Alzheimer's Disease Associated Presenilin-1 Holoprotein and Its 18−20 kDa C-Terminal Fragment Are Death Substrates for Proteases of the Caspase Family†

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ABSTRACT: Mutations in the presenilin (PS) genes are linked to early onset familial Alzheimer's disease (FAD). PS-1 proteins are proteolytically processed by an unknown protease leading to the formation of two stable fragments of ∼30 and ∼20 kDa [Thinakaran, G., et al. (1996) *Neuron 17*, 181−190]. In addition to the conventional fragments, alternative cleavage products were observed as well. Here we characterize an alternative proteolytic pathway of PS-1 which involves proteases of the caspase superfamily. Caspase mediated cleavage occurs between aspartate 345 and serine 346 C-terminal to the conventional cleavage determined previously [Podlisny, M., et al., (1997) *Neurobiol. Dis. 3*, 325−337]. Full-length PS-1 can serve as a substrate for caspase-like proteases, as demonstrated by the generation of the alternative C-terminal fragment in cells expressing PS-1 containing the Δexon 10 deletion which is known to accumulate as a full-length molecule [Thinakaran, G., et al. (1996)]. By inhibition of de novo protein synthesis in untransfected cells we demonstrate that the conventional C-terminal fragment of PS-1 is a substrate for caspase-like proteases as well. Therefore full-length and the conventional C-terminal fragment of PS-1 can serve as potential death substrates. Due to the fact that very little full-length PS-1 is expressed *in vivo*, the much more abundant C-terminal fragment and not the full-length precursor is the major *in vivo* substrate for the alternative cleavage of PS-1 by proteases of the caspase superfamily.

Alzheimer's disease (AD)[1] is characterized by the invariant accumulation of senile plaques which are composed predominantly of amyloid β-peptide (Aβ) (for review, see ref *3*). Aβ is derived from a high molecular mass precursor, the β-amyloid precursor protein (βAPP) (*4*). AD frequently occurs as a sporadic disease; however, in about 10% of the cases AD is inherited as an autosomal dominant trait (familial Alzheimer's disease (FAD) (for review, see ref *3*). FAD-causing mutations were so far found in three genes, the βAPP gene (for review, see ref 3), the PS-1 gene, and the PS-2 gene (*5*−*7*; for review see refs *8* and *9*). Mutations in all three genes cause an increased production of Aβ terminating after amino acid 42 (Aβ42) (*10*−*16*). Aβ42 is now widely believed to play a pivotal role in AD as well as FAD (*3*). Of the mutations causing FAD, those within the PS-1 gene occur most frequently (for review, see refs *8* and *9*). The cell biology as well as the pathobiology of PS proteins is therefore of great interest for the understanding of the molecular mechanisms causing AD. The two homologous PS proteins are expressed as 45−50 kDa proteins upon overexpression in cultured cells or transgenic mice (*1, 2, 17*−*19*). It appears that endogenous full-length (fl) PS is present in very small amounts, whereas large amounts of proteolytic products of the PS proteins were detected (*1, 2*). Proteolytic processing of PS-1 and PS-2 results in the accumulation of two fragments, a ∼30 kDa N-terminal fragment (NTF) and a ∼20 kDa C-terminal fragment (CTF) (*1, 2, 15, 19*). Proteolytic processing of PS-1 is known to occur within the domain encoded by exon 10, since the naturally occurring FAD-associated Δexon10 mutation (*20*) abolishes proteolytic processing (*1*). Proteolytic cleavage within that domain is highly regulated, and only small amounts of these fragments are produced even after overexpression of PS in transfected cells or transgenic mice (*1*). However, these fragments appear to be very stable and therefore accumulate *in vivo* in cultured cells and all tissues analyzed (*1, 2*).

Very little is known about the biological and pathological function of proteolytic processing. Early work suggested that FAD-associated PS-1 mutations abolish PS processing in PC12 cells (*19*). On the other hand, expression of mutant PS-1 in transgenic mice results in the hyperaccumulation of proteolytic fragments (*21*). However, proteolytic processing appears not to be affected in brains of several patients with PS-1 mutations (*2*). A functional analysis in the nematode *Caenorhabditis elegans* revealed that proteolytic processing of PS-1 is not an absolute prerequisite to rescue the mutant *sel-12* [the PS-1 homologous gene in *C. elegans* (*22*)]

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[1] Abbreviations: Aβ, amyloid β-peptide; AD, Alzheimer's disease; βAPP, β-amyloid precursor protein; CTF, C-terminal fragment; ER, endoplasmic reticulum; FAD, familial Alzheimer's disease; NTF, N-terminal fragment; PS, presenilin; STS, staurosporine; TM, transmembrane domain.

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phenotype (*23*, *24*; for review, see ref *9*). Whereas full-length PS-1 can functionally replace defective *sel-12* in *C. elegans* (*23*, *24*), it is not clear so far if proteolytic processing is required for the physiological activity of PS-1 in higher organisms.

In addition to the highly regulated proteolytic processing pathway, it appears that a large fraction of PS-2 and probably PS-1 as well is degraded by an ubiquitin-dependent pathway, which seems to involve the proteasome (*25*; for review, see ref *9*).

Besides the ~30 kDa NTF and ~20 kDa CTF, additional abundant proteolytic fragments are detected in a variety of tissues and cell lines (C. Haass and J. Grünberg, unpublished data). Podlisny et al. (*2*) described the detection of a 10−14 kDa C-terminal fragment as well as several alternative NTFs. These fragments occur not only in transfected cell lines but also in neurons and human brain tissue. We (*26*) and others (*27*) have recently described the production of alternative proteolytic fragments in primary hippocampal neurons. Generation of these fragments was found to be developmentally regulated. In fully differentiated neurons, a substantial increase of a larger NTF (*26*, *27*) and a smaller CTF was observed (*26*). Moreover, Hartmann et al. (*27*) also found similar proteolytic fragments in brain tissue from rat and humans. Therefore it appears that *in vivo* an additional, alternative proteolytic pathway might generate these fragments independent of the conventional pathway. We now characterized the proteolytic mechanism involved in the generation of the alternative fragments in detail. We found that the alternative cleavage occurs between aspartate 345 and serine 346 of PS-1, C-terminal to the originally described cleavage in the domain encoded by exon 10 (*1*, *2*). Overexpression of caspase 3 (CPP32), a protease of the caspase super family, caused an increased alternative cleavage, whereas inhibition of caspases completely blocked the generation of alternative fragments but not the generation of the conventional fragments. Taken together, our data strongly indicate that proteases which are essential for apoptosis play an important role in the generation of alternative proteolytic fragments of PS-1.

MATERIALS AND METHODS

Cell Culture. All cell lines were cultured in Dulbecco's minimal essential medium supplemented with 10% FCS. Transient transfections were carried out using DOTAP (Boehringer Mannheim). All cDNA constructs used are encoding the VRSQ motif (*5*).

Antibodies. The polyclonal antibodies 2953, 3027, and 4627 used in this study were described previously (*2*, *26*, *28*, *29*). The polyclonal antibody 4627 was raised to amino acids 457−467 of PS-1 (*2*, *28*).

The polyclonal antibody 2953 (*26*) was raised to a fusion protein containing amino acids 2−81 of PS-1 N-terminus. The respective coding region was amplified by PCR using the primer pair 5′-CCGGATCCACAGAGTTACCTGCACC-3′ and 5′-TGCGGTCGACATGCTTGGC-GCCATATT-3′. Human PS-1 (*5*) cDNA was used as a template. The PCR products were cloned into the vectors pMAL-c2 (New England Biolabs, Beverly, MA) and pGEX-4T-1 (Pharmacia, Uppsala, Sweden). The glutathione *S*-transferase (GST) and the maltose binding protein (MBP) fusion proteins were expressed in *Escherichia coli* DH5α. GST/MBP fusion proteins were purified using glutathione Sepharose-4B (Pharmacia) or amylose resin (New England Biolabs) according to the supplier's instructions. MBP fusion protein was inoculated into rabbits. The specificity was tested using the PS-1 N-terminus fused to GST as well as protein lysates from PS transfected cells (*26*).

The polyclonal antibody 3027 was raised to the loop domain (amino acids 263−407) of PS-1 (*29*). The coding region was amplified by PCR using the primers 5′-CCGAATTCTGTCCGAAAGGTCCA-3′ and 5′-CCGGATC-CCTAGGTTGTGTTCCAGTC-3′. The resulting DNA was cloned into the vectors pMAL-c2 (New England Biolabs) and pGEX-4T-1 (Pharmacia). The GST and the MBP fusion proteins were expressed in *E. coli* DH5α. GST/MBP fusion proteins were purified using glutathione Sepharose-4B (Pharmacia) or amylose resin (New England Biolabs) according to the supplier's instructions. MBP fusion protein was inoculated into rabbits. The specificity was tested using the PS-1 C-terminus fused to GST as well as protein lysates from PS transfected cells (*29*).

Immunoprecipitation. Cells were lysed in STEN buffer (50 mM Tris-HCl, pH 7.6; 150 mM NaCl; 2 mM EDTA; 0.2% NP-40) containing 1% Triton X-100 for 20 min at 4 °C, 48 h after transfection. The insoluble pellet was extracted with STEN buffer containing 1% SDS at 4 °C for 20 min. Triton X-100 and SDS lysates were centrifuged at 15 000 rpm at 4 °C for 20 min. Supernatants were carefully removed and transferred to new Eppendorf tubes. For immunoprecipitation, the SDS concentration was diluted to 0.1% with STEN lysis buffer. The immunoprecipitations were carried out as described by Haass et al. (*30*), except that the immunoprecipitates were heated to 42 °C for 15 min in sample buffer containing 8 M urea before they were separated on 10−20% Tris/Tricine polyacrylamide gels (*31*). Immunoblotting was carried out as described before (*30*). Proteins were transferred to PVDF membranes (Millipore) at 400 mA for 1 h at 4 °C in 1× electrophoresis buffer without SDS.

Site-Directed Mutagenensis. In vitro mutagenesis was carried out by PCR. For the PCR we used standard conditions with 500 ng of template and 12 amplification cycles to prevent artifacts during DNA amplification by Taq polymerase. For the first PCRs the following primer pairs were used: (1) PS1/187F (5′-CCGAATTCAAGAAAGAAC-CTCAA-3′) and D345NR (5′-CCTAGATGACTGTTC-CTCTGGGCTTC-3′), (2) D345NF (5′-GAAGCCCAGAG-GAACAGTCATCTAGG-3′) and PS1/1660R (5′-CCGGAT-CCGCAAATATGCTAGAT-3′, (3) PS1/187F and D373NR (5′-CCTTTCCTCTGGGTTTTCACCAGCGAGG-3′), (4) D373NF (5′-CCTCGCTGGTGAAAACCCAGAGGAAAGG-3′) and PS1/1660R, (5) PS1/187F and D385NR (5′-GTA-GAAAATGAAATTTCCCAATCCAAG-3′), and (6) D385NF (5′-CTTGGATTGGGAAATTTCATTTTCTAC-3′) and PS1/1660R. Human PS-1 cDNA was used as a template (*5*). After gel purification the primary PCR products were mixed (1 and 2; 3 and 4; 5 and 6) and a second PCR using the outside primers (PS1/187F and PS1/1660R) was performed. The resulting fragments were cloned into the *Eco*RI/*Bam*HI site of pSG5 (Stratagene). All cDNAs were sequenced to prove successful mutagenesis.

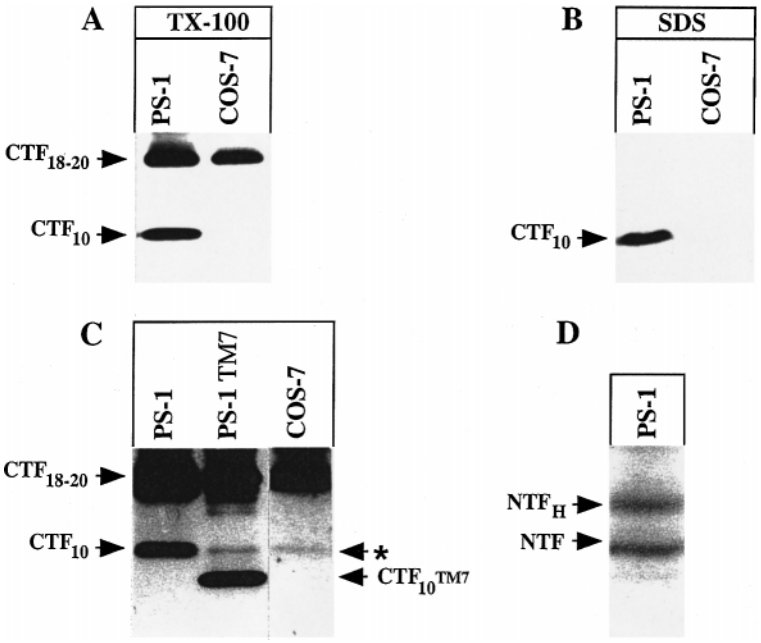

FIGURE 1: Identification of an alternative proteolytic processing pathway for PS-1. (A) PS-1 transfected or untransfected COS-7 cells were lysed in a buffer containing Triton X-100. CTFs of PS-1 were immunoprecipitated with antibody 4627 to the C-terminus of PS-1 (*2, 28*). Precipitated CTFs were detected by immunoblotting using antibody 3027 which is known to be specific for PS-1 (*29*). The alternative CTF_{10} was strongly augmented upon overexpression. Longer exposure reveals the endogenous CTF_{10} (see also 1C). (B) Same as in A except that the insoluble material from the Triton X-100 extraction was solubilized with SDS. Immunoprecipitation followed by immunoblotting reveals CTF_{10} but not CTF_{18-20} in the Triton X-100 insoluble fraction. (C) Expression of PS-1 with a stop codon inserted after TM7 (*28*) leads to the production of a smaller, truncated CTF_{10} which proves the identity of the novel fragment observed in A and B (further proof is presented by the mutagenesis of the amino acid at the P_1 position shown in Figure 3D). As a control, cell lysates from COS-7 cells and cells transfected with the PS-1 cDNA were run in parallel. Note the endogenous CTF_{10} in untransfected COS-7 cells as well as in COS-7 cells transfected with the PS-1 TM7 cDNA (arrow labeled with an asterisk). In addition the faster migrating truncated version of CTF_{18-20} is detected (additional peptide migrating just below endogenous CTF_{18-20}). Immunoprecipitation and immunoblotting were carried out with antibody 3027, since the epitope for antibody 4627 is deleted in the PS-1 TM7 recombinant protein. (D) An NTF of higher molecular mass was detected as well. Immunoprecipitated NTFs were detected by immunoblotting using antibody 2953 (*28*). In addition to the conventional 30 kDa NTF, a higher molecular mass NTF (NTF_H) which most likely represent the N-terminal cleavage product corresponding to CTF_{10} was detected.

Inhibition of Caspases. Two highly selective inhibitors were used: biotinyl-Asp-Glu-Val-aspartic acid aldehyde (Bio-DEVD-CHO, Bachem) and Ac-Tyr-Val-Ala-Asp-2,6-dimethylbenzoyloxymethyl ketone (Ac-YVAD-DMB, Bachem). Ac-YVAD-DMB is highly selective for caspase 1, 4, and 5 and Bio-DEVD-CHO for CPP32 like caspases. Inhibitors were solubilized as recommended by the manufacturer. Cells were incubated with a 170 μM solution of the corresponding inhibitor or the vehicle alone for 12 h. Cell lysates were prepared as described above. CTFs were detected by immunoprecipitation with antibody 4627 or 3027, followed by immunoblotting with antibody 3027.

Inhibition of Protein Synthesis and Staurosporine-Induced Apoptosis. Untransfected COS-7 cells were incubated in the presence of cycloheximide (Biomol; 20 mg/mL) either with or without staurosporine (Biomol; 1 mM) for 15 h. Inhibition of protein biosynthesis was controlled by determining the incorporation of [^{35}S]methionine during incubation with cycloheximide. Cells were collected by centrifugation at 800*g* for 5 min after being scraped from culture dishes and were then lysed in a buffer containing 1% SDS and 1% Triton X-100. Lysates were centrifuged at 14000*g* for 10 min, and proteolytic fragments of PS-1 were isolated and detected as described above.

RESULTS

Identification of Alternative Cleavage Products of PS-1. To analyze the generation of alternative proteolytic processing products of human PS-1 we used COS-7 cells. COS cells as well as a variety of other non-neuronal cells were used frequently to analyze the effect of FAD associated mutations on Aβ generation (*11, 14*−*16, 31*−*33*), demonstrating that these cell lines can provide extensive and invaluable information about βAPP processing and the influence of FAD associated mutations. Moreover, βAPP metabolism and the effects of βAPP and PS mutations originally sorted out in peripheral cell lines were confirmed in neuronal cells, primary cell cultures, human and mouse brain tissue, cerebrospinal fluid, and plasma (*10*−*13, 31, 34*).

COS-7 cells were transfected with the PS-1 cDNA, and cell lysates were immunoprecipitated with an antibody to the C-terminal domain of PS-1 (antibody 4627) (*2, 28*). The precipitate was separated on a 10−20% Tris/Tricine gel, transferred to PVDF membranes, and analyzed with antibody 3027 to the large loop of PS-1 (*29*). We detected the previously described (*1*) 18−20 kDa C-terminal fragments (CTF_{18-20}) (Figure 1A) which are not overexpressed upon transfection as expected (*1*). In addition to this fragment, we reproducibly obtained a smaller fragment of approximately 10 kDa (CTF_{10}) (Figure 1A). CTF_{10} was detected in the Triton X-100 soluble phase as well as in the insoluble phase extracted by SDS, whereas the CTF_{18-20} was exclusively observed in the Triton X-100 soluble fraction (Figure 1A and B). The detection of CTF_{10} in the insoluble pellet indicates that large amounts of this fragment may be overlooked using mild extraction methods. This might also

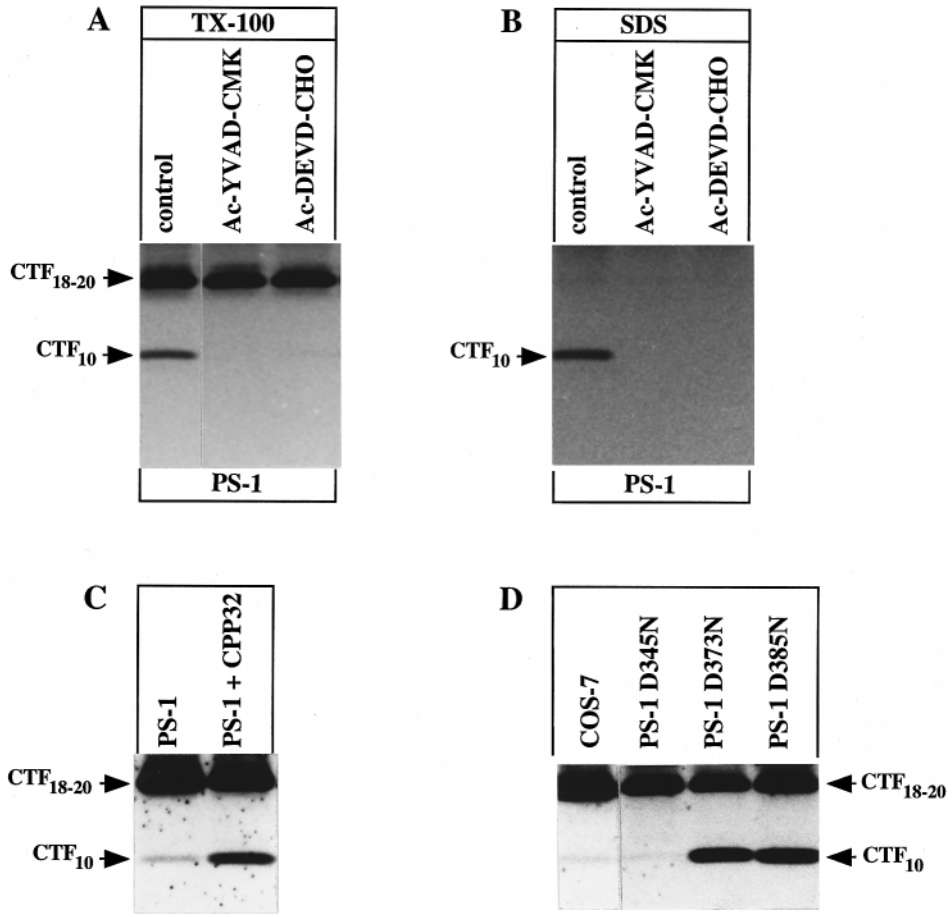

FIGURE 2: Alternative proteolytic processing involves proteases of the caspase cascade. (A and B) Two highly specific inhibitors to caspases (Ac-YVAD-DMB and Bio-DEVD-CHO) block the alternative cleavage. COS-7 cells transfected with the PS-1 cDNA were treated with and without the inhibitors for 12 h. CTFs were detected as described in Figure 1. Upon incubation with the inhibitors, CTF_{10} is not detected in the Triton X-100 (A) or SDS soluble fraction (B). (C) Overexpression of a member of the caspase protease family induces CTF_{10} production. COS-7 cells were transfected with the PS-1 cDNA alone or with the PS-1 cDNA and the cDNA encoding caspase 3 (CPP32). Upon cotransfection a marked increase of the CTF_{10} production is observed. CTFs were isolated and detected as described in Figure 1. (D) The alternative cleavage of PS-1 occurs between aspartate 345 and serine 346. COS-7 cells were transfected with PS-1 D345N, PS-1 D373N, and PS-1 D385N and CTFs were isolated as described in Figure 1. Exchanging aspartate 345 to asparagine selectively inhibits PS-1 alternative cleavage with no effect on conventional processing.

explain the slight variability in the amount of CTF_{10}, which could be due to its inefficient extraction by Triton X-100.

In contrast to CTF_{18-20}, CTF_{10} was strongly augmented in transfected cells as compared to untransfected cells (Figure 1A and B). Longer exposures also revealed endogenous CTF_{10} in untransfected COS-7 cells (Figure 1C). The endogenous CTF_{10} was also detected within the Triton X-100 insoluble pellet as well (data not shown). To further prove the identity of the novel polypeptide, we transfected a cDNA construct encoding a PS-1 protein which is deleted after trans-membrane domain (TM) 7 (PS-1 TM7) (*28*). Expression of PS-1 TM7 led to the production of a C-terminal peptide which migrated at a lower molecular mass as the CTF_{10} derived from the wt PS-1 gene (Figure 1C). The identity of the novel PS-1 fragment therefore is proven by its strong augmentation after overexpression of PS-1 as well as by an expected molecular mass shift upon expression of a C-terminal deleted recombinant PS-1 molecule (further proof is provided by the mutagenesis of the cleavage site; see Figure 2D).

In addition to the novel CTF_{10}, we detected an alternative NTF of higher molecular mass (34 kDa) (Figure 1D, NTF_H) as the conventional fragment (30 kDa) (Figure 1D NTF, see also Figure 4). In contrast to the conventional 30 kDa NTF, the 34 kDa NTF_H is also recognized by antibody 3027 (raised to the large loop), indicating that this peptide is generated by an alternative cleavage C-terminal to the conventional cleavage site (data not shown). The detection of CTF_{10} together with NTF_H strongly indicates that full-length PS-1 can serve as a substrate for alternative cleavage (see below).

PS-1 Is Cleaved by Proteases of the Caspase Superfamily between Aspartate 345 and Serine 346. Recently it was reported that PS proteins might participate in apoptosis (*35*−*37*; for review, see ref *9*). We therefore tested if proteases of the caspase family, which are known to play a fundamental role in apoptosis (*38*−*40*), might be involved in CTF_{10} generation. Caspases can be inhibited by peptide aldehydes and peptide methyl ketones, which were shown previously to block individual members of the protease family with very high specificity *in vivo* (*38*, *39*). COS-7 cells were transfected with the PS-1 cDNA and incubated in the presence or absence of the inhibitors Ac-YVAD-DMB and Bio-DEVD-CHO. Ac-YVAD-DMB is known to specifically block caspases 1, 4, and 5 (*38*−*40*). Bio-DEVD-CHO blocks CPP32 like caspases (*38*−*40*). Incubating transfected cells with Ac-YVAD-DMB or Bio-DEVD-CHO for 12 h led to a

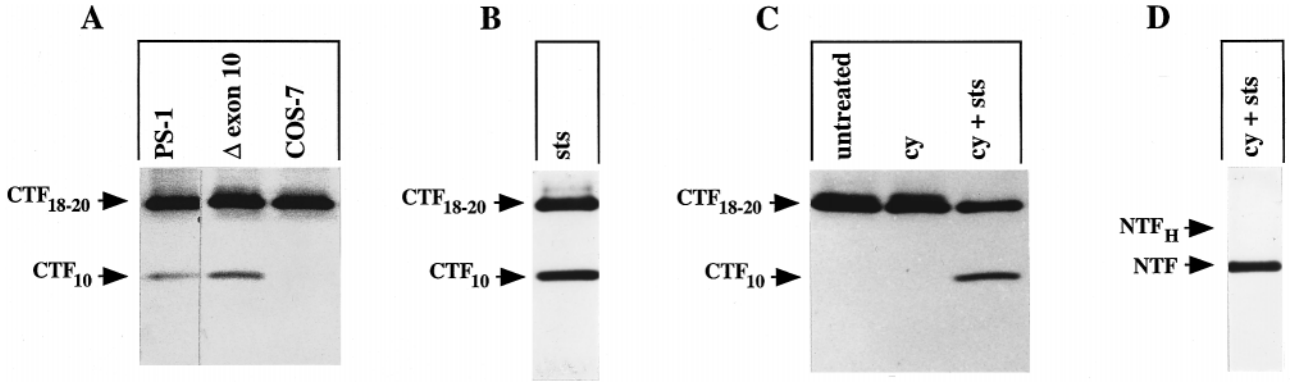

FIGURE 3: Full-length PS-1 and CTF_{18-20} can be the substrate for caspase mediated cleavage. (A) PS-1 not cleaved at the conventional site can be a substrate for caspase mediated cleavage. COS-7 cells, COS-7 cells transfected with the PS-1 cDNA, or the PS-1Δexon10 cDNA was immunoprecipitated as described in Figure 1. CTFs were detected by immunoblotting with antibody 3027. Deletion of exon 10 still allows the production of CTF_{10}. CTF_{18-20} observed in cells transfected with the PS-1Δexon10 cDNA represents the endogenous fragment (in a fraction of cells which do not overexpress PS-1). (B) Untransfected cells were treated with staurosporine (sts) to induce apoptosis. Under these conditions, high levels of CTF_{10} are produced. (C) After inhibition of de novo synthesis of PS-1 by cycloheximide (cy), staurosporine (sts) induced apoptosis still allows the generation of large amounts of CTF_{10} demonstrating caspase mediated cleavage of CTF_{18-20}. Total cell lysates from untreated cells, cells treated with cycloheximide (cy) alone (note that this has no influence on the accumulated CTF_{18-20}), and cycloximide plus staurosporine (cy + sts) were immunoprecipitated as described above. (D) Lysates from cells treated with staurosporine and cycloheximide (sts + cy) were immunoprecipitated with antibody 2953 to isolate N-terminal fragments of PS-1 as described in Figure 1. Note that only the conventional NTF is observed, whereas the higher molecular mass NTF_H is not detected (compare Figures 1D and 3D). In all experiments, cell lysates were prepared in the presence of SDS to allow quantitative extraction of CTF_{10}.

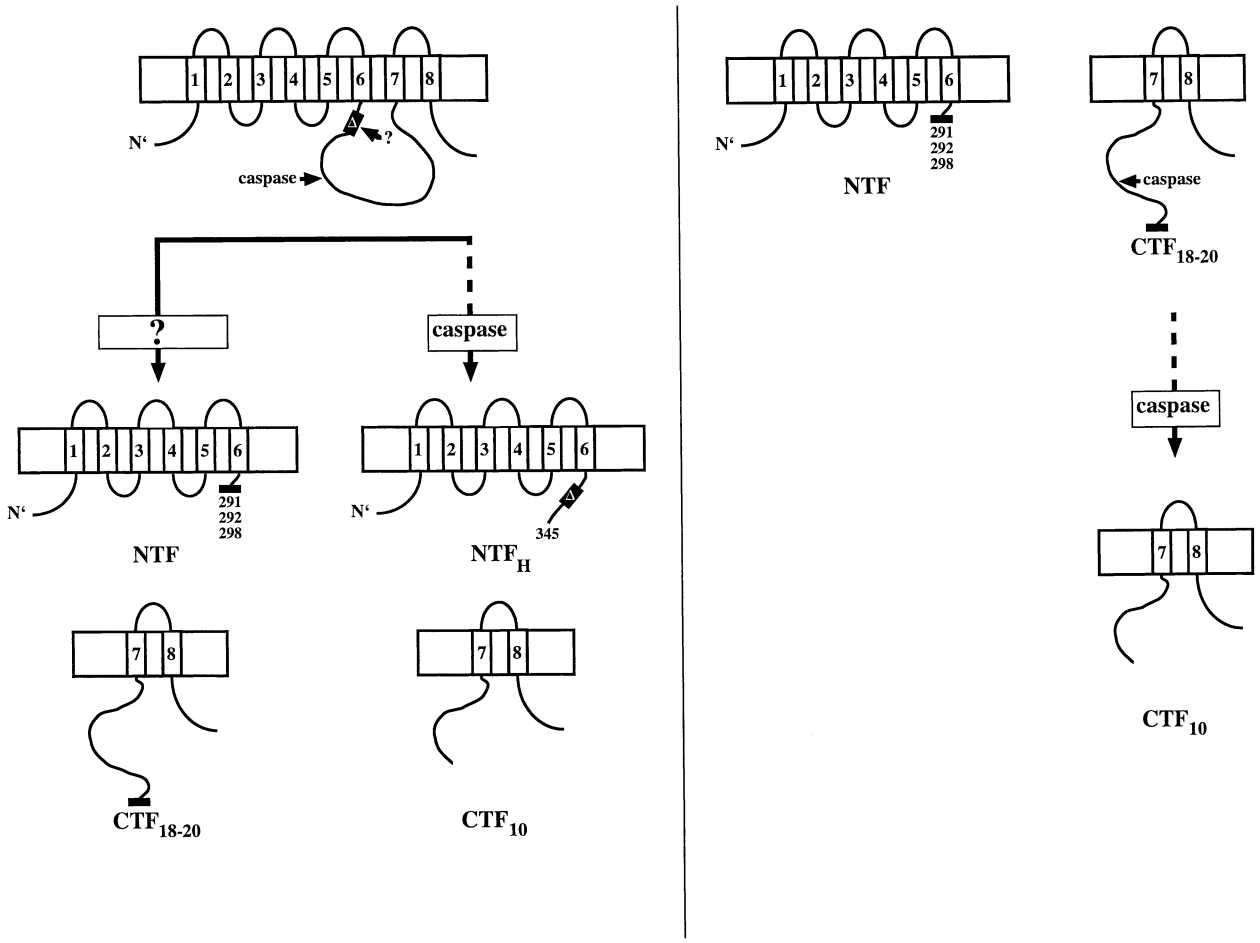

FIGURE 4: Schematic summary of proteolytic processing of PS-1. PS-1 is proteolytically processed by the conventional cleavage within exon 10 (*1*). The caspase mediated cleavage occurs between aspartate 345 and serine 346 and generates CTF_{10} and NTF_H upon cleavage of full-length PS-1 in transfected cells (left panel). Without overexpression of PS-1, CTF_{18-20} is the substrate for caspase mediated cleavage (in that case no NTF_H is generated; right panel). Δ = domain encoded by exon 10. Arrows indicate the cleavage sites of the two processing pathways.

marked inhibition of CTF_{10} generation in the soluble as well as the insoluble fraction (Figure 2A and B). Interestingly, we found that the formation of the CTF_{18-20} is not inhibited by either Ac-YVAD-DMB or Bio-DEVD-CHO (Figure 2A). This result therefore supports the notion that PS-1 can be proteolytically processed by two different pathways (see also

Figure 4). Since both inhibitors blocked production of CTF_{10}, one can assume that CTF_{10} is generated by a protease which is activated by the caspase cascade. To prove this further, COS-7 cells were cotransfected with the human PS-1 cDNA and the cDNA encoding caspase 3 (CPP32), a protease which is inhibited by Bio-DEVD-CHO and known to induce apoptosis upon overexpression (*40*). As shown in Figure 2C, coexpression of PS-1 and caspase 3 (CPP32) lead to marked augmentation of CTF_{10} production, again demonstrating that caspases are involved in alternative processing of PS-1. Similar results were obtained when apoptosis was induced in untranfected COS-7 cells by incubation with staurosporine (Figure 3B).

Caspases are known to selectively cleave substrates after aspartates. Mutagenesis of aspartate at the P_1 position of the cleavage site results in the inhibition of proteolytic cleavage (*38*−*40*). We therefore mutagenized aspartate residues at positions which upon cleavage could give rise to CTFs of the expected molecular mass. Three aspartate residues at positions 345, 373, and 385 of PS-1 were selected and mutagenized to asparagine. The corresponding cDNA clones (PS-1 D345N, PS-1 D373N, and PS-1 D385N) were transfected into COS-7 cells, and the proteolytic processing products of the respective proteins were analyzed by immunoprecipitation followed by immunoblotting. Exclusively the mutation at aspartate 345 abolished CTF_{10} formation, whereas mutations at aspartate residues 373 and 385 had no influence (Figure 2D). These results therefore clearly demonstrate that PS-1 is cleaved by proteases of the caspase superfamily between aspartate 345 and serine 346.

CTF_{18-20} Is the Major in Vivo Substrate for the Alternative Cleavage. To learn if the full-length PS-1 molecule by itself can indeed serve as a substrate for the caspase cleavage, we transfected COS-7 cells with a cDNA encoding the PS-1 Δexon 10 mutation (*20*). This naturally occurring FAD-associated mutation is known to inhibit the conventional PS-1 cleavage due to the lack of the recognition sequence for the corresponding protease between amino acids 290 and 319 (*1*). As shown in Figure 3A, expression of PS-1 Δexon 10 does not inhibit the generation of CTF_{10}, demonstrating that PS-1 molecules which are not cleaved at the conventional site can serve as a substrate for the caspase mediated cleavage. CTF_{18-20} observed in cells transfected with the PS-1Δexon10 cDNA represents the endogenous fragment (in a fraction of cells which do not overexpress PS-1) (*1, 29*). In cells stably transfected with the PS-1Δexon10 cDNA the endogenous CTF_{18-20} is significantly reduced (*1, 29*); however, production of CTF_{10} is still observed (data not shown).

Since very little full-length PS-1 can be detected *in vivo* (*1, 2, 28, 29, 41*), we analyzed if the endogenous CTF_{18-20} can serve as a substrate for the caspase cleavage as well. For this purpose we used untransfected COS-7 cells. As with any other cell line analyzed so far, very little if any endogenous full-length PS-1 has been detected in these cells (*1, 2, 28, 29, 41*). To induce apoptosis, COS-7 cells were incubated with staurosporine (*40*). As shown in Figure 3B, incubation with staurosporine results in a robust generation of CTF_{10}. This result indicates that CTF_{18-20} might be the substrate for the caspase mediated cleavage in untransfected cells since very little full-length PS-1 is available (*1, 2, 28, 29, 41*). To prove this possibility directly we inhibited de novo synthesis of endogenous PS-1 by incubating cells with cycloheximide. Under these conditions equivalent amounts of CTF_{18-20} can still be detected (Figure 3C). This is consistent with the previous finding that CTF_{18-20} is very stable over long time periods (*1, 2*). As shown in Figure 3C, CTF_{10} is still produced during co-incubation of cells with cycloheximide and staurosporine. Moreover, the reduced levels of CTF_{18-20} and the correspondingly increased levels of CTF_{10} emphasize a precursor product relationship. If CTF_{18-20} is the *in vivo* substrate for caspase mediated cleavage, one should not detect NTF_H in untransfected cells upon stimulation of apoptosis (Figure 1D). We therefore isolated N-terminal PS-1 fragments from cells incubated with staurosporine and cycloheximide. This results in the identification of robust amounts of the conventional PS-1 NTF, while no NTF_H could be observed (compare Figures 3D and 1D). These data demonstrate that CTF_{18-20}, not full-length PS-1, is the major *in vivo* substrate for the caspase mediated cleavage. This is further supported by the fact that *in vivo* very little full-length PS-1 can be detected, whereas the conventional fragments (NTF and CTF_{18-20}) accumulate at high levels (*1, 2, 28, 29, 41*).

DISCUSSION

The results presented in this paper demonstrate that PS-1 proteins can be proteolytically processed by an alternative mechanism observed in a variety of cell lines such as COS-7, U373, and human kidney 293 cells (Figures 1−3, and data not shown). Therefore at least two different pathways influence the fate of full-length PS proteins (Figure 4, left panel). Conventional proteolytic processing occurs within the domain encoded by exon 10 leading to the production of the previously described fragments (*1*). Besides this pathway, an alternative pathway exists *in vivo*, which is characterized by a single proteolytic cleavage between aspartate 345 and serine 346. This pathway can be induced by apoptosis as demonstrated by overexpression of caspase 3 (CPP32) and induction of apoptosis by staurosporine.

In vivo, where very little full-length PS-1 can be detected (*1, 2, 28, 29, 41*), CTF_{18-20} is the major substrate for the caspase mediated cleavage (Figure 4, right panel). This is supported by our finding that induction of apoptosis in untransfected cells induces the production of high levels of CTF_{10}, although very little if any full-length PS-1 is detected. Moreover, inhibition of de novo synthesis of PS-1 still allows efficient cleavage of the accumulated CTF_{18-20}. The latter occurs without generation of NTF_H therefore again demonstrating that *in vivo* CTF_{18-20} is the substrate for caspase mediated cleavage (Figure 4, right panel).

Interestingly, alternative cleavage of PS-1 is blocked by two highly selective inhibitors of caspases, proteases which are known to be dependent on aspartate residues at the P_1 position. Moreover, overexpression of caspase 3 (CPP32) caused an increased production of the alternative C-terminal fragment. Our data therefore demonstrate that proteases of the caspase family are involved in the generation of the alternative fragments of PS-1. However, it seems unlikely that caspase 3 (CPP32) is the protease which cleaves PS-1 directly, since the sequence of the PS-1 cleavage site (AQRD) is not homologous to the consensus sequence of caspase 3 [(CPP32) DXXD (*40*)]. We therefore suggest that

another member of the caspase cascade, which is activated by overexpressed caspase 3 (CPP32), is involved in alternative cleavage. Currently 10 different caspases of three subgroups are known, all of them induce apoptosis upon overexpression (*40*). Members of the caspase protease family are activated by sequential proteolytic cleavage. Finally, a member of the caspase family or other proteases are cleaving their death substrates such as lamin, actin, poly(ADP)ribose polymerase, rho-GDI, SREBP, and DNA dependent protein kinase which then results in the morphological changes typical for programmed cell death (*40*). Therefore, PS-1 represents a novel death substrate, most likely for one of the proteases of the caspase superfamily. During the time this manuscript was prepared, similar data were obtained for alternative cleavage of PS-2 as well (*42*, *43*). Both PS proteins therefore represent novel death substrates. It is also very likely that the conventional C-terminal fragment of PS-2 is the *in vivo* death substrate for alternative cleavage, similar to the situation for PS-1. Our data therefore indicate that induction of apoptosis results in the rapid cleavage of CTF_{18-20} by caspase-like proteases *in vivo*.

If it indeed turns out that the conventional fragments are the pathologically and functionally active form of PS (*21*), caspase mediated cleavage would result in the immediate inactivation of processed PS.

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