HtrA2 Regulates β-Amyloid Precursor Protein (APP) Metabolism through Endoplasmic Reticulum-associated Degradation

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Henri J. Huttunen‡§1, Suzanne Y. Guénette‡§1, Camilla Peach‡§, Christopher Greco‡§, Weiming Xia*, Doo Yeon Kim‡§, Cory Barren‡§, Rudolph E. Tanzi‡§, and Dora M. Kovacs‡§2

From the ‡Neurobiology of Disease Laboratory and the §Genetics and Aging Research Unit, Massachusetts General Institute for Neurodegenerative Disease (MIND), Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts 02129 and the ¶Center for Neurologic Diseases, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts 02115

Alzheimer disease-associated β-amyloid peptide is generated from its precursor protein APP. By using the yeast two-hybrid assay, here we identified HtrA2/Omi, a stress-responsive chaperone-protease as a protein binding to the N-terminal cysteine-rich region of APP. HtrA2 coimmunoprecipitates exclusively with immature APP from cell lysates as well as mouse brain extracts and degrades APP in vitro. A subpopulation of HtrA2 localizes to the cytosolic side of the endoplasmic reticulum (ER) membrane where it contributes to ER-associated degradation of APP together with the proteasome. Inhibition of the proteasome results in accumulation of retrotranslocated forms of APP and increased association of APP with HtrA2 and Derlin-1 in microsomal membranes. In cells lacking HtrA2, APP holoprotein is stabilized and accumulates in the early secretory pathway correlating with elevated levels of APP C-terminal fragments and increased Aβ secretion. Inhibition of ER-associated degradation (either HtrA2 or proteasome) promotes binding of APP to the COPII protein Sec23 suggesting enhanced trafficking of APP out of the ER. Based on these results we suggest a novel function for HtrA2 as a regulator of APP metabolism through ER-associated degradation.

A major neuropathological hallmark of Alzheimer disease (AD) is the accumulation of amyloid β-peptide (Aβ) in senile plaques (1). Aβ is generated from β-amyloid precursor protein (APP) by sequential proteolytic cleavages mediated by β- and γ-secretases (2). An alternative nonamyloidogenic α-secretase cleavage cuts APP in the middle of the Aβ region (3). APP is a ubiquitous type 1 transmembrane glycoprotein with many putative functions related to cell adhesion and migration. Like most plasma membrane proteins, APP is synthesized and N-glycosylated in the endoplasmic reticulum (ER) and transported to the Golgi complex for maturation before transport to the cell surface. Several molecular chaperones that interact with APP have been identified including BiP/GRP78 (4) and calreticulin (5).

Genetic evidence shows that duplication of the APP gene locus is sufficient to cause early-onset AD (6). APP gene dosage is doubled in Down syndrome (DS), and all adults over the age of 40 years develop AD (7). Furthermore, analysis of the human genome suggests that copy number variations play a significant role in the etiology of complex diseases such as AD (8) highlighting the importance of cellular regulatory mechanisms. Although proteolytic events resulting in the generation of Aβ from APP are well characterized, the regulation of APP protein levels in cells remains poorly understood. Altered maturation, processing, and degradation of APP holoprotein may regulate the availability of APP for Aβ generation or affect the ratio of Aβ42 to Aβ40, both of which appear to be important in the pathogenesis of AD. Several recent reports have suggested that events in the early secretory pathway strongly affect APP metabolism and Aβ generation (9–11).

HtrA2, a novel APP-interacting chaperone protease, was recently reported to cleave APP in the mitochondria (12). HtrA proteins are oligomeric, ATP-independent serine proteases widely found from bacteria to mammals with important roles in protein quality control (13). In general, HtrA proteins contain a protease domain and one or two regulatory PDZ domains that recognize partially folded or misfolded proteins. Of the four human HtrA family members HtrA2 is the only one with an intracellular localization (13), and there is strong evidence showing that HtrA2 plays a role in the regulation of apoptosis (14–16). However, the neurodegenerative phenotype of the HtrA2 knock-out mice and increased susceptibility of HtrA2−/− cells to ER stress suggest that HtrA2 might play an important role in protein quality control (17). HtrA2 has been shown to bind through its PDZ domain to both Aβ (18) and the C termini of the presenilins (PS1 and PS2), key components of the γ-secretase complex (19).

Our current results suggest that HtrA2 has a more general role in the regulation of APP metabolism. Partial localization of HtrA2 to the cytosolic side of ER membranes together with the finding that HtrA2 exclusively binds to the immature form of...
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APP indicate that the HtrA2-APP interaction occurs in the early secretory pathway. Lack of HtrA2 in cells results in stabilization of APP holoprotein, its accumulation in microsomes as well as increased association of immature APP with the COPII protein Sec23. Furthermore, we show that APP is retrotranslocated from the ER to the cytosol and is a substrate for the ER-associated degradation (ERAD) pathway. Based on these data, we propose that HtrA2 collaborates with the proteasome in ERAD to regulate cellular levels of APP.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Assay—Identification of APP N-terminal domain-binding proteins was performed as described previously for the APP cytoplasmic domain (20). Briefly, the N-terminal cysteine-rich region (38–187 amino acids) of human APP was cloned in-frame in the LexA plasmid. The yeast strain EGY48 that contained LexAop-LEU2, LexAop-LacZ, and LexA-APP (38–187) was transformed with DNA from a galactose-inducible activation domain-cDNA fusion library constructed from 22-week-old human fetal brain (a gift from Dimitri Krainc, Massachusetts General Hospital, Boston). We identified and confirmed 63 independent positives from this screen. Southern blot analysis of the cDNA inserts showed that 61 of these clones were highly conserved sequences from six classes of overlapping cDNA clones. A full-length 458 amino acid open reading frame for the newly identified human gene was assembled from the two hybrid library clone containing the longest cDNA insert, a human adult brain Lambda Zap cDNA library (B616), a human kidney lambda phage cDNA library (Clontech) and a human cosmid library (a gift from Marcy Macdonald, Massachusetts General Hospital, Boston).

Cell Culture and Transfection—Parental CHO, H4, CHOAPP751, H4APP751, and mouse embryonic fibroblasts (MEF; wild-type or HtrA2−/− (17)) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals), 2 mM l-glutamine (Lonza), 100 units/ml penicillin, and 100 µg/ml streptomycin (Lonza). Stable cell lines were selected and maintained in G418 (Calbiochem). Cells were cultured at 37 °C in a water-saturated air/5% CO2 atmosphere. MEF cells were transiently transfected using the Amaxa Nucleofector system according to the manufacturer’s instructions (Amaxa Biosystems).

Antibodies, Peptides, and Recombinant Proteins—Recombinant human HtrA2 (rec-HtrA2), His-tagged recombinant BACE1 and HtrA2 antibody were from R&D Systems. The following APP antibodies were used: 22C11 (N-terminal; Chemicon), A8717 (C-terminal; Sigma), 6E10 (Aβ 1–17, Signet) and NAB228 (Aβ 1–11, Covance). C-66 is an affinity-purified rabbit polyclonal antibody raised to the C-terminal 20 amino acids of human APP (751–770) by the same protocol (BioSource) that was used for the widely used C7 antibody (21). Calreticulin and syntaxin-6 from BD Biosciences are used for the widely used C7 antibody (21). Calreticulin and syntaxin-6 from BD Biosciences are used for the commercially available antibodies and Alexa Fluor-conjugated secondary antibodies.

Confocal Fluorescence Microscopy—CHO cells were grown on coverslips coated with poly-D-lysine/laminin mixture. The cells were fixed with 3% paraformaldehyde/PBS, permeabilized with 0.1% Triton X-100/PBS for 10 min and blocked first with 10 mM NH4Cl/PBS and then with 2% BSA/PBS. Endogenous HtrA2, EIC1, syntaxin-6, and APP (rabbit C-terminal (Chemicon) or 6E10 mA (S ignited)) were detected with corresponding antibodies and Alexa Fluor-conjugated secondary antibodies.

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(Molecular Probes/Invitrogen). Mitochondria were stained with MitoTracker Red and nuclei with Hoechst 33342 (Molecular Probes/Invitrogen). Confocal fluorescence images were obtained using an Olympus DSU/IX70 spinning disc confocal microscope and processed using IPlab software (Scanalytics/BD Biosciences).

In Vitro Cleavage Assay—Rec-HtrA2 was incubated with purified recombinant APP751, APP695, nectin-1 or BACE1 in a reaction buffer containing 50 mM Tris-HCl, pH 7.0, 0.5 mM EDTA, 1 mM dithiothreitol. Depending on the assay, duration and temperature of incubation varied between 15 and 90 min and 37–42 °C. Reaction was stopped by adding 1× LDS gel-loading buffer (Invitrogen) and heating for 10 min at 70 °C. Occasionally, the 15 amino acid peptide corresponding to the presenilin-1 C terminus was included in the reaction mixture at 10–100 μM (19).

Retrotranslocation Assay—Retrotranslocation of proteins to the cytosol was analyzed as described previously (24). Briefly, cells in monolayer were detached from tissue culture plates with PBS containing 5 mM EDTA. After centrifugation at 1,000 × g for 5 min, the cell pellet was resuspended in 100 μl of DPB buffer (0.04% digitonin (Sigma), 50 mM Hepes, pH 7.5, 150 mM NaCl, 2 mM CaCl₂, protease inhibitor mixture (Roche Applied Sciences)), incubated on ice for 10 min and centrifuged at 16,000 × g for 10 min at 4 °C. The supernatant was removed (cytosol), and the membrane pellet was washed with 500 μl of cold PBS and extracted in 150 μl of DEB buffer (1% digitonin, 25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture). Alternatively, microsomal membranes rehomogenized in 10 mM Hepes, pH 7.4, 0.25 M sucrose, 1 mM EDTA with or without 1% Triton X-100 were used as substrate material.

Pulse Chase—Nearly confluent cells grown on 100-mm plates were first preincubated in methionine/cysteine-free medium for 1 h. 100 μCi of [35S]methionine/cysteine (MP Biomedicals) was then added per plate for 15 min (pulse). Then, cells were washed in PBS containing 5 mM EDTA and temperature of incubation varied between 15 and 90 min and 37–42 °C. Reaction was stopped by adding 1× LDS gel-loading buffer (Invitrogen) and heating for 10 min at 70 °C. Occasionally, the 15 amino acid peptide corresponding to the presenilin-1 C terminus was included in the reaction mixture at 10–100 μM (19). Alternatively, microsomal membranes rehomogenized in 10 mM Hepes, pH 7.4, 0.25 M sucrose, 1 mM EDTA with or without 1% Triton X-100 were used as substrate material.

Statistical Methods—Statistical comparisons were done using unpaired t tests or analysis of variance with significance placed at p < 0.05. Data are expressed as mean ± S.D.

RESULTS

HtrA2 Binds and Degrades Immature APP—To identify proteins that interact with the cysteine-rich, N-terminal domain of APP, we performed a yeast two-hybrid assay with the APP cysteine-rich domain as a bait sequence (depicted in Fig. 1A). The smallest APP-interacting cDNA contained 162 C-terminal amino acids of HtrA2/Omi chaperone-protease including the active site motif, GNSGG, and the PDZ domain (data not shown). Furthermore, the HtrA2 two-hybrid fusion proteins failed to induce lacZ expression when the APLP2 cysteine-rich region (56–203 amino acids) was used in the yeast two-hybrid assay, indicating that HtrA2 binding is specific for the APP Cys-rich region.

Next, we performed reciprocal immunoprecipitations with HtrA2 and APP antibodies to verify the yeast two-hybrid findings. As shown in Fig. 1B, only immature form of APP interacts

![HtrA2/OMI interacts with the ectodomain of immature APP.](image)
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with HtrA2 in naive and APP751-overexpressing CHO cells. A similar interaction of HtrA2 with immature APP was found in both parental H4 human neuroglioma cells and H4 cells overexpressing APP751 (data not shown). To confirm that the HtrA2 interaction with APP occurs in vivo we immunoprecipitated HtrA2 and APP from mouse brain extracts. Similar to our findings in cultured cells, the immature form of APP holoprotein compared with the control samples containing no HtrA2.

although HtrA family proteases do not display strict cleavage site requirements in general, optimal substrate cleavage sites for HtrA2 have been reported (25). Sequence analysis of APP suggests that there are several potential HtrA2 cleavage sites in the APP ectodomain (data not shown). To analyze degradation of APP by HtrA2 in vitro, we incubated purified V5/His-tagged APP with rec-HtrA2 in different conditions: (A) increasing concentrations of HtrA2 at 42 °C (15 or 45 min), (B) 132 nM HtrA2 at 37 °C (60 min) in a series of increasing buffer pH, (C) APP751-V5/His or APP695-V5/His were incubated with HtrA2 at 37 °C, pH 7.0 (60 min). D, 0.5 μg of nectin-1-V5/His or BACE1-His were incubated with increasing concentrations of HtrA2 at 37 °C (60 min). Western blots were stained with APP C-terminal antibodies, V5 or His antibodies and subjected to densitometric analysis. The results are displayed as percentage of remaining APP holoprotein compared with the control samples containing no HtrA2.

However, the resulting 25-kDa C-terminal fragment (presumably APP-C161) was present in the APP751 but not in APP695 samples suggesting that the presence of the KPI domain somehow affects the degree of completion to which HtrA2 degrades APP. Recombinant V5/His-tagged nectin-1, a type 1 transmembrane protein (29), and His-tagged ectodomain of BACE1 (30) were used to control for specificity of HtrA2 proteolytic activity. As shown in Fig. 2D, HtrA2 was not able to cleave either nectin-1 or BACE1 in these assay conditions suggesting that APP is a specific substrate for the HtrA2 protease.

In addition to APP, HtrA2 has been reported to interact with presenilin-1 (PS1) and presenilin-2 (PS2), both implicated in Alzheimer disease. PS1 is a critical component of the γ-secretase complex that generates Aβ from APP (31) and the C terminus of PS1 interacts with the regulatory PDZ domain of HtrA2 activating the protease (19). Thus, we tested whether a 15-amino acid C-terminal peptide of PS1 could regulate HtrA2 proteolytic activity toward APP. In the presence of 100 μM PS1 C-terminal peptide, HtrA2 activity is roughly doubled and only 31% of full-length APP remains in the reaction mixture compared with 66% in the control reaction without the PS1 peptide (supplemental Fig. S1). Altogether, the in vitro cleavage data suggest that HtrA2 is capable of specifically degrading APP, and that the degree of APP degradation is controlled by conditions that affect the regulatory PDZ domain of HtrA2 as well as the presence of a protease inhibitor domain in the substrate.

HtrA2 Localizes to Mitochondria, ER, and Cytosol—Original reports described mammalian HtrA2 as an ER- and nucleus-
localized protease (32, 33). However, it has become evident that the majority of cellular HtrA2 is localized to mitochondria (14, 15). Because of these contradictory reports, we used both subcellular fractionation and confocal immunofluorescence imaging to study subcellular localization of HtrA2. Crude cell fractionation by differential centrifugation confirmed that the majority of HtrA2 was localized to the mitochondrial fractions. However, a significant amount of HtrA2 was also found in the microsomal fraction and a small amount in the cytosolic fraction under normal growth conditions (Fig. 3A). To analyze the microsomal fraction in detail, we resolved the postmitochondrial supernatant in an OptiPrep density gradient. Clearly, most of the extramitochondrial HtrA2 colocalized with the ER-chaperone BiP/GRP78 (Fig. 3B). Interestingly, an alkali carbonate extraction of both microsomal (Fig. 3A) and pooled ER fractions (data not shown) washed out most if not all HtrA2 but none of the integral membrane proteins (APP and calreticulin in fractions P5 and S5 in Fig. 3A) or ER lumenal proteins (GRP78/BiP, data not shown). This suggests that HtrA2 associates peripherally to the cytosolic side of the microsomal/ER vesicles.

Confocal microscopy of CHO cells stained with HtrA2 antibodies together with MitoTracker Red, PDI or syntaxin-6 confirmed that HtrA2 mainly colocalized with the mitochondrial marker (Fig. 3C). However, less frequent but still significant colocalization of HtrA2 with the ER marker PDI was detected (Fig. 3D). No colocalization was observed with the Golgi marker, syntaxin-6 (Fig. 3E). Costaining of HtrA2 and APP showed colocalization of the two proteins in ER-like structures (Fig. 3F). Given a couple of recent reports indicating mitochondrial localization of APP (12, 34), it should be noted that in these experiments we could not detect any colocalization of APP with mitochondrial markers in our cell lines (Fig. 3G). Taken together, our subcellular localization data suggest that HtrA2 interacts with immature APP in the early secretory pathway.

**APP Can Be Retrotranslocated and Degraded by the Proteasome and HtrA2**—Several studies have implied that a degradation pathway associated with the ER participates in the regulation of APP metabolism (4, 35–37). Because of the localization of extramitochondrial HtrA2 to the cytosolic side of the ER/microsomal membranes, access of HtrA2 to its binding sites in the extracellular/lumenal domain of APP would require dislocation of APP from the ER to the cytosol. To assess whether APP is retrotranslocated from the ER, we used a retrotranslocation assay (24) to collect cytosols from semipermeabilized cells. Treatment of CHO APP751 cells with epoxomicin, a selective and irreversible proteasome inhibitor (38), resulted in cytosolic accumulation of immature APP and a smaller, 95-kDa form of APP (Fig. 4A). Neither form of APP was ubiquitinated suggesting that the cytosolic forms of APP are degraded by the proteasome in an ubiquitin-independent manner. Interestingly, the 95-kDa form of APP appeared to be N-terminally truncated as C-terminal (A8717, NAB228) but not N-terminal (22C11) APP antibodies recognized it in Western blots (Fig. 4B).
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To rule out the possibility that only overexpressed APP is retrotranslocated, we used naïve B104 neuroblastoma cells in the retrotranslocation assay. Although free cytosolic, endogenous APP forms could not be detected in epoxomicin-treated B104 cells, we noticed an abundant pool of peripherally associated APP molecules that could be detached from microsomal membranes with an alkali carbonate buffer (Fig. 4C). These data show that endogenous APP can be retrotranslocated from the ER but remains associated with the cytosolic side of ER membranes.

Next, we tested whether HtrA2 could degrade the retrotranslocated, cytosolic APP. When cytosols collected from epoxomicin-treated CHOAPP751 cells were incubated with rec-HtrA2, APP was efficiently degraded (Fig. 4D). This suggests that HtrA2 may collaborate with the proteasome to degrade retrotranslocated APP. Remarkably, when microsomal membranes isolated from epoxomicin-treated cells were immunoprecipitated with HtrA2, a dramatic increase in the association of both immature and 95-kDa forms of APP with HtrA2 was observed (Fig. 4E). Microsomal APP-immunoprecipitates from epoxomicin-treated cells also contained more retrotranslocation protein Derlin-1 than the controls (Fig. 4E) suggesting that Derlin-1 is involved in retrotranslocation of APP. Together with the ability of HtrA2 to N-terminally trim APP in vitro (Fig. 2B), these data suggest that HtrA2 may serve as a shuttling factor delivering and preparing ERAD substrates for proteasomal degradation.

Inhibition of the proteasome by epoxomicin caused a 29% increase in the microsome-associated HtrA2 (supplemental Fig. S2A). Interestingly, when microsomal membranes isolated from epoxomicin-treated cells were incubated with rec-HtrA2, HtrA2 not only cleaved APP (data not shown) but also decreased the amount of total polyubiquitinated proteins by about 25% (supplemental Fig. S2B). This suggests that when proteasomal activity is compromised, HtrA2 may be recruited to the ER membrane to assist in the disposal of accumulating proteasome substrates.

APP Metabolism Is Altered in Cells Lacking HtrA2—To better understand the physiological relationship between HtrA2 and APP, we next analyzed metabolism of endogenous APP in fibroblasts derived from HtrA2−/− mouse embryos (17). As compared with fibroblasts derived from wild-type embryos, HtrA2−/− cells had only slightly elevated total levels of APP holoprotein (by ~10%; Fig. 5A). However, the APP C-terminal fragment levels were more significantly increased in HtrA2−/− cells. After normalization to APP holoprotein levels, HtrA2−/− cells harbored 218% higher levels of APP-C99 and 171% higher levels of APP-C83 than the HtrA2+/+ cells (Fig. 5, A and B). The elevated APP-C99 level correlated with a 41% increase in the amount of Aβ in the conditioned media from HtrA2−/− cells as compared with wild-type cells (Fig. 5C).

As HtrA1, a secreted Htra-family member, was previously suggested to be an APP-CTF degrading protease (39) and HtrA2 has been shown to bind to Aβ and APP-C99 (18), we tested whether HtrA2 could also be an APP-CTF degrading protease. However, HtrA2 was unable to directly degrade APP-C99 in vitro (supplemental Fig. S3) suggesting that even though both HtrA2 and HtrA1 are capable of binding APP they may exhibit different cleavage site specificities. Therefore, the increased levels of APP-CTFs in HtrA2−/− cells are more likely explained by reduced degradation of APP holoprotein rather than altered degradation of the C-terminal fragments. To further investigate this, we performed pulse-chase assays with [35S]methionine/cysteine in wild-type and HtrA2−/− fibroblasts. As shown in Fig. 5, D and E, the lack of HtrA2 increases
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APP holoprotein levels were significantly increased in microsomes prepared from HtrA2<sup>−/−</sup> cells (Fig. 6A). We could not detect accumulation of immature or 95-kDa APP forms in the cytosols prepared from HtrA2<sup>−/−</sup> or HtrA2<sup>+/+</sup> cells in the retrotranslocation assay. However, similarly to B104 cells, microsomes prepared from HtrA2<sup>−/−</sup> cells contained a pool of peripherally associated APP molecules that was detached from microsomal membranes with an alkali carbonate buffer (Fig. 6A). A transmembrane ER chaperone calreticulin did not dissociate from the microsomes, whereas a significant fraction of Sec23, a peripherally associated COPII transport protein, shifted to the wash fraction. Importantly, the carbonate wash had little effect on microsomal APP in HtrA2<sup>+/+</sup> cells showing that retrotranslocated APP accumulates in HtrA2<sup>−/−</sup> cells and remains peripherally associated with the microsomal membranes. Thus, lack of HtrA2 in fibroblasts results in accumulation of immature APP in the early secretory pathway further suggesting that HtrA2 plays a role in ERAD of APP.

Recently, Kincaid et al. (40) showed that ERAD and ER exit pathways compete for substrates that have ER exit signals. Therefore, lowered ERAD capacity may result in increased ER exit of (misfolded) secretory proteins such as APP. Altered ER exit dynamics could be indicated by differential binding of APP to COPII cargo selection complex Sec23/Sec24 (41, 42). Supporting this idea, immunoprecipitation of Sec23 pulled down a significantly higher amount of immature APP from HtrA2<sup>−/−</sup> cells as compared with the wild-type cells (Fig. 6B). Similarly, epoxomicin treatment of H4 cells resulted in increased binding of immature APP to Sec23 (Fig. 6C) suggesting that suppression of HtrA2 or proteasome activities have similar accelerating effects on ER exit of APP molecules.

β- and γ-secretase activities are known to be concentrated in lipid rafts, cholesterol-enriched membrane microdomains implicated in protein trafficking, proteolytic processing, and signal transduction (23, 43). As most of the secreted Aβ is thought to be generated in lipid rafts in post-Golgi compartments, we tested whether APP association with lipid rafts is altered in HtrA2<sup>−/−</sup> cells. As shown in Fig. 6D, more APP holoprotein is associated with flotillin-positive lipid raft fractions

the half-life of APP holoprotein by ~175% (from 27.1 ± 2.7 to 47.5 ± 2.3 min for immature APP and from 27.3 ± 5.0 to 47.4 ± 0.9 min for mature APP).

Next, we tested if retransfection of HtrA2 back to the HtrA2<sup>−/−</sup> cells could reverse the alterations in APP metabolism. FLAG-tagged mouse HtrA2 was transfected by electroporation into the wild-type and HtrA2<sup>−/−</sup> fibroblasts. In wild-type MEFs, overexpression of HtrA2 had no significant effect on the half-life of APP holoprotein by 47.5 ± 2.3 min for immature APP and 47.4 ± 0.9 min for mature APP).
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DISCUSSION

Mammalian cells have evolved sophisticated mechanisms for recognition, retrotranslocation, and degradation of misfolded or unassembled ER and secretory proteins (44, 45). Several studies have suggested that a degradation pathway associated with ER participates in the regulation of APP metabolism (4, 35–37). Early events in the maturation of amyloid precursor protein (APP) seem to determine its entry into one of several alternative processing pathways, one involving proteasomal degradation in the cytosol. We have characterized an interaction between the stress-responsive chaperone protease HtrA2 and APP in the early secretory pathway. The present results are summarized in Fig. 7 and suggest that ERAD plays a role in the regulation of APP metabolism, and that by increasing the amount of APP available for β- and γ-secretase cleavages defective or impaired ERAD pathways could contribute to pathogenesis of AD.

A prerequisite for HtrA2-APP interaction is retrotranslocation of APP from the ER membrane. Several studies have shown that APP holoprotein is degraded by the proteasome under various conditions: proteasomal degradation of APP is enhanced by interrupted N-glycosylation of APP (36) and overexpression of modifier of cell adhesion (MOCA, Dock3) (35) whereas overexpression of peroxisome proliferator-activated receptor-γ (PPARγ) induces accumulation of polyubiquitinated APP (46). On the other hand, induction of ER stress results in retention of APP in the early secretory pathway and decreases generation of Aβ (47). Our results showing that (1) immature APP as well as N-terminally truncated form of APP are retrotranslocated to the cytosol, and (2) both retrotranslocated forms of APP can be degraded by the proteasome and HtrA2, provide a mechanistic explanation for the previously reported findings and identify APP as a novel substrate for ERAD. Although we noticed retrotranslocated immature APP peripherally associated with microsomal membranes in HtrA2−/− cells, we were unable to detect the N-terminally truncated form of APP suggesting that it may be generated by HtrA2 during the retrotranslocation process. This is supported by the in vitro cleavage data where HtrA2 truncated APP N-terminally.

Most retrotranslocated ERAD substrates carry polyubiquitin chains that are trimmed by shuttling factors such as ataxin-3 to facilitate proteasomal degradation (48). As retrotranslocated APP appears to be degraded in a ubiquitin-independent manner, it is tempting to speculate that HtrA2 could serve as a shuttling chaperone between retrotranslocation machinery and proteasome using its proteolytic activity to trim the core protein of nonubiquitinated ERAD substrates before proteasomal degradation. The finding that cells lacking HtrA2 are more...
susceptible to ER stress (17) strongly supports our current results and may be partially explained by accumulation of ERAD substrates in the ER. Inhibition of proteasomal function induced association of HtrA2 with APP as well as Derlin-1, a putative retrotranslocation channel forming ER protein. This suggests that Derlin-1 is involved in retrotranslocation of APP and that HtrA2 may get recruited to Derlin-1 containing retrotranslocation complexes to assist in ERAD. Interestingly, exogenously added HtrA2 is able to degrade ubiquitinated proteins in proteasome-inhibited microsomes suggesting a more general role for HtrA2 in ERAD.

Recently, it was reported that ERAD competes with ER-exit pathways for substrates that have ER exit signals (40). Thus, inhibition of ERAD in ER may result in increased ER exit of (misfolded) secretory proteins (as depicted in Fig. 7). Immature APP holoprotein is clearly stabilized in HtrA2−/− cells, and this may result in increased ER exit of APP. Accordingly, we found increased binding of immature APP to Sec23, a COPII cargo-loading protein, in both HtrA2−/− cells and proteasome inhibitor-treated cells supporting the idea of altered ER exit dynamics. This also suggests that increased generation of APP C-terminal fragments and Aβ in HtrA2−/− cells could be explained by accelerated trafficking of APP out of ER. Increased levels of lipid raft-associated APP in HtrA2−/− results do not exclude the possibility that in addition to the ER, HtrA2 could interact with APP when localized to mitochondria (12, 34). However, in our cell lines we could not detect APP in the mitochondria suggesting that this may be cell type dependent. We also noticed a residual amount of HtrA2 in the cytosol of normal, nonapoptotic cells under standard cell culture conditions. It is possible that to prevent induction of apoptosis, HtrA2 is recruited from the cytosol to ER by HtrA2-interacting proteins such as presenilins (19). Presenilins are abundant in the ER and have been suggested to have γ-secretase independent functions in trafficking and unfolded protein response (UPR) in the early secretory pathway (51–53). Thus, it is tempting to speculate that presenilin C termini could regulate the association of HtrA2 with the ER membranes. This could also be relevant regarding the role of cytosolic HtrA2 in apoptosis, especially in light of the antiapoptotic roles attributed to presenilin C-terminal fragments (54). Interestingly, proteolytic activity of HtrA2 can be regulated through phosphorylation at S212 by Akt kinase, a member of a key antiapoptotic pathway, adding another layer of complexity to HtrA2 biology (55).

The current view of mostly mitochondrial localization of HtrA2 (13, 50) argues against HtrA2 being a part of the protein quality control/degradation machinery of the ER. However, our localization studies by both subcellular fractionation and confocal microscopy are supported by two previous studies (32, 33) and suggest that although the majority of HtrA2 is localized to the mitochondrial intermembrane space, there is a fraction of cellular HtrA2 that associates peripherally with ER membranes and colocalizes with APP. It should be noted that our

![HtrA2 collaborates with the proteasome in degradation of APP in the early secretory pathway.](image)

FIGURE 7. HtrA2 collaborates with the proteasome in degradation of APP in the early secretory pathway. A schematic model summarizing our hypothesis based on the findings of this study. A, in normal cells, most newly synthesized APP molecules fold correctly and are exported from the ER in COPII vesicles after correct folding. Occasional misfolded APP molecules are retained in the ER, engaged with the ERAD machinery, and retrotranslocated to the cytosol for degradation by the proteasome and HtrA2. B, in HtrA2−/− cells, shutting of ERAD substrates from the retrotranslocation machinery to the proteasome is impaired, resulting in accumulation of retrotranslocated immature APP on the ER membrane. C, in cells treated with a proteasome inhibitor, ERAD substrates are retrotranslocated and shuttled to proteasome but fail to be degraded and accumulate in the cytosol. More HtrA2 is recruited to the ER membrane to compensate for the lost ERAD capacity. Similar to HtrA2−/− cells, reduced ERAD capacity results in increased flow of APP from the ER to Golgi as reflected by increased association of APP with Sec23. The size of the red arrows reflects the number of molecules passing through each pathway.

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analysis of these HtrA2−/− mice resulted in a conclusion that despite of the suggested role of HtrA2 as a mitochondrial regulator of apoptosis, mammalian HtrA2 is likely involved in protection against cell stresses that involve protein misfolding. Notably, loss of function mutations in HtrA2 gene result in neurodegenerative phenotype with Parkinsonian features in both humans and in mice. Furthermore, S276C loss of function mutation in HtrA2 is responsible for the neurodegenerative phenotype in mnd2 (motor neuron degeneration 2) mice (56). Recently, two mutations in HtrA2 gene (G399S and A1415S) were reported in German Parkinson disease (PD) patients (57). Both mutations resulted in defective induction of the proteolytic activity of HtrA2. In this regard, our findings identify yet another PD-associated protein as a component of the protein quality control and proteasome-related disposal machinery. Our results raise interesting new questions on the neuronal roles of HtrA2, especially in the light of the neurodegenerative phenotypes of mnd2 and HtrA2 knock-out mice. Being a part of protein quality control systems in both mitochondria and ER puts HtrA2 in a central position in terms of pathophysiology of neurodegeneration.

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