previously (26). Other antibodies used for immunoblot (IB) analyses were anti-APP (Sigma), anti-Myc (Santa Cruz Biotechnology), anti-FLAG (Sigma), anti-HSP60 (Santa Cruz Biotechnology), and anti-caspase-3 (Upstate).

Plasmid Construction—Plasmids encoding the various forms of HtrA2 and APP were previously described (25–27). To express the GST-APP (aa 1–695) fusion protein with the C-terminal Myc epitope tag in Escherichia coli, the pGST-APP695M plasmid was generated by digesting pCMV-APP695M with appropriate restriction enzymes, followed by inserting into the pGEX4T-3 plasmid (Amersham Biosciences). The pGST-C390M plasmid encoding the GST-APP (aa 306–695) fusion protein with the C-terminal Myc tag was generated by self-ligation after digesting the pGST-APP695M construct with Sall (5’) and Xhoi (3’) restriction enzymes. The pGST-C109F plasmid encoding the GST-APP (aa 587–695) fusion protein with the C-terminal FLAG tag was generated by PCR from the pcDNA-LexA-APP751 plasmid and subcloned into the pGEX4T-1 plasmid. The pGST-C100 plasmid encoding APP (aa 596–695) was generated by digesting the pBS-C100 plasmid with BamHI (5’) and SalI (3’). To construct the pGST-Asub-syn plasmid, the putative substrate portion (aa 449–598 of APP695) was generated by PCR from pCMV-APP695M plasmid as a template, and the amplified fragment was inserted into the pGEX-β-synuclein (aa 88–134) plasmid in-frame between the GST epitope and the region of β-synuclein.

To express APP (aa 533–695) as the C-terminal Myc-tagged or untagged proteins in mammalian cells, pCMV-C161M and pCMV-C161 plasmids were generated by PCR from pCMV-APP695M and pCMV-APP751 plasmids, respectively. The resulting amplified fragments were digested with BamHI (5’) and Xhoi (3’) and inserted into the pcDNA3.0 plasmid (Invitrogen). The APLP plasmids (pcDNA-APLP1–650 and APLP2–751) were kindly provided by D. J. Selkoe.

For all plasmid constructs, sequence integrity was verified by DNA sequencing with ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), and expression was determined by IB analysis with the specific Abs. Details of all plasmid constructs and all primer sequences are available upon request.

Purification of GST Fusion Proteins—GST fusion proteins expressed in E. coli were purified by selective binding to glutathione-Sepharose 4B beads (Amersham Biosciences) as described previously (26). The bead-bound GST fusion proteins were resuspended in 1× SDS loading buffer (60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, 0.1% bromphenol blue) and resolved by 15% SDS-PAGE. For purification of GST-Δ133, the protein-bound beads were resuspended in 100 µl of elution buffer (50 mM Tris-HCl (pH 7.6), 1 mM dithiothreitol) containing 5 mM reduced glutathione. The GST-Δ133 protein was eluted from glutathione beads by incubation for 5 min at 37 °C. The purified GST-Δ133 protein was stored for endoproteolytic cleavage assays at −70 °C in a final concentration of 20% glycerol and 7 mM dithiothreitol.

In Vitro Transcription and Translation—[35S]Met-labeled APP and APLP proteins were generated by using the in vitro TnT T7 Quick Coupled Transcription/Translation System (Promega) according to the manufacturer’s instructions. The pCMV-APP and pcDNA-APLP plasmids were linearized with an appropriate restriction enzyme and incubated with 40 µl of TnT Quick master mixture containing 2 µl of [35S]methionine (10 mCi/ml) in a total volume of 50 µl for 90 min at 30 °C.

In Vitro Proteolytic Cleavage Assays—For in vitro cleavage, [35S]Met-labeled APP or recombinant GST-APP proteins were incubated in the presence of 1–3 µg of recombinant GST-Δ133 in cleavage buffer (50 mM Tris-HCl (pH 7.6), 1 mM dithiothreitol) at 37 °C for 3 h. The reactions were stopped by adding 1× SDS loading buffer. Cleavage products were resolved by 15% SDS-PAGE and detected either by IB assay or autoradiography.

N-terminal Amino Acid Sequencing—Amino acids at the cleavage site were identified by N-terminal amino acid sequencing. The processed APP proteins were transferred electrophoretically onto a polyvinylidene difluoride membrane, and the transferred proteins were stained with Ponceau S solution. The cleaved fragment was excised from the membrane, and its N-terminal amino acid residues were identified by using the Procise 491 protein sequencer (Applied Biosystems) (Korea Basic Science Institute).

Site-directed Mutagenesis—The QuickChange Site-directed Mutagenesis Kit (Stratagene) was used according to the manufacturer’s instructions to generate a cleavage site mutant (APP695(R/R)M) of APP. The sequence integrity of the mutant plasmid construct was verified by DNA sequencing.

Cell Culture and Transfection—Human embryonic kidney (HEK) 293 and HeLa cells (American Type Culture Collection, Manassas, VA) were maintained at 37 °C in a humidified 5% CO2 incubator in Dulbecco’s modified Eagle’s minimum essential medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen), 50 units/ml penicillin, and 50 µg/ml streptomycin (Invitrogen). For all
transfections into HEK293 and HeLa cells, the Lipofectamine reagent (Invitrogen) was used according to the manufacturer’s instructions.

IB Assay—Transfected cells and mouse brain tissues were lysed in digitonin lysis buffer (0.2% digitonin, 20 mM HEPES (pH 7.5), 100 mM KCl, 10 mM CaCl₂, 50 mM MgCl₂) or RIPA
buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate) containing protease inhibitors (10 µg/ml leupeptin, 10 µg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride) and incubated for 1 h on ice. The samples were analyzed by 12 or 15% SDS-PAGE and transferred onto a nitrocellulose membrane (Protran). Blots were blocked for 1 h at room temperature in 5% nonfat dried milk in TBST (TBS buffer + 0.1% Tween 20). Membranes were incubated with primary Abs for 1 or 3 h, followed by processing with secondary Abs for 1 h at room temperature. The antigen-antibody complex was detected with the enhanced chemiluminescence (ECL) kit (Amersham Biosciences).

**Immunofluorescence Assay**—Transfected cells were briefly washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 5 min at room temperature, and followed by brief rinse with PBS. Cells were quenched for 10 min in PBS containing 50 mM NH4Cl and washed three times with PBS. For permeabilization, cells were incubated for 5 min in PBS containing 0.1% Triton X-100 and washed with PBS. Cells were blocked for 1 h with PBS containing 2% bovine serum albumin at room temperature. After blocking, cells were incubated with anti-Myc Ab (1:1,000) or anti-HtrA2 Ab (1:1,000) in blocking solution for 2 h at room temperature, followed by incubating with Cy3-coupled anti-mouse IgG (1:500) or Alexa 488 anti-rabbit IgG (1:500) at room temperature, respectively. Between incubations, cells were washed three times with blocking solution at room temperature. After final washes with blocking solution, cells were washed with PBS for 5 min and incubated with 2 µg/ml 4',6-diamidino-2-phenylindole solution for 5 min. Subcellular localization of APP and HtrA2 was analyzed by inverted fluorescence microscopy (Carl Zeiss).

**Subcellular Fractionation**—Cells were broken by passing 10 times through a G26 needle in buffer D (250 mM sucrose, 20 mM HEPES (pH 7.5), 10 mM KCl, 5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 10 mM Tris-HCl (pH 7.5)) containing protease inhibitors and 0.02% digitonin. Cell extracts were centrifuged at 900 × g for 2 min at 4 °C to recover pellets (Mito, the mitochondria-containing fraction) and supernatants (Cyto, the cytoplasmic fraction). The subcellular fractions were processed for IB analysis with anti-HtrA2, APP, HSP-60, or caspase-3 Abs.

**RESULTS**—HeLa cells were plated at 7 × 10^4 cells/well on 6-well plates. Cells were transfected with double-stranded small interfering RNA by using Lipofectamine reagent according to the manufacturer's instructions. Small interfering RNA oligonucleotides were made by Ambion and had the following sequences: siHtrA2 sense, 5'-r(GGGAGAUGUUGUUGC-CA)d(TT)-3'; and siHtrA2 antisense, 5'-r(UGGCAACAAAC-ACUCCC)d(TT)-3' corresponding to residues 254–260 (24).

**RNA Interference**—HeLa cells were plated at 7 × 10^4 cells/well on 6-well plates. Cells were transfected with double-stranded small interfering RNA by using Lipofectamine reagent according to the manufacturer’s instructions. Small interfering RNA oligonucleotides were made by Ambion and had the following sequences: siHtrA2 sense, 5'-r(GGGAGAUGUUGUUGC-CA)d(TT)-3'; and siHtrA2 antisense, 5'-r(UGGCAACAAAC-ACUCCC)d(TT)-3' corresponding to residues 254–260 (24).

**RESULTS**—HtrA2 Directly Cleaves the APP/APLP Family Members—Both APP751 and APP695 isoforms that contain chimeric endoplasmic reticulum and mitochondrial targeting domains were used in an *in vitro* cleavage assay (Fig. 1A). To establish an in *vitro* assay for HtrA2 cleavage of the APP/APLP family members, we purified wild-type or mutant, mature HtrA2 as GST fusions by selective binding to glutathione-Sepharose 4B beads (GST-Δ133 or GST-Δ133 (S306A)) (Fig. 1, B and C). During expression of GST-Δ133 protein in E. coli and purification, protein fragments with lower molecular masses ranging from 30 to...
50 kDa were detected in an SDS-PAGE system (Fig. 1C, left panel) (26). N-terminal amino acid sequencing of these fragments showed that GST-Δ133 proteolytically cleaved amino acid residues in the C-terminal region of GST fused to the N terminus of Δ133 (data not shown), whereas the GST itself was not affected by the proteolytic activity of HtrA2 (Fig. 1C, middle panel). We reconfirmed the serine protease activity of GST-Δ133 against β-casein as an exogenous substrate (Fig. 1C, right panel).

To investigate the notion of whether APPs are directly cleaved by HtrA2, [35S]Met-labeled APP695M or APP751 was incubated with proteolytically active GST-Δ133 or inactive GST-Δ133 (S306A) (Fig. 1D). After a 3-h incubation, APP751 containing the kunitz-serine protease inhibitor domain, as well as APP695M were almost completely cleaved by GST-Δ133 (Fig. 1D, left panel). Subsequently, to clearly detect the HtrA2-cleaved APP fragments, we used a 3-fold amount of APP695M relative to that of the reaction shown in the left panel (Fig. 1D, right panel). The fragments of APP695M generated by HtrA2 proteolytic activity were further characterized by analogy with the previous study on APP cleavage by caspase-3. The 110-kDa APP was processed to three smaller polypeptides of ~85–90 kDa by active caspase-3, whereas two major fragments of ~32 and 50 kDa were generated by active HtrA2.

It is well known that the APP family consists of three members: APP, APLP1, and APLP2. Alignment of amino acid sequences of the APP family shows that both APLPs are significantly homologous to APP: APP is 39.3 and 52.2% identical to APLP1 and APLP2, respectively (PIR: Protein Information Resource). Moreover, a recent study has shown that secretases cleave APP and APLPs in a similar manner (5). To determine whether APLP1 and APLP2 can also be cleaved by HtrA2, we incubated [35S]Met-labeled APLP1 and APLP2 with proteolytically active or inactive recombinant HtrA2 (Fig. 1E). After incubation, the 80-kDa APLP1 was processed into two major fragments of 65 and 67 kDa, and the 115-kDa APLP2 was processed into cleaved fragments of 103 and 109 kDa by active HtrA2 (Fig. 1E). These results indicate that the APP family members may serve as substrates for the human serine protease HtrA2.

Identification of the HtrA2 Cleavage Site in APP—Although two major HtrA2-cleaved fragments of APP were detected by an in vitro cleavage assay using [35S]Met-labeled APP as a substrate, more feasible assays need to be developed for identifying the exact HtrA2-cleavage site in APP. To accomplish our purpose, we generated a series of GST-APP fusion constructs, and the GST-APP fusion proteins expressed in E. coli were purified and utilized to characterize the precise cleavage site through IB assay and N-terminal amino acid sequencing (Figs. 2 and 3).

In an in vitro cleavage assay using GST-APP695M as a substrate, we observed a band with the relative molecular mass of 32 kDa that was detectable by IB assay with anti-Myc Ab, indicating that the 32-kDa fragment is the C-terminal region of
β-Amyloid Precursor Protein Is a Direct Cleavage Target

A

CMV  1  Aβ  Myc  pCMV-APP695M

CMV  535  Aβ  Myc  pCMV-C161M

CMV  695  Aβ  695  pCMV-C161

B

| Construct                  | CMV | HtrA2 | 1133 | 1133 (S306A) | APP695M | C161M |
|---------------------------|-----|-------|------|-------------|---------|-------|
| pCMV-HtrA2                | -   | -     | -    | -           | +       | +     |
| pCMV-Δ1133                | -   | +     | -    | -           | -       | -     |
| pCMV-Δ1133 (S306A)        | +   | -     | +    | +           | -       | -     |
| pCMV-APP695M              | +   | +     | -    | +           | +       | -     |
| pCMV-C161M                | -   | -     | +    | -           | -       | -     |

C

| Construct                  | CMV | HtrA2 | 1133 | 1133 (S306A) | Total | Cyto | Mito | HtrA2 | p50 | p36 | Cas-3 | HSP60 |
|---------------------------|-----|-------|------|-------------|------|------|------|-------|-----|-----|-------|-------|
| pCMV-HtrA2                | +   | -     | -    | -           | +    | -    | -    | -     | +   | -   | +     | -     |
| pCMV-HtrA2 (S306A)        | -   | +     | -    | -           | -    | -    | -    | -     | -   | -   | +     | +     |

D

Images showing localization of HtrA2, Mitochondria, APP, and DAPI staining.
APP with the Myc epitope tag (Fig. 2B, bottom). Nonetheless, we were not able to discern the 32-kDa cleaved fragment from other APP fragments generated by proteolysis in E. coli by staining with Coomassie Brilliant Blue dye (Fig. 2B, top). Because of the aberrant expression pattern of full-length APP in E. coli, we analyzed APP with the PESTfind analysis program at the European Molecular Biology Network (EMBnet), leading to the identification of the presence of a PEST motif in amino acid residues 179–276 of APP. Full-length APP containing this motif seems likely to be highly susceptible to proteolysis in E. coli expression systems.

To construct a suitable form for an in vitro APP processing assay and to narrow the HtrA2 cleavage site in APP, we generated pGST-C390M plasmid, an N-terminal truncated form of APP, by removing the N-terminal region of APP containing the possible PEST motif. The purified GST-C390M protein was incubated with GST-Δ133 or GST-Δ133 (S306A) (Fig. 2C, bottom). We observed the 32-kDa fragment similar in size and antigenicity to the band derived from the HtrA2-mediated cleavage of GST-APP695M (Fig. 2C, bottom), but not from GST-C109F and GST-C100M (data not shown). The result indicates that the HtrA2-cleavage site is located within the region comprising aa 306–588 of APP.

Although GST-C390M was less susceptible to proteolysis in E. coli than GST-APP695M and was almost completely processed by HtrA2, the 32-kDa cleaved fragment was hard to discern as Coomassie-stained bands (Fig. 2C, top). Because the Aβ region (aa 597–638 of APP695) located within the 32-kDa fragment may reduce protein solubility, the cleaved fragment might be prone to being extremely insoluble and thus barely detectable under nondenaturing conditions. To overcome the insolubility problem encountered with the HtrA2-cleavage fragment containing the Aβ region, we subsequently generated a GST-Asub-syn construct that contains the putative cleavage site by fusing β-synuclein to the cDNA fragment (aa 449–598 of APP695) in which the C-terminal region with the Aβ region was truncated (Fig. 2D). β-Synuclein was not cleaved by HtrA2 in an in vitro cleavage assay and was soluble under nondenaturing conditions (data not shown). After incubation of the soluble GST-Asub-syn with GST-Δ133, the reaction mixtures were resolved by 15% SDS-PAGE and visualized by staining with Coomassie Brilliant Blue dye. We selected the C-terminal 23-kDa fragment containing β-synuclein (Fig. 2D, asterisk). The amino acid residues of the C-terminal side of the scissile bond (P1′–P6′) were identified as NGEFSL (aa 535–540 of APP695) by N-terminal amino acid sequencing, representing that the observed 32-kDa fragment was verified as the 161-residue C-terminal APP fragment comprising aa 535–695 of APP, designated C161. The amino acid residues surrounding the cleavage site within APP are identical among human, mouse, and rat (Fig. 2D), suggesting that HtrA2 catalytic cleavage of APP may be evolutionially conserved among mammals.

To verify the HtrA2-cleavage site of APP, proline (P) and valine (V) at positions 533 and 534 were replaced with arginine (R) residues (named APP695(R/R)) (Fig. 2E). Production of the C161 fragment was almost abrogated in the APP695(R/R)M, indicating that HtrA2 can cleave APP on the C-terminal side of Val-534.

HtrA2-mediated APP Proteolysis Is Associated with the Mitochondria in Mammalian Cells—To determine whether APP is cleaved by HtrA2 in mammalian cells, a plasmid expressing either wild-type or mutant HtrA2 (pCMV-Δ133 or pCMV-Δ133 (S306A)) was transiently transfected with a plasmid encoding full-length APP with a C-terminal Myc tag (pCMV-APP695M) (Fig. 3, A and B). Upon overexpression of APP with proteolytically active HtrA2 in HEK293 cells, a 32-kDa fragment of APP was also detected by IB analysis with anti-APP Ab that recognizes the epitope corresponding to aa 676–695 of APP695. On the other hand, the proteolytic activity of HtrA2 on APP was abolished by substitution of serine 306 for alanine at the conserved active site of HtrA2. To verify whether the HtrA2-mediated proteolytic fragment of APP observed in HEK293 cells is the same size as C161, the pCMV-C161M plasmid encoding C161 with a C-terminal Myc tag was transiently transfected as the size control (Fig. 3, A and B). The APP-cleaved fragments generated by co-expressing mature or the full-length form of HtrA2 (pCMV-Δ133 or pCMV-HtrA2, respectively) and C161M were specific to anti-Myc Ab, and molecular mass of both cleaved fragments was remarkably similar with that of C161, being ~32 kDa (Fig. 3B).

Interestingly, we found that not only mature HtrA2 but also full-length HtrA2 can process APP into C161 (Fig. 3B). Several studies have shown that full-length HtrA2 is predominantly targeted to the mitochondria where it is autoproteolytically processed into the matured form of HtrA2 (18, 19, 21, 22, 26). To characterize the subcellular localization of full-length HtrA2 in our system, we transiently transfected into HEK293 cells the pCMV-HtrA2 plasmid encoding full-length HtrA2 with a C-terminal FLAG epitope tag, and the localization of different forms of HtrA2 was analyzed by subcellular fractionation experiments (Fig. 3C). Mature 36-kDa HtrA2 processed (termed p36) from its precursor HtrA2 (termed p50) was detected only in the mitochondrial fraction (Mito). Using an immunofluorescence assay, we assessed the possibility whether the mitochondrial HtrA2 colocalizes with APP. HEK293 cells were transfected with the pCMV-APP695M and pCMV-HtrA2 plasmids for 24 h, followed by double immunostaining with anti-HtrA2 and anti-Myc Abs (Fig. 3D). Consistent with our
β-Amyloid Precursor Protein Is a Direct Cleavage Target

biochemical fractionation assays, the expression pattern of HtrA2 showed punctuate structures that are typical for mitochondrial localization (18, 26). The immunofluorescence staining with anti-Myc Ab exhibited extranuclear granule structures in conjunction with APP expression, some of which was colocalized with HtrA2. We can infer the notion that APP partially colocalizes with HtrA2 in the mitochondria to allow an appropriate subcellular location for accurate and efficient proteolytic cleavage of APP by HtrA2.

HtrA2 Efficiently Cleaves APP in the Normal Physiological Condition—APP seems to be predominantly processed by mature mitochondrial HtrA2 (p36), because endoproteolytic activity of full-length HtrA2 (p50) was 20 times less than that of p36 (Fig. 3, B and C) (27).

Caspase-mediated APP cleavage has been shown to increase during staurosporine- or etoposide-induced apoptosis (28). A number of recent studies have reported that HtrA2 is released from the mitochondria into the cytoplasm in response to apoptotic stimuli, and the cytosolic mature HtrA2 promotes apoptosis through antagonizing XIAP anti-caspase activity and regulating its serine protease activity in the cytoplasm (18, 19, 21, 22). We therefore investigated whether HtrA2-mediated APP cleavage can be increased by cytosolic mature HtrA2 that is released from mitochondria during apoptosis (Fig. 4). After transiently expressing HtrA2 and APP695M in HEK293 cells for 24 h, the cells were induced by 1 μM staurosporine for 4 or 7 h. The ratio of C161M/APP band intensities is accompanied by decreases rather than an increased production level of C161M during apoptosis, showing decreases of 29 (4 h) and 64.9% (7 h) compared with their normal level. Moreover, the ratio of C161M/APP also decreased 66% during 40 μM etoposide-induced apoptosis compared with the non-apoptotic condition (data not shown). The result suggests that the APP cleavage event by HtrA2 is naturally occurring under normal physiological conditions.

Serine Protease Activity of HtrA2 Is Essential for the Production of the C161 Fragment—Previous biochemical and immunological studies have shown that the N terminus of APP is located inside the mitochondrial membrane, whereas the C terminus is exposed outside the mitochondria (16). To define localization of the processed C161 in intracellular organelles, we carried out subcellular fractionation experiments using HEK293 cells expressing both HtrA2 and APP (Fig. 5). The C161M fragment processed from APP695M was mainly detected in the cytoplasmic fraction (Cyto). Moreover, we detected an additional band specific to the anti-APP Ab with a molecular mass of ~28 kDa in both active and inactive HtrA2-transfected cells (Fig. 5). The 28-kDa fragment recognized by anti-APP antibody was observed in cells transfected with the pCMV plasmid as a mock vector (Fig. 6A), but not in the APP-deficient B103 neuronal cells (data not shown). To verify the identity of the 28-kDa fragment, we constructed the pCMV-C161 plasmid encoding untagged C161 and transiently expressed it in HEK293 cells, followed by IB assays with anti-APP or -Myc Abs. The transiently expressed untagged C161 was only recognized by anti-APP Ab, but not by anti-Myc Ab, and its molecular mass was also identical with that of the endogenous 28-kDa fragment (Fig. 6A).

Because proteolytically active HtrA2 pre-exists in the mitochondria, it seems that endogenous mitochondrial APP is naturally processed into C161. Upon overexpression of APP in HEK293 cells, it was also partly cleaved to C161 by endogenous HtrA2 (Fig. 6B). Suppression of endogenous HtrA2 by HtrA2-specific small interfering RNA resulted in almost 20–30% reduction of the C161 production (Fig. 6C). Additionally, a...
recent study has demonstrated that the protease activity of HtrA2 was almost completely abolished in mnd2 mice carrying the missense mutation S276C in the protease domain of HtrA2 (23). We confirmed the definitive visible phenotypes of the mnd2 mice, as shown previously, and the serine to cysteine (S to C) substitution at residue 276 in mnd2 mice was identified by direct DNA sequencing (Fig. 6D).

DISCUSSION
We have described here that the processing of mitochondrial APP is executed by the mitochondrial serine protease HtrA2. As shown by the results from both in vitro and in vivo cleavage assays, HtrA2 directly cleaved APP in the mitochondria to produce the C161 fragment. Additionally, we found that endogenous HtrA2 cleaves APP to produce C161 in mouse brains as well as in cultured cells without any apoptotic stimuli.

Unexpectedly, we found that both extramitochondrial HtrA2 (termed H9004) and full-length HtrA2, which is targeted to the mitochondria, cleaved APP to generate the C161 fragment. Moreover, the immunofluorescence and subcellular fractionation data showed that autoproteolytically processed and proteolytically active HtrA2 is localized predominantly in the mitochondria (18, 26), where APP is partly colocalized with HtrA2. From the results, we postulate that apoptotic stimuli or the release of mitochondrial HtrA2 into the cytosol are not responsible for mitochondrial APP processing. A recent study has shown that the N-terminal portion of mitochondrial APP exists through the mitochondrial membranes, and the C-terminal portion of APP is exposed to the cytosol (16). However, the topology of the middle portion of APP localized between the mitochondrial intermembrane space and outer membrane remains unclear. When plasmids encoding full-length HtrA2 and APP were cotransfected into HEK293 cells, we could detect the C161 fragment in the cytosolic fraction. Our results indicate that the HtrA2 cleavage site
between Val-534 and Asn-535 may be localized in the intermembrane space, and the resulting C161 fragment is likely to be released into the cytosol after cleavage of APP by HtrA2 (Fig. 7). Although the mechanism by which C161 is released from the mitochondria into the cytosol remains to be elucidated, the C161 release was not accompanied by the release of cytochrome c. In addition, overexpression of cytosolic C161 did not induce the release of cytochrome c and HtrA2 from the mitochondria (data not shown).

We have previously demonstrated that HtrA2 interacts with the Aβ region of APP through the PDZ domain of HtrA2 (25). Nevertheless, HtrA2 directly and efficiently cleaved the GST-Asub-syn protein that does not contain the Aβ region. HtrA2 lacking the PDZ domain also cleaved APP to yield the C161 fragment (data not shown). These results led us to the conclusion that the interaction between HtrA2 and APP is not necessarily required for recognition of APP as the HtrA2 substrate. Therefore, it is possible that HtrA2 localized in the intermembrane space cleaves mitochondrial APP, which exposes the C terminus containing the Aβ domain into cytosol.

Several lines of evidence have demonstrated that the secretase-mediated Aβ production, as well as caspase-mediated APP processing occur much more frequently under apoptotic conditions (6–9). Cleavage of most of the substrates of HtrA2 was also detected in apoptotic cells (29–32). In contrast, we found that the levels of C161 production significantly decreased under apoptotic conditions, and we could not detect any apoptotic cells in HEK293 cells that overexpress C161M or C161 (data not shown).

Additionally, α-secretase cleaves APP (aa 1–695) on the C-terminal side of lysine 612 within the Aβ region to release the non-amyloidogenic C83 fragment and competes with β-secretase for cleavage of APP under normal physiological conditions, thereby precluding the formation of amyloidogenic Aβ (Figs. 1A and 6C) (33, 34). Thus, it seems likely that overall Aβ production is barely modulated by HtrA2-mediated APP processing under normal physiological conditions.

The studies using HtrA2 knock-out mice and mnd2 mice delineate the notion that the role of HtrA2 exerted through its serine protease activity within the mitochondria might be responsible for cell survival and mitochondrial physiological functions (23, 24). Moreover, a recent report revealed that accumulation of APP in the mitochondria results in mitochondrial dysfunction and impaired energy metabolism (16). We found that the level of C161 is greatly reduced in the brain of mnd2 mice, indicating that APP and other substrates of HtrA2 may accumulate in the mitochondria, and protein accumulation can be a fatal cause of cell death observed in the HtrA2 knock-out and mnd2 mice. Under normal physiological conditions, HtrA2 seems to prevent the accumulation of mitochondrial APP through APP cleavage by its serine protease activity in the mitochondria.
In this study, we found that APP family proteins are direct substrates for HtrA2. Our study is the first demonstration of the physiologically relevant substrate for HtrA2 in the mitochondria under normal conditions. Some reports suggest that mitochondria play a central role in neurodegeneration including AD through modulation of cellular energy, calcium levels, and reactive oxygen species (35). Therefore, well designed biochemical and biological studies, such as measurement of the mitochondrial energy metabolism impairment in APP overexpressing wild-type or mnd2 mouse embryonic fibroblast cell lines, can provide definitive evidence that the serine protease activity of HtrA2 mediates mitochondrial dysfunction induced by APP accumulation and the role of HtrA2 in AD pathogenesis.

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REFERENCES

1. Yuan, J., and Yankner, B. A. (2000) Nature 407, 802–809
2. Selkoe, D. J. (1991) Neuron 8, 465–478
3. Vassar, R., and Citron, M. (2000) Neuron 27, 419–422
4. Citron, M. (2000) Annu. N. Y. Acad. Sci. 920, 192–196
5. Eggert, S., Paliga, K., Soba, P., Evin, G., Masters, C. L., Weidemann, A., and Beyreuther, K. (2004) J. Biol. Chem. 279, 18146–18156
6. Siman, R., Card, J. P., and Davis, L. G. (1990) J. Neurosci. 10, 2400–2411
7. Barnes, N. Y., Li, L., Yoshikawa, K., Schwartz, L. M., Oppenheim, R. W., Aebi, U., and Milligan, C. E. (1998) J. Neurosci. 18, 5869–5880
8. Weidemann, A., Paliga, K., Durrwag, U., Reinhard, F. B., Schuckert, O., Evin, G., and Masters, C. L. (1999) J. Biol. Chem. 274, 5823–5829
9. Gervais, F. G., Xu, D., Robertson, G. S., Vaillancourt, J. P., Zhu, Y., Huang, J., LeBlanc, A., Smith, D., Rigby, M., So, S. K., van der Ploeg, L. H., Ruffolo, S. C., Thornberry, N. A., Xanthoudakis, S., Zamboni, R. J., Roy, S., and Nicholson, D. W. (1999) Cell 97, 395–406
10. Caswell, M. D., Mok, S. S., Henry, A., Cappai, R., Klug, G., Beyreuther, K., Masters, C. L., and Small, D. H. (1999) Eur. J. Biochem. 266, 509–516
11. Grau, S., Baldi, A., Bussani, R., Tian, X., Stefanescu, R., Przybylski, M., Richards, P., Jones, S. A., Shridhar, V., Clausen, T., and Ehrmann, M. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 6021–6026
12. Nishimura, I., Utseki, T., Dami, S. U., Ohsawa, Y., Ito, S., Okamura, H., Uchiyama, Y., and Yoshikawa, K. (1998) J. Neurosci. 18, 2387–2398
13. Utseki, T., Takemoto, K., Nishimura, I., Okamoto, M., Niinobe, M., Momoi, T., Miura, M., and Yoshikawa, K. (1999) J. Neurosci. 19, 6955–6964
14. Kienlen-Campard, P., Tasiaux, B., and Octave, J. N. (2000) Exp. Gerontol. 35, 843–850
15. Grant, S. M., Shankar, S. L., Chalmers-Redman, R. M., Tatton, W. G., Szyf, M., and Cuello, A. C. (1999) Neuroreport 10, 41–46
16. Anandatheerthavarada, H. K., Biswas, G., Robin, M. A., and Avadhani, N. G. (2003) J. Cell Biol. 161, 41–54
17. Uren, R. T., Dewson, G., Bonzon, C., Lithgow, T., Newmeyer, D. D., and Kluck, R. M. (2005) J. Biol. Chem. 280, 2266–2274
18. Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., and Takahashi, R. (2001) Mol. Cell 8, 613–621
19. 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
20. Martins, L. M. (2002) Cell Death Differ. 9, 699–701
21. Verhagen, A. M., Silke, J., Ekert, P. G., Pakusch, M., Kaufmann, H., Connolly, L. M., Day, C. L., Tiko, A., Burke, R., Wrobel, C., Moritz, R. L., Simpson, R. J., and Vaux, D. L. (2002) J. Biol. Chem. 277, 445–454
22. Suzuki, Y., Takahashi-Niki, K., Akagi, T., Hashikawa, T., and Takahashi, R. (2004) Cell Death Differ. 11, 208–216
23. 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
24. Martinis, M. L., Morrison, A., Klupsch, K., Fedele, V., Moisoi, N., Teismann, P., Abuin, A., Grau, E., Geppert, M., Livi, G. P., Creasy, C. L., Martin, A., Hargreaves, I., Heales, S. J., Okada, H., Brandner, S., Schulz, J. B., Mak, T., and Downward, J. (2004) Mol. Cell. Biol. 24, 9848–9862
25. Park, H. J., Seong, Y. M., Choi, J. Y., Kang, S., and Rhim, H. (2004) Neurosci. Lett. 357, 63–67
26. Seong, Y. M., Choi, J. Y., Park, H. J., Kim, K. J., Ahn, S. G., Seong, G. H., Kim, I. K., Kang, S., and Rhim, H. (2004) J. Biol. Chem. 279, 37588–37596
27. Seong, Y. M., Park, H. J., Seong, G. H., Choi, J. Y., Yoon, S. J., Min, B. R., Kang, S., and Rhim, H. (2004) Protein Expression Purif. 33, 200–208
28. Tesco, G., Koh, Y. H., and Tanzi, R. E. (2003) J. Biol. Chem. 278, 46074–46080
29. 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
30. Sekine, K., Hao, Y., Suzuki, Y., Takahashi, R., Tsu Rao, T., and Naito, M. (2005) Biochem. Biophys. Res. Commun. 330, 279–285
31. Cilenti, L., Soundarapandian, M. M., Kyriazis, G. A., Stratico, V., Singh, S., Gupta, S., Bonventre, J. V., Alnemri, E. S., and Zervos, A. S. (2004) J. Biol. Chem. 279, 50295–50301
32. Trinc, A., Fiory, F., Maitan, M. A., Bonventre, J. V., Alnemri, E. S., and Zervos, A. S. (2005) Proc. Natl. Acad. Sci. U. S. A. 96, 3922–3927
33. Skovronsky, D. M., Moore, D. B., Milla, M. E., Doms, R. W., and Lee, V. M. (2000) J. Biol. Chem. 275, 2568–2575
34. Skovronsky, D. M., Moore, D. B., Milla, M. E., Doms, R. W., and Lee, V. M. (2000) J. Biol. Chem. 275, 2568–2575
35. Swerdlow, R. H., and Khan, S. M. (2004) Med. Hypotheses 63, 8–20