Expression of Alzheimer’s Disease-associated Presenilin-1 Is Controlled by Proteolytic Degradation and Complex Formation*

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Numerous mutations causing early onset Alzheimer’s disease have been identified in the presenilin (PS) genes, particularly the PS1 gene. Like the mutations identified within the β-amyloid precursor gene, PS mutations cause the increased generation of a highly neurotoxic variant of amyloid β-peptide. PS proteins are proteolytically processed to an N-terminal (NTF) and a C-terminal ~20-kDa fragment (CTF20) that form a heterodimeric complex. We demonstrate that this complex is resistant to proteolytic degradation, whereas the full-length precursor is rapidly degraded. Degradation of the PS1 holoprotein is sensitive to inhibitors of the proteasome. Formation of a heterodimeric complex is required for the stability of both PS1 fragments, since fragments that do not co-immunoprecipitate with the PS complex are rapidly degraded by the proteasome. Mutant PS fragments not incorporated into the heterodimeric complex lose their pathological activity in abnormal amyloid β-peptide generation even after inhibition of their proteolytic degradation. The PS1 heterodimeric complex can be attacked by proteinases of the caspase superfamily that generate an ~10-kDa proteolytic fragment (CTF10) from CTF20. CTF10 is rapidly degraded most likely by a calpain-like cysteine proteinase. From these data we conclude that PS1 metabolism is highly controlled by multiple proteolytic activities indicating that subtle changes in fragment generation/degradation might be important for Alzheimer’s disease-associated pathology.

Alzheimer’s disease (AD) is the most common dementia worldwide. A pathological hallmark of AD is the invariant accumulation of numerous senile plaques in certain areas of the brain. Senile plaques are composed of the amyloid β-peptide (Aβ), a proteolytic derivative of the β-amyloid precursor protein (βAPP; see Ref. 1). In the majority of cases, AD occurs as a sporadic disease with an increasing risk during aging (2). However, in about 10–15% of the cases AD is caused by autosomal dominant mutations within three genes (2). A very limited set of families was identified carrying mutations in the βAPP gene (2). These mutations occur at, or close to, the cleavage sites of the βAPP-processing enzymes, the secretases (1). All βAPP mutations analyzed so far cause an enhanced production of Aβ, specifically the highly pathogenic 42-amino acid variant, Aβ42 (2).

By far the highest number of FAD-associated mutations were identified within the PS gene (see Ref. 3 and for review see Ref. 4). Two mutations were also observed in the highly homologous PS2 gene (5, 6). PS proteins are integral membrane proteins, which form 6 to 8 trans-membrane domains with the N-terminal domain, the large loop, and the C-terminal domain located within the cytoplasm (7, 8). Both PS proteins are predominantly expressed within the endoplasmic reticulum, the nuclear envelope, and the early Golgi (7, 9–12).

Like βAPP mutations, mutant PS proteins are involved in aberrant Aβ generation. All PS1 and PS2 mutations analyzed so far affect the cleavage of βAPP at the C terminus of the Aβ domain, resulting in a significant increase of Aβ42 generation (13–20). Very recent work by De Strooper et al. (21) on Aβ generation in PS1+/− mice suggests that the wt PS1 protein activates the cleavage at the C terminus of the Aβ domain by a mechanism that might be reminiscent to the clip promoted by the sterol regulatory element binding protein cleavage-activating protein (22).

PS proteins are proteolytically processed (Fig. 1A; see Refs. 23 and 24 and for review see Ref. 4). Two processing pathways can be discriminated. In the predominant pathway, proteolytic processing occurs by a putative protease, called the presenilinase (Ref. 23; see Fig. 1A). A naturally occurring mutation that results in the deletion of exon 9 due to a splicing error (25) blocks proteolytic processing by the presenilinase (23), indicating that the cleavage occurs within the domain encoded by exon 9 (Fig. 1A). Indeed a major cleavage site has been identified after amino acid 298 (26). PS processing by the presenilinase results in the generation of ~30-kDa N-terminal and ~20-kDa C-terminal fragments (NTF and CTF20; see Refs. 23 and 24). A very similar processing pathway is also observed for PS2 (27). The resulting NTF and the CTF20 of PS1 and PS2 bind to each other and form a heterodimeric complex (28–30). In contrast to the wt PS1 holoprotein, which does not participate in complex formation, PS1Δexon9 substitutes the PS1 complex as a holoprotein (28).

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§ The abbreviations used are: AD, Alzheimer’s disease; Aβ, amyloid β-peptide; βAPP, β-Amyloid precursor protein; CTF, C-terminal fragment; CTF10, caspase generated 10-kDa CTF; CTF20, presenilinase generated CTF; FAD, familial Alzheimer’s disease; NTF, N-terminal fragment; PARP, poly(ADP) ribose polymerase; PS, presenilin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; wt, wild type; ELISA, enzyme-linked immunosorbent assay; LLaL, N-acetyl-L-leucinyl-L-leucinyl-L-norleucine.
In addition to processing by the presenilinase, both PS pro-
teins are substrates for proteinases of the caspase superfamily (31–34). In this pathway the 20-kDa CTF₂₀ serves as a sub-
strate for caspase cleavage (33) which generates a smaller
~10-kDa C-terminal fragment (Fig. 1A). Caspase-mediated
cleavage appears to be enhanced by a FAD mutation occurring
within the PS₂ gene (31).

Formation of CTF₂₀ is highly regulated. Overexpression of
PS results in the replacement of endogenous PS fragments but
not in a linear accumulation of the overexpressed fragments
(23, 35). Moreover, overexpression of PS₁ proteins not only
results in a replacement of the endogenous PS₁ fragments but
also blocks the synthesis of PS₂ fragments and vice versa (23,
35). The very tight regulation of PS processing and fragment
accumulation indicates that a limited factor binds to PS pro-
teins (35). Indeed, as described above PS proteins form a com-
plex, which contains the PS heterodimer (28–30, 36) and prob-
ably binding protein(s) as well. Moreover, slight changes in the
tightly balanced fragment formation might be pathologically
relevant (see Refs. 24, 37, and 38; for review see Ref. 39). We
therefore analyzed the stability of the PS precursor and its
proteolytic fragments in transfected and untransfected cells and
found that PS₁ expression is highly controlled by multiple
proteolytic degradation pathways. The formation of a het-
erodimeric complex protects the fragments from their rapid
degradation, and complex formation appears to be required for
the pathological activity of PS on Aβ₄₂ generation. Our data
suggest that proteolytic degradation plays a major role in a
balanced and equal expression of PS fragments. These data fur-
ther indicate that slight changes in the stability of excess
amounts of PS fragments might be pathologically relevant.

EXPERIMENTAL PROCEDURES

Cell Culture and Cell Lines—All cell lines were cultured in Dulbec-
cóo’s minimal essential medium (Dulbecco’s minimal essential medium/
Glutamax; Life Technologies, Inc.) supplemented with 10% fetal calf
serum, and 1% penicillin/streptomycin (40). K293 cell lines stably
transfected with βAPP or PS₁ cDNA constructs were described
previously (15, 28, 40, 41, 44).

The anti-PS antibodies used are described previously (12, 33, 42). The epitopes of the antibodies used are shown in Fig. 1A.
The anti-PARP antibody was obtained from Boehringer Mannheim.
Anti-β-catenin antibodies were obtained from Sigma. The C7 antibody
to the C terminus of βAPP and the detection of C-terminal βAPP
fragments were described previously (40).

Treatment of Cells with Proteinase Inhibitors and Other Com-
ponds—The following concentrations of proteinase inhibitors and other compounds were used: lactacystin, 10 μM; LLA(L, 50 μM; M;
MDL28170, 50 μM; leupeptin, 10–100 μM; NaN₃, 25 mM; E64, 50 μM;
Z-VAD, 50 μM; staurosporine, 1 μM. All proteinase inhibitors except lactacystin (Biomol) were obtained from Sigma. The calpain inhibitor
MDL28170 was synthesized by Boehringer Ingelheim KG. Proteasome
and calpain inhibitors were dissolved in Me₂SO at 10 or 10 mM (lact-
cystin). Leupeptin (10 mg/ml) was dissolved in H₂O, E64 (50 mM) in 50%
EtOH, and NaN₃ (5 mM) in H₂O. Inhibition of caspases was performed
using Z-VAD (Z-VAD-FMK, Calbiochem) dissolved at 50 mM in Me₂SO.
Apoptosis was induced with 1 μM staurosporine (Biomol) dissolved at 1
mM in Me₂SO. Treatment with inhibitors was performed in 4 ml of
culture medium for the indicated times using cells grown to 80–100%
confluence in 10-cm dishes.

Analysis of PARP Cleavage as a Biological Marker for Apoptosis—
Analysis of apoptosis in cell culture upon treatment with proteinase
inhibitors was monitored in lysates of HeLa cells by the identification of
the caspase-generated cleavage product of PARP (31).

Preparation of Cell Extracts and Immunoprecipitation—Preparation
of cell lysates and immunoprecipitations were carried out as described
previously (12, 33, 42). To detect the PS₁ CTF₂₀, cell extracts were
prepared by lysis in STEN buffer (50 mM Tris-HCl, pH 7.6, 150 mM
NaCl, 2 mM EDTA, 0.2% Nonidet P-40) containing 1% SDS, 1% Triton
X-100 in the presence of 1 mM phenylmethylsulfonyl fluoride. For
immunoprecipitation SDS extracts were diluted 10× in STEN buffer con-
taining 1% Triton X-100.

Immunoblotting—Immunoblotting was carried out as described (40)
using the enhanced chemiluminescence kit (ECL, Amersham Pharma-
cia Biotech).

Pulse-Chase Experiments—Stably transfected K293 cell lines were
grown to confluence in 10-cm dishes. After starvation for 2 h in 4 ml of
methionine- and serum-free medium (MEM, L-glutamine, 1% penicillin/ streptomycin), cells were metabolically labeled with 500–700 μCi of
³⁵S)Methionine (Amersham Pharmacia Biotech Promix) in 4 ml of
methionine-free minimal essential medium for 20–30 min as described
under “Results.” The cold chase was performed by replacing the labeling
medium with 4 ml of chase medium (Dulbecco’s minimal essential
medium, 10% fetal calf serum, 1% penicillin/streptomycin, 0.8 mM L-
methionine) for the indicated periods. Cell extracts were prepared and
immunoprecipitated as described (42). Proteinase inhibitors were
added during starvation, labeling, and cold chase periods where
indicated.

Isolation of Membrane Proteins—K293 cells were grown to confluence.
Cells of five 10-cm dishes were scraped in phosphate-buffered saline
and pelleted. The cell pellet was washed three times in phos-
phate-buffered saline. Cells were then resuspended in 5 ml of RSB
buffer (10 mM Tris, pH 7.5, 20 mM KCl, 1.5 mM MgAc₂) containing
proteinase inhibitors as described (28) and homogenized by passing the
cell suspension 10 times through a 23-gauge needle. To prepare a
postnuclear supernatant, the homogenate was centrifuged at 1000 × g
for 15 min at 4 °C. Membranes from the postnuclear supernatant were
then recovered by ultracentrifugation for 1 h at 100,000 × g. Membrane
fractions were washed in a high salt HEPES buffer (1 M KCl, 20 mM
HEPES, pH 7.2, 2 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol)
containing proteinase inhibitors. The purified membranes were
extracted with 2% CHAPS in HEPES buffer (100 mM KCl, 20 mM
HEPES, pH 7.2, 2 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol), containing
proteinase inhibitors as described (28) for 1 h on ice. Membrane extracts
were cleared by ultracentrifugation for 1 h at 100,000 × g at 4 °C. Incubation with PS₁ antibodies was performed as described (28).
Immunoprecipitations were washed 4 times for 20 min in CHAPS washing buffer (0.5% CHAPS, 200 mM NaCl, 50 mM HEPES, pH 7.6) prior to SDS-polyacrylamide gel
electrophoresis.

Detection of Aβ₄₀ and Aβ₄₂—Cells were plated at equal density in
6-well plates. After reaching confluence, 2 ml of conditioned media were
collected for 18 h. Media were centrifuged to remove cell fragments, and
 aliquots were then used to determine the ratios of Aβ₄₀ to total Aβ
(Aβ₄₀ and Aβ₄₂). A highly specific sandwich ELISA employing mono-
clonal antibodies specific for the detection of Aβ₄₀ or Aβ₄₂ was
developed according to previous protocols (43).

RESULTS
To determine the cellular fate of the PS₁ holoprotein and its
proteolytic fragments, we used human kidney 293 cells (K293)
stably transfected with the βAPP and PS₁ cDNAs (15, 26, 44).
The K293 cell line is highly appropriate for the analysis of the
biochemistry of the FAD-associated proteins (βAPP and PS₁/
PS₂), since βAPP metabolism and the effects of βAPP and PS
mutations originally sorted out in K293 cells (41, 44–46) and
other peripheral cell lines such as COS (19) and Chinese ham-
ter ovary (20) were completely confirmed in neuronal cells,
primary cell cultures, human and mouse brain tissue, cerebro-
spinal fluid, and plasma (12, 15–18, 47). Co-expression of mut-
ant βAPP/PS₁ allows the highly sensitive detection of Aβ₄₀/42
(16–17).

The PS₁ Precursor but Not the NTF and CTF₂₀ Is Rapidly
Degraded by a Pathway That Is Sensitive to Inhibitors of the
Proteinase—Untransfected K293 cells or K293 cells stably
transfected with wt PS₁ (15, 26) were metabolically labeled for
30 min with [³⁵S]Methionine and chased for 4 h in the presence
of excess amounts of unlabeled methionine. Cell lysates were
immunoprecipitated with antibody 3027 (Fig. 1A) to detect the
full-length protein. Immediately after pulse labeling robust
amounts of PS₁ holoprotein were detected in transfected cells
(Fig. 1B). Interestingly, we detected the 20-kDa CTF₂₀ upon a
Degradation of the PS1 holoprotein is reduced (but not blocked) by LLnL and lactacystin, whereas the same inhibitors do not affect the levels of the PS1 precursor, whereas lactacystin did (Fig. 1A). As shown in Fig. 1B, the CTF20 is rapidly degraded. K293 cells stably transfected with PS1 were pulse-labeled for 30 min and chased for 4 h with and without the indicated protease inhibitors. Cell lysates were immunoprecipitated with antibody 3027 to detect the full-length PS1 protein as well as CTF 20. Degradation of the PS1 holoprotein is reduced (but not blocked) by LLnL and lactacystin, whereas the same inhibitors do not affect the levels of CTF20, C, steady state levels of NTF and the CTF20 (1st and 2nd panels) are resistant to proteolytic degradation. K293 cells were treated with and without the indicated protease inhibitors for 18 h (leupeptin and NH4Cl) or 24 h (LLnL and lactacystin). Both fragments were detected by a combined immunoprecipitation/immunoblotting protocol (26, 33, 35). Minor differences in the levels of the corresponding fragments are due to experimental variations. The activity of proteasome inhibitors was confirmed by the detection of stabilized C-terminal fragments of βAPP (40, 53). Aliquots of cell lysates from the experiment described in C were immunoblotted with the anti-β-catenin antibody and with anti-C7 to the C terminus of βAPP (40). D, rapid degradation of de novo synthesized PS1Δexon9. K293 cells stably transfected with PS1Δexon9 were pulse-labeled for 30 min and chased for 4 h with and without the indicated protease inhibitors. Cell lysates were immunoprecipitated with antibody 3027 to detect the PS1Δexon9 protein. Degradation of the PS1Δexon9 protein is reduced but not blocked by LLnL and lactacystin.

FIG. 1. The PS1 precursor, but not the NTF and CTF20, is rapidly degraded. A, schematic representation of PS1. The epitopes of all anti-PS1 antibodies used are shown as bars. The proteases involved in proteolytic processing of PS1 are indicated in italics. Numbers in parentheses indicate the site of cleavage. aa, amino acid. The black box indicates the domain encoded by exon 9. B, the wt PS1 holo-protein, but not the proteolytic fragments, are rapidly degraded. K293 cells stably transfected with PS1 were pulse-labeled for 30 min and chased for 4 h with and without the indicated protease inhibitors. Cell lysates were immunoprecipitated with antibody 3027 to detect the full-length PS1 protein as well as CTF20. Degradation of the PS1 holoprotein is reduced (but not blocked) by LLnL and lactacystin, whereas the same inhibitors do not affect the levels of CTF20. C, steady state levels of NTF and the CTF20 (1st and 2nd panels) are resistant to proteolytic degradation. K293 cells were treated with and without the indicated protease inhibitors for 18 h (leupeptin and NH4Cl) or 24 h (LLnL and lactacystin). Both fragments were detected by a combined immunoprecipitation/immunoblotting protocol (26, 33, 35). Minor differences in the levels of the corresponding fragments are due to experimental variations. The activity of proteasome inhibitors was confirmed by the detection of stabilized β-catenin (52). Arrowhead indicates the high molecular weight variants of β-catenin observed after inhibition of the proteasome (3rd panel). Inhibition of lysosomal proteinases (4th panel) was proved by the detection of stabilized C-terminal fragments of βAPP (40, 53). Aliquots of cell lysates from the experiment described in C were immunoblotted with the anti-β-catenin antibody and with anti-C7 to the C terminus of βAPP (40). D, rapid degradation of de novo synthesized PS1Δexon9. K293 cells stably transfected with PS1Δexon9 were pulse-labeled for 30 min and chased for 4 h with and without the indicated protease inhibitors. Cell lysates were immunoprecipitated with antibody 3027 to detect the PS1Δexon9 protein. Degradation of the PS1Δexon9 protein is reduced but not blocked by LLnL and lactacystin.
leupeptin or NH$_2$Cl did not result in significant changes in the amounts of CTF$_{20}$ detected (Fig. 1C). To prove that the proteasome and endosomal/lysosomal inhibitors were indeed active, we analyzed the turnover of β-catenin and C-terminal fragments of βAPP within the same samples. As reported previously (52) we observed the high molecular weight ubiquitinated variants of β-catenin in the samples that were treated with proteasome inhibitors but not in samples treated with inhibitors of the endosomal/lysosomal pathway (Fig. 1C). In contrast, treatment with endosomal/lysosomal inhibitors stabilized the C-terminal fragments of βAPP as described previously (40, 53). In addition DTT but not lactacystin lead to an augmentation of the βAPP fragments, which is again in agreement with previous results (54). We therefore conclude from these data that the PS1 NTF and CTF$_{20}$ are not degraded by the proteasome or endosomal/lysosomal proteinases as well as other cysteine proteinases but are rather stable over long periods.

By having established the degradation of the wt PS1 holoprotein, we next wanted to investigate the proteolytic degradation pathway of a mutant uncleaved PS1 derivative. In order to study this process, we used K293 cells stably expressing PS1Δexon9 (15), which is known to accumulate as an uncleaved holoprotein due to the lack of the cleavage site within exon 9 (23). The cell line was pulse-labeled with [35S]methionine for 30 min and chased for 4 h in the presence of excess amounts of unlabeled methionine. Cell lysates were immunoprecipitated with antibody 3027 to the C terminus of PS1. Robust amounts of a PS1 holoprotein were detected in the cells stably transfected with the PS1Δexon9 cDNA (Fig. 1D). During the chase period the PS1Δexon9 protein was rapidly degraded (Fig. 1D). As observed for the wt holoprotein, only proteasome inhibitors reduced the degradation of PS1Δexon9. Again, only a fraction of PS1Δexon9 could be stabilized by proteasome inhibitors indicating the potential participation of an additional proteolytic activity, which is insensitive to the inhibitors used (see “Discussion”).

**NTFs Not Incorporated into the Heterodimeric Complex Are Degraded by the Proteasome and Do Not Affect the Pathological Generation of Aβ42 upon Proteasome Inhibition**—Previously it has been observed that a recombinant NTF (NTF$_{298}$) of PS1 artificially produced due to a stop codon inserted at amino acid 298 (the predominant cleavage site of PS1; see Ref. 26) can only be detected upon a short pulse labeling but not by steady state metabolic labeling (44). To test if that phenomenon can be explained by proteolytic degradation, we performed a pulse-chase experiment. K293 cells stably expressing NTF$_{298}$ (44) were metabolically labeled with [35S]methionine for 20 min and chased in the presence of excess amounts of unlabeled methionine for 2 h. Cell lysates were immunoprecipitated with antibody 2953. This revealed very high levels of the recombinant NTF$_{298}$ during the short pulse (Fig. 2A). In agreement with the previous results, endogenous NTF could not be labeled during the pulse period (Ref. 44 and data not shown). Upon a 2-h cold chase, NTF$_{298}$ was quantitatively removed, indicating very efficient proteolytic degradation of this fragment (Fig. 2A). To identify the proteasome involved, we treated cells during the labeling and cold chase period with proteasome inhibitors. Whereas proteasome inhibitors efficiently blocked the degradation of NTF$_{298}$, all other inhibitors did not affect the rapid removal of the recombinant fragment (Fig. 2A). Thus, the proteasome is involved in the degradation of the recombinant NTF$_{298}$.

We next asked the question, why endogenous NTFs derived from proteolytic processing of the holoprotein are resistant to the proteasome, whereas the recombinant NTF terminating after amino acid 298 and the full-length protein are highly sensitive to proteasome-mediated degradation. Previously, we and others (28–30) have shown that the CTF$_{20}$ and NTF bind to each other and form a heterodimeric complex. To investigate whether the endogenous NTF can be replaced by the recombinant NTF$_{298}$ within the heterodimeric complex, we performed co-immunoprecipitation experiments. In order to avoid artifacts due to the addition of an epitope tag, which might interfere with protein-protein interactions, we first investigated if the endogenous NTF can be discriminated from the recombi-
nant NTF<sub>298</sub>. Cell lysates from either untransfected cells or NTF<sub>298</sub>-transfected cells treated with and without LLnL were immunoprecipitated with antibody 2953 and detected with antibody PS1N. Inhibition of proteasomal activity was used to allow the efficient detection of the recombinant NTF<sub>298</sub> (see above). Under these conditions the steady state levels of endogenous PS1 fragments are detected (44), in contrast to the pulse-chase experiment shown in Fig. 2A. Immunoblotting with antibody PS1N revealed in untransfected cells a predominant endogenous NTF and a much less intense band migrating just below it (Fig. 2B). In cells stably transfected with NTF<sub>298</sub>, the lowest band was slightly elevated under control conditions, indicating expression of a transgene-derived gene product. Moreover, upon treatment with LLnL this band was strongly augmented, whereas the higher molecular weight band was not affected (Fig. 2B). This is consistent with our finding that NTF<sub>298</sub> but not the endogenous NTF is stabilized by inhibition of the proteasome. Therefore, the lower band corresponds predominantly to the recombinant NTF<sub>298</sub>, and the upper band represents the endogenous NTF. The differential migration might be explained by post-translational modifications of the long lived endogenous NTF leading to an increased molecular weight. After demonstrating that both fragments can be efficiently and reproducibly discriminated based on their differential migration behavior on SDS gels, membrane fractions of LLnL-treated cells stably expressing NTF<sub>298</sub> were subjected to immunoprecipitation (Fig. 2C, upper panel). In parallel, endogenous PS fragments were immunoprecipitated from membrane fractions prepared from untransfected cells that were also treated with LLnL (Fig. 2C, lower panel). Membrane fractions were immunoprecipitated with antibody 3027 (to the large loop) or 2953 (to the N terminus) and immunoblotted with antibody PS1N to the N terminus of PS1. Antibody 3027 co-immunoprecipitated the endogenous NTF (Fig. 2C; upper and lower panel, 2nd lane) which is consistent with data observed previously (28–30). Again, we detect two predominant polypeptides in the cell line expressing NTF<sub>298</sub> (Fig. 2C, upper panel). The lower band corresponds to NTF<sub>298</sub>, whereas the higher band is equivalent to the endogenous NTF. Note that the lower band is strongly augmented upon immunoprecipitation with antibody 2953 but not with antibody 3027, suggesting that the recombinant NTF<sub>298</sub> might not be co-immunoprecipitating with the heterodimeric complex. We then wanted to deplete the cell lysate from the heterodimeric complex and to analyze if in such a depleted extract the recombinant NTF<sub>298</sub> is retained. We therefore re-immunoprecipitated the cell lysates with antibody 3027 (Fig. 2C). After two rounds of consecutive re-immunoprecipitations we observed very low amounts of the co-immunoprecipitating NTF indicating at least a partial depletion of the lysate from the heterodimeric complex (Fig. 2C). The depleted lysate was then immunoprecipitated with an antibody to the N-terminal domain of PS1 (antibody 2953). This revealed a very prominent NTF in the transfected cell line but not in untransfected control cells (Fig. 2C, compare last lane in the upper and lower panel). In contrast to the immunoprecipitation with the antibody 2953 before depletion, we now observe predominantly the lower molecular weight band (Fig. 2C, upper panel; compare last lane with 1st lane), which corresponds to the recombinant NTF<sub>298</sub> (Fig. 2B). When the same blot was decorated with antibodies to the large loop of PS1 (antibody 3027), we detected almost no CTF<sub>10</sub> in the final immunoprecipitation (data not shown), again demonstrating that the majority of NTF<sub>298</sub> is not bound to endogenous PS fragments. Therefore, the overexpressed NTF<sub>298</sub> does not replace the endogenous NTF within the heterodimeric complex but can be immunoprecipitated as a free fragment after the PS complex has been largely removed. These results indicate that heterodimer formation is required for the observed stability of endogenous PS1 fragments. Fragments lacking their binding partners are rapidly degraded by the proteasome.

Previously it was demonstrated that NTF<sub>298</sub> containing a FAD-associated point mutation does not cause an increased generation of Aβ42 (44). However, here we demonstrated that this fragment is highly unstable, and most of it is rapidly removed by the proteasome. Therefore, an accurate determination of its pathological effects on Aβ42 formation might not be possible without inhibition of its proteasomal degradation. In order to investigate if stabilized mutant PS1 NTF<sub>298</sub> could affect the rate of Aβ42 generation, cells were treated with and without LLnL (which efficiently stabilizes NTF<sub>298</sub> as shown in Fig. 2A), and the conditioned media were analyzed for the ratio of Aβ42:total Aβ (Aβ40 and Aβ42) using a sandwich ELISA which allows the quantitative and specific detection of both peptides. Four cell lines expressing either the Swedish mutant βAPP alone (45) or together with the wt NTF<sub>298</sub>, the mutant NTF<sub>298</sub>Y115H (44), or PS1<sup>exon9</sup> (as a positive control for enhanced production of Aβ42 caused by PS mutations) were used (15). Swedish mutant βAPP was co-expressed to facilitate Aβ detection as described previously (15). Fig. 3 demonstrates that as expected the PS1<sup>exon9</sup> mutation results in an increased ratio of Aβ42:total Aβ (Aβ40 and Aβ42) (see Refs. 13–15), whereas the expression of NTF<sub>298</sub>Y115H had no effect on Aβ42 generation as demonstrated before (44). As expected from previous results (55, 56), treatment with LLnL results in an increase of the ratio of Aβ42:total Aβ (Aβ40 and Aβ42), which is due to the inhibition of calpains (56). This is not only observed in cells expressing endogenous PS1 but also in cells lines expressing mutant and wt NTF<sub>298</sub> as well as PS1<sup>exon9</sup> (Fig. 3). However, stabilization of mutant NTF<sub>298</sub> by proteasome inhibition did not result in any increase of the ratio of Aβ42:total Aβ (Aβ40 and Aβ42) as compared with wt NTF<sub>298</sub> (Fig. 3). From these data we conclude that a mutant recombinant NTF does not contribute to Aβ42 production even when its degradation is blocked by proteasomal inhibitors. Moreover, these results might indicate that complex formation is required for the pathological activity of PS1 (see “Discussion”).

Endogenous CTF<sub>20</sub> Is Proteolytically Degraded upon Previous Caspase Cleavage—As shown above, the endogenous PS1 CTFs are very stable (Fig. 1C). However, we and others (31–34) have shown previously that the endogenous PS1 CTF<sub>20</sub> can be an in vivo substrate for an additional cleavage mediated by a protease of the caspase superfamily even without the induction of apoptosis (Fig. 1A). Therefore, we next wanted to analyze if the cleavage of the PS1 CTF<sub>20</sub> by caspases makes the resulting CTF<sub>10</sub> accessible for further proteolytic degradation. We investigated the levels of CTF<sub>10</sub> within the samples shown in Fig. 1C (where K293 cells were treated with LLnL, lactacystin, NH<sub>4</sub>Cl, or leupeptin) by immunoblotting with antibody 3027, which efficiently detects CTF<sub>10</sub> (33). As shown in Fig. 4A, treatment of cells with LLnL caused a marked accumulation of CTF<sub>10</sub> that was barely detectable in the untreated cells suggesting that CTF<sub>10</sub> is rapidly removed by proteolysis. The specific proteasome inhibitor lactacystin also enhanced the levels of CTF<sub>10</sub> but to a significantly lesser extent than LLnL (Fig. 4A), suggesting that the proteasome might only play a minor role in the degradation of CTF<sub>10</sub>. Interestingly, treatment of cells with leupeptin led to a pronounced increase of CTF<sub>10</sub>, whereas NH<sub>4</sub>Cl had almost no effect (Fig. 4A). From these results it appeared that a cysteine protease activity might

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FIG. 3. Mutant NTF_{\text{agg}} does not increase the production of A\textbeta}42 even upon its stabilization. K293 cells stably expressing Swedish βAPP (Ctr.) or Swedish βAPP with the PS1\text{exon9} mutation (PS1\text{exon9}) (15) were incubated for 18 h (upper panel) with and without LLnL. The ratios of A\textbeta}42 to total A\textbeta} (A\textbeta}42 and A\textbeta}40) were determined by a sandwich ELISA according to Suzuki et al. (43). Bars represent means ± S.E. of three independent experiments. As described previously (13–17), the PS1\text{exon9} mutation causes a significant increase of A\textbeta}42 production demonstrating the specificity of the ELISA. Lower panel, K293 cells stably expressing Swedish βAPP and NTF_{\text{agg}} with (NTF_{\text{agg}}Y115H) and without the Y115H mutation were treated with LLnL or the vehicle alone. Note, that the mutant NTF_{\text{agg}} (NTF_{\text{agg}}Y115H) does not induce A\textbeta}42 generation even upon its stabilization with LLnL. Treatment with LLnL increases the ratio of A\textbeta}42 to total A\textbeta} as observed previously (56).

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contribute to the final degradation of the PS1 CTF. Since high concentrations of leupeptin (100 μM) might inhibit the proteasome as well (57), we titrated the concentration of leupeptin. As shown in Fig. 4B, leupeptin inhibited degradation of CTF_{10} at concentrations as low as 10 μM, making it unlikely that leupeptin was inhibiting the proteasome. In addition, immunoblotting with anti-β-catenin antibodies failed to detect high molecular weight variants of this protein upon treatment with leupeptin (see Fig. 1C) further demonstrating that leupeptin does not inhibit the proteasome under the conditions used in this experiment. Moreover, E64, a specific cysteine proteinase inhibitor that does not inhibit the proteasome (58) also blocked degradation of CTF_{10} (Fig. 4C). Taken together, these data therefore demonstrate that CTF_{10} is predominantly degraded by a cysteine proteinase. Since calpains are cysteine proteinases that can also be inhibited by LLnL (see above and see Ref. 48), we also treated cells with the calpain inhibitor MDL28170. As observed for leupeptin or LLnL treatment, MDL28170 significantly stabilized the CTF_{10} (Fig. 4C), therefore indicating that calpain-like proteinases are most likely involved in its degradation. Previously, we and others (32–34) have noticed that the amounts of CTF_{10} are highly variable depending on the cell line or tissue analyzed. We therefore analyzed CTF_{10} degradation in another cell type. HeLa cells were treated with the cysteine proteinase inhibitor leupeptin and the proteasome inhibitor lactacystin. Although HeLa cells expressed higher levels of CTF_{10}, under control conditions (Fig. 4D), treatment with leupeptin still allowed its augmentation (Fig. 4D). Again, as observed for K293 cells, the effect of lactacystin on CTF_{10} stabilization was only modest if detectable at all, although the drug was active in HeLa cells as judged from the accumulation of high molecular weight β-catenin variants (see Fig. 4E). The same result was obtained when the cysteine proteinase inhibitor E64 was used in these experiments (not shown). Based on the results in Fig. 4, A—C, we could not exclude that treatment with the variety of inhibitors used might have induced apoptosis resulting in an enhanced production of CTF_{10} rather than increased stabilization. We therefore investigated the possible induction of apoptosis by monitoring cleavage of poly(ADP-ribose) polymerase (PARP) (31) within the samples analyzed above. Whereas staurosporine as positive control for the induction of apoptosis caused a quantitative cleavage of PARP and an enhanced generation of CTF_{10}, no PARP cleavage was observed in the cells treated with the proteinase inhibitors (Fig. 4D), demonstrating that apoptosis was not the cause of the observed CTF_{10} accumulation. As a further control we treated HeLa cells with Z-VAD, an inhibitor of caspase-1 like proteinases, to inhibit de novo production of CTF_{10} by blocking the caspase activation cascade (Refs. 31–34; for review see Ref. 59). Treatment of HeLa cells with Z-VAD resulted in a significant reduction of CTF_{10} (Fig. 4E). However, when cells were treated with Z-VAD and leupeptin simultaneously, CTF_{10} could still be detected in amounts that were increased compared with untreated cells (Fig. 4E), despite the inhibition of de novo production of CTF_{10}. Simultaneous treatment of Z-VAD and lactacystin did not lead to a stabilization of CTF_{10} further supporting the observation that a cysteine proteinase rather than the proteasome plays the major role in CTF_{10} degradation. The activity of lactacystin was monitored by the detection of high molecular weight variants of β-catenin, stabilized upon proteasome inhibition (Fig. 4E). Taken together, these data rule out that treatment with the various proteinase inhibitors induced apoptosis resulting in an accumulation of CTF_{10} due to enhanced caspase cleavage. Moreover, these data demonstrate that the CTF_{10} is predominantly degraded by a cysteine proteinase and, depending on the cell line used, possibly also to a lesser extent by the proteasome. The much higher levels of CTF_{10} detected in HeLa cells as compared with K293 cells
derivatives of lactacystin was monitored by the detection of high molecular weight additional caspase cleavage at an aspartic acid residue N-terminal to apoptosis. This band most likely represents a CTF that is generated by inhibiting its degradation or its enhanced production upon induction of 24 h with Z-VAD alone to inhibit caspase-mediated generation of CTF10. Upon parallel treatment with leupeptin. HeLa cells were treated for 18 h. CTF10 was isolated and detected as described (see Fig. 1C). B, titration of the leupeptin concentration required for the inhibition of CTF10 degradation. C, the cysteine protease inhibitor E64, which does not inhibit proteasomal degradation, efficiently blocks degradation of CTF10. MDL28170 inhibits degradation of CTF10 as well. CTF10 was isolated and detected as described in A. D, HeLa cells were treated with and without the indicated inhibitors for 18 h. CTF10 was isolated and detected as described (A). Under control conditions HeLa cells contain high levels of CTF10. However, the levels of CTF10 can be further increased upon treatment with leupeptin. Staurosporine was used to induce apoptosis and therefore the production of CTF10 (31–33). E, inhibition of de novo synthesis of CTF10 still allows its stabilization upon parallel treatment with leupeptin. HeLa cells were treated for 24 h with Z-VAD alone to inhibit caspase-mediated generation of CTF10. Simultaneous incubations of Z-VAD with leupeptin, but not with lactacystin, inhibited degradation of CTF10. * indicates a C-terminal fragment, which is, like CTF10, specifically detected after stabilization by inhibiting its degradation or its enhanced production upon induction of apoptosis. This band most likely represents a CTF that is generated by additional caspase cleavage at an aspartic acid residue N-terminal to the major caspase cleavage site at amino acid 345. Upon reprobing of the same blot with antibody APS18 only CTF10 is detected (lower panel). ** indicates the position of CTF10, not detectable with APS18. B, comparison of the molecular mass of CTF20 and CTF20. CTF20 was isolated from untransfected K293 cells treated with the indicated inhibitors for 24 h, and CTF20 was isolated from K293 cells stably expressing PS1Δexon9 as described in A. The fragments were detected using the monoclonal antibody APS18. As expected from the results shown in Fig. 1C, LLnL and lactacystin do not affect levels of CTF20. Note that expression of PS1Δexon9 inhibits the formation of the endogenous CTF20 (23, 42, 60).

Fig. 4. The caspase-generated CTF10 is predominantly degraded by a cysteine proteinase. A, untransfected K293 cells were treated with and without the indicated inhibitors as described in Fig. 1C. CTF10 was detected by reprobing the blot shown in Fig. 1C with antibody 3027. LLnL and leupeptin stabilize large amounts of CTF10, whereas the proteasome-specific inhibitor lactacystin has less effect. The activity of proteasome inhibitors within these samples was confirmed (see Fig. 1C). B, titration of the leupeptin concentration required for the inhibition of CTF10 degradation. C, the cysteine protease inhibitor E64, which does not inhibit proteasomal degradation, efficiently blocks degradation of CTF10. MDL28170 inhibits degradation of CTF10 as well. CTF10 was isolated and detected as described in A. D, HeLa cells were treated with and without the indicated inhibitors for 18 h. CTF10 was isolated and detected as described (A). Under control conditions HeLa cells contain high levels of CTF10. However, the levels of CTF10 can be further increased upon treatment with leupeptin. Staurosporine was used to induce apoptosis and therefore the production of CTF10 (31–33). E, inhibition of de novo synthesis of CTF10 still allows its stabilization upon parallel treatment with leupeptin. HeLa cells were treated for 24 h with Z-VAD alone to inhibit caspase-mediated generation of CTF10. Simultaneous incubations of Z-VAD with leupeptin, but not with lactacystin, inhibited degradation of CTF10. * indicates a C-terminal fragment, which is, like CTF10, specifically detected after stabilization by inhibiting its degradation or its enhanced production upon induction of apoptosis. This band most likely represents a CTF that is generated by additional caspase cleavage at an aspartic acid residue N-terminal to the major caspase cleavage site at amino acid 345. Upon reprobing of the same blot with antibody APS18 only CTF10 is detected (lower panel). ** indicates the position of CTF10, not detectable with APS18. B, comparison of the molecular mass of CTF20 and CTF20. CTF20 was isolated from untransfected K293 cells treated with the indicated inhibitors for 24 h, and CTF20 was isolated from K293 cells stably expressing PS1Δexon9 as described in A. The fragments were detected using the monoclonal antibody APS18. As expected from the results shown in Fig. 1C, LLnL and lactacystin do not affect levels of CTF20. Note that expression of PS1Δexon9 inhibits the formation of the endogenous CTF20 (23, 42, 60).
PS1 complex, since our previous work demonstrated that the PS1Δexon9 holoprotein substitutes the heterodimeric complex and forms a complex by itself (28). CTF$_{59}$ might thus behave like a "free" fragment (similar to NTF$_{298}$) that is subjected to rapid proteolysis by the proteasome.

**DISCUSSION**

The observation that many PS1 mutations cluster close to the cleavage sites of the presenilinase (3, 4) indicates that proteolytic processing of PS1 might be very important for its physiological and pathological function. Moreover, fragment formation appears to be highly regulated. Overexpression of PS1 inhibits synthesis of endogenous PS1 and the homologous PS2 fragments (23, 35). Furthermore, overexpression of PS proteins does not result in a linear increase of fragment formation. Instead, endogenous fragments are replaced by the transgene-derived fragments at approximately the same levels (23, 35). Although the available data regarding the effect of mutations on PS processing are quite controversial (see Refs. 24, 37, and 38; for review see Ref. 39), it appears that proteolytic processing of PS proteins might be disturbed by FAD-associated mutations. Cell type specificity may be responsible for these controversial observations; however, in the majority of cell lines including primary neurons and in all human tissues analyzed, PS proteins are proteolytically processed in the presence and absence of FAD mutations (4). Interestingly, FAD-associated mutations also affect proteolytic processing via the caspase-mediated pathway. In that case the Volga-German mutation causes an increased alternative cleavage of PS2 by a member of the caspase superfamily (31). Together with the highly regulated fragment formation, these data indicate that subtle changes in the balanced fragment formation may result in early onset AD. One would therefore expect physiological control mechanisms that prevent aberrant accumulation of PS fragments and/or its precursor. We therefore searched for proteolytic enzymes that are involved in the degradation of the PS holoprotein as well as its proteolytically processed fragments. PS fragments were found to be very stable when they are generated by proteolytic processing from the holoprotein (Fig. 6). This might be explained by the recent finding that PS fragments bind to each other to form a heterodimeric complex (Fig. 6; see Refs. 28–30 and 36). Upon complex formation, PS fragments are then either transported to a compartment that protects them from proteolytic degradation or undergo structural changes that make the dimer resistant to proteolysis. In that regard it is interesting to note that NTF$_{298}$ is highly unstable (data shown in this work; see also Refs. 44 and 60). This is due to the rapid removal by the proteasomal degradation pathway (Fig. 6). The sensitivity of NTF$_{298}$ to proteolysis...
can be explained by its inability to be incorporated into the PS1 heterodimeric complex (Fig. 6) as demonstrated by the lack of co-immunoprecipitation of NTF$_{296}$ with the PS1 heterodimeric complex. Rapid proteolysis of such free fragments is further supported by the proteasomal degradation of CTF$_{39}$ which is most likely derived from an alternative presenilinase cleavage of the PS1$\Delta$exon9 holoprotein (which forms a complex by itself; see Ref. 28) and thus represents a free CTF (Fig. 6). In the presenilinase pathway PS proteins are cleaved within the domain encoded by exon 9 of PS1 (23). The cleavage is heterogeneous and occurs after amino acids 291, 292, and 298 (26). The heterogeneous cleavage of PS1 might suggest the involvement of an endoproteinase with relaxed sequence specificity. This is further supported by our surprising finding that PS1$\Delta$exon9 can undergo a presenilinase-like cleavage, although the original cleavage site within the domain encoded by exon 9 is absent. Relaxed sequence specificity is also observed for other proteases, such as the $\alpha$-secretase (61). The cleavage site of $\alpha$-secretase appears to be determined by its distance to the cell membrane (61), a characteristic phenomenon that might be very similar to the PS-cleaving enzyme. On the other hand, the observed cleavage of PS1$\Delta$exon9 might also represent a less frequently used cleavage site. CTF$_{39}$, in contrast to the uncleaved PS1$\Delta$exon9 protein (28), is not incorporated into the complex and therefore is sensitive to proteolytic degradation like NTF$_{296}$. If we assume that CTF$_{39}$ represents a free CTF$_{20}$, it appears that both PS fragments are rapidly removed by the proteasome as soon as they are not incorporated into the PS1 complex. Interestingly, both fragments are not attacked by the leupeptin-sensitive cysteine protease that degrades CTF$_{10}$ (see below), demonstrating differential degradation of PS-derived peptides.

The stable CTF$_{20}$ that is incorporated into the heterodimeric PS complex can be proteolytically attacked by caspases (data presented in this work and see Ref. 33). Caspases cleave CTF$_{20}$ and generate the alternative CTF$_{10}$ (Fig. 6). CTF$_{10}$ is then predominantly removed by a cysteine protease activity. This protease can be inhibited by LLNl, MDL28170, E64, and leupeptin but not by NH$_4$Cl. Furthermore, treatment with the proteasome-specific inhibitor, lactacystin, only stabilizes the caspase-generated fragment to a minor extent. Therefore, it is likely that CTF$_{10}$ is degraded by a calpain-like cysteine protease.

PS1 holoproteins (wt as well as PS1$\Delta$exon9) are rapidly degraded by a pathway that is sensitive to inhibitors of the proteasome similar to the PS2 holoprotein (31). Moreover, inhibition of the proteasome results in the accumulation of polyubiquitinated PS1 holoproteins (data not shown; see Ref. 55). As previously observed for the cystic fibrosis transmembrane conductance regulator protein (62), inhibition of proteasomal degradation did only partially prevent the degradation of the PS1 holoproteins. Thus, it is possible that an additional protease activity that is not inhibited by any of the compounds used in this study is involved in the degradation of PS1 holoproteins. This protease activity might be related to the recently identified proteolytic system that can compensate for the loss of proteasome function (63).

In addition to Ratovitski et al. (60), who demonstrated a long-lived PS1$\Delta$exon9 pool, we show that most of the de novo synthesized PS1$\Delta$exon9 is rapidly removed. This might again be due to the fact that excess amounts of PS1$\Delta$exon9 do not assemble into the PS1$\Delta$exon9 complex characterized previously (28). Thus, uncomplexed PS1$\Delta$exon9 holoprotein is probably unstable like the PS1 holoprotein that is not incorporated into the PS complex (28).

Interestingly, a mutant NTF$_{296}$ does not cause an increased production of Aβ42 even after its stabilization by inhibition of the proteasome. Furthermore, we have previously shown that a highly similar recombinant NTF is not sufficient to rescue the phenotype of a mutant PS-homologue (sel-12) in Caenorhabditis elegans (64). This suggests that either the PS heterodimeric complex or the full-length protein is required for the pathological and biological activity. However, in respect to the extremely low amounts of the full-length protein and its instability, we find it more likely that the heterodimeric complex (28–30, 36) is biologically and pathologically active. The fact that PS1$\Delta$exon9 is pathologically and biologically active as an uncleaved protein is explained by the recent finding that this protein but not the wt PS1 holoprotein forms a PS complex by itself (28). The recently described PS1 $\Delta$exon 4 mutation (65), which results in the production of an N-terminally truncated PS1 molecule, also does not cause the enhanced production of Aβ42. In addition, we have recently demonstrated that N-terminally truncated PS2 species derived from alternative splicing do not induce Aβ42 generation as well (66). These data again support our hypothesis that the heterodimeric complex or the full-length protein is required for the pathological activity.

Taken together, our data demonstrate that in addition to the highly balanced fragment formation, several proteolytic systems including the proteasomes, caspases, and a leupeptin-sensitive cysteine proteinase degrade PS to avoid aberrant accumulation of excess amounts of PS fragments and holoprotein (Fig. 6). Efficient removal of the PS holoprotein and its proteolytic fragments might be very important to avoid aberrant Aβ generation specifically in the light of the recent findings that the lack of PS1 expression leads to significant reduction of Aβ40 and Aβ42 generation (21). The pathological mechanism of FAD-associated mutations might therefore be closely related to the half-life of the PS precursor or its proteolytic fragments. Future work on PS processing should therefore carefully determine whether FAD-associated PS mutations exert their pathological activity due to subtle changes in PS stability.

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