Alzheimer’s disease is characterized by neurodegeneration and deposition of βA4, a peptide that is proteolytically released from the amyloid precursor protein (APP). Missense mutations in the genes coding for APP and for the polytopic membrane proteins presenilin (PS) 1 and PS2 have been linked to familial forms of early-onset Alzheimer’s disease. Overexpression of presenilins, especially that of PS2, induces increased susceptibility for apoptosis that is even more pronounced in cells expressing presenilin mutants. Additionally, presenilins themselves are targets for activated caspases in apoptotic cells. When we analyzed APP in COS-7 cells overexpressing PS2, we observed proteolytic processing close to the APP carboxyl terminus. Proteolytic conversion was increased in the presence of PS2-I, which encodes one of the known PS2 pathogenic mutations. The same proteolytic processing occurred in cells treated with chemical inducers of apoptosis, suggesting a participation of activated caspases in the carboxy-terminal truncation of APP. This was confirmed by showing that specific caspase inhibitors blocked the apoptotic conversion of APP. Sequence analysis of the APP cytosolic domain revealed a consensus motif for group III caspases (IVL)ExD. Mutation of the corresponding cytosolic domain revealed a consensus motif for group II/III caspases (IVL)ExD). Mutation of the corresponding Asp residues abolished cleavage, thereby identifying APP as a target molecule for caspase-like proteases in the pathways of programmed cellular death.

Neurodegeneration in selected brain regions is one of the pathological features of Alzheimer’s disease, together with amyloid deposition in extracellular plaques, congophilic angiopathy, and intracellular tangles. Amyloid deposited in senile plaques is mainly composed of βA4, a peptide consisting of 40–42 residues, which derives by proteolytic processing from its cognate precursor protein termed APP1 (1). Missense mutations in the APP gene cause a rare form of early-onset familial Alzheimer’s disease (FAD) by raising the levels of total βA4 or by increasing the ratio of the longer form of βA4 that ends at residue 42 to the form ending at residue 40 (2–5). Additionally, the expression of APP carrying those mutations has been reported to induce apoptosis in cells, suggesting a probable link between apoptotic pathways and neurodegeneration in Alzheimer’s disease (6–8). Mutations causative for the majority of FAD cases have been identified in genes encoding presenilin (PS) 1 and PS2 (9–11). Missense mutations in these genes were also found to alter APP processing in a pathological manner by increasing the relative concentration of βA4 ending at residue 42 (12–16). Both proteins are endoproteolytically converted by two alternative pathways: (i) in the first pathway, cleavage occurs within a highly hydrophobic region of the presenilins (16–18), and (ii) in the second pathway, presenilins become converted by caspase-3 family proteases at about 30 residues distal to the normal cleavage site (19–20). The corresponding caspase-derived fragments have been detected in all tissues and stages of animal development, suggesting a role of PS processing in the cellular response to apoptotic signals (19, 21). Furthermore, overexpression of PS2 in transfected cells has been shown to increase susceptibility to apoptotic cell death. The latter is even more pronounced in cells expressing mutant PS2-I that encodes one of the known PS2 missense mutations changing Asn141 into Ile (22, 23).

When we analyzed carboxyl-terminal fragments of APP in cells coexpressing PS2 or PS2-I, we observed proteolytic processing of APP within the cytoplasmic domain that was augmented for mutated PS2-I (see below). To determine whether this result could be attributed to apoptotic pathways and whether APP proteolysis was mediated by caspases, cells expressing full-length APP and amino-terminal-truncated fragments were analyzed after the induction of apoptosis with different compounds including staurosporine and doxorubicin. We report here that proteolytic conversion of APP within its cytoplasmic domain is increased in apoptotic cells and is inhibited by the addition of specific caspase inhibitors. These results suggest that APP is a target for caspase-like proteases and is involved in apoptotic pathways.

**EXPERIMENTAL PROCEDURES**

The cloning of PS2 and construction of mutant PS2-I have been described previously (24). The point mutations in the cytoplasmic domain of SPA4CT (25) (SPA4CT<sub>PS2a</sub> and SPA4CT<sub>PS2del664</sub>) were introduced by polymerase chain reaction and cloned into pBluescript SK+ (Stratagene). The open reading frames of the resulting constructs were verified by sequencing, followed by cloning into the expression vector pHIV-1

acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Bis-Tris, bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane.
processing of APP by caspases

induction of apoptosis is accompanied by proteolytic processing of SPA4CT and APP within the cytoplasmic domain—structurally, APP represents a type I integral membrane protein with a large ectodomain, a transmembrane domain, and a short cytoplasmic tail (Fig. 1; numbered according to Ref. 1). In COS-7 cells stably transfected with APP, immunoprecipitated APP was detected as a band of 12 kDa (Fig. 2, lane 3). This band was not recognized, suggesting that the APP carboxyl terminus was cleaved in apoptotic cells (Fig. 2, lanes 6–8).

For further analysis, the last 103 carboxyl-terminal residues of APP, which extend from the amino terminus of βA4 to the very end of the APP cytoplasmic domain and mimic the β-secretase carboxyl-terminal product (Fig. 1, A4CT), were expressed in COS-7 cells. This amino-terminal-truncated derivative of APP allowed a more accurate investigation of the site of cleavage because of the lower molecular weight and the higher resolution of the corresponding peptide on SDS gels. To ensure correct membrane insertion, a signal peptide was fused to the amino terminus (SPA4CT (25)). After metabolic labeling of the cells, the expressed peptide was immunoprecipitated with mAb W02, which is directed to βA4 residues 1–16 (26). The antibody detected SPA4CT as a band of 12 kDa (Fig. 2, lane 3), a result consistent with earlier studies (25). Additionally, a second band of about 8 kDa was detected in nonapoptotic cells after immunoprecipitation with this antibody, suggesting that this band represents a carboxyl-terminal-truncated species lacking the last 20–30 residues of the APP sequence as deduced from the differences in the relative molecular masses. Treatment of cells with staurosporine resulted in a strong increase in the relative intensity of the 8-kDa species (Fig. 2B, lane 2), suggesting elevated processing of SPA4CT within its cytoplasmic domain in apoptotic cells.

cosexpression of PS2 and PS2-I with the SPA4CT fragment stimulates cleavage within the APP cytoplasmic domain—because overexpression of PS2 has been reported to stimulate apoptotic pathways in cells, we analyzed the conversion of SPA4CT in cells that were cotransfected with a PS2-encoding expression plasmid. Additionally, PS2-I was coexpressed, which encodes one of the two known missense mutations (Asn141 to Ile) within the PS2 gene causative for early-onset Alzheimer’s disease (10, 11). Expression of PS2 and PS2-I was monitored by immunoprecipitating lyases from radiolabeled cells with anti-PS2 antisera, as described previously (Ref. 24; data not shown).

In the first experiment, both PS2 and SPA4CT DNA-encoding plasmids were transiently cotransfected in an equal ratio. Here, the relative intensity of the 8-kDa band compared with the 12-kDa band was increased (Fig. 2C, lane 2). A similar result was obtained with the cells cotransfected with PS2-I (lane 3). In a second experiment, PS2 cDNA and SPA4CT cDNA at a ratio of 0.1:1 were expressed in the cells. Under these conditions, mutant PS2-I was still able to induce an increased conversion of SPA4CT into the 8-kDa species.
lanes 1 containing media ($\text{SPA4CT cDNA}$) (lane 8) treated for 8 h with 2 $\mu$M staurosporine and doxorubicin (Fig. 3A). After induction with staurosporine for 9 h, about 50% of SPA4CT was converted into the 8-kDa fragment (lane 2). Incubation for 30 h caused a decrease in the intensities of both SPA4CT and the 8-kDa fragment, a result attributed to the lower synthesis of SPA4CT in dying cells (lane 3). A similar precursor-product relationship between SPA4CT and its 8-kDa fragment was observed in doxorubicin-treated cells (lanes 4–6).

To determine whether a caspase was involved in the cleavage of the APP cytoplasmic domain under apoptotic conditions, a subset of caspase inhibitors was used such as a caspase-1-type inhibitor (YVAD-CHO), a caspase-3-type inhibitor (DEVD-CHO), a caspase-8-type inhibitor (IETD-CHO), and a broad spectrum inhibitor (zVAD-fmk). Of the three tetrapeptide inhibitors, only IETD-CHO showed a clear inhibition of the conversion of SPA4CT into the 8-kDa species (Fig. 3B, lanes 3–5). zVAD-fmk (lane 6) was the most potent inhibitor, probably because it can block all caspase activity by also inhibiting the upstream caspases in the proteolytic activation cascade, whereas IETD-CHO, due to its relative specificity, blocks the total activity of the group III caspases less effectively. Therefore, zVAD-fmk was then tested at lower concentrations. Reduced proteolytic conversion of SPA4CT into the 8-kDa fragment was observed at concentrations ranging between 3.3 and 100 $\mu$M (Fig. 3C). This result again demonstrates the participation of activated caspases in the apoptotic processing of APP.

In Vitro Conversion of the APP Carboxyl Terminal Domain by Recombinant, Activated Caspase-8—To directly demonstrate the ability of activated caspases to cleave the APP carboxyl terminus, SPA4CT immunoprecipitated from radioactively labeled COS-7 cells was incubated with purified, recombinant caspase-3 and caspase-8 (Fig. 3D). Incubation with buffer alone was used as a control and showed no cleavage (lane 1). Incubation with caspase-8 resulted in the formation of two fragments of about 8 and 6 kDa (lane 3). After a 30-min incubation, a 75% conversion of SPA4CT was observed in the presence of caspase-8 (lane 3), whereas a conversion of about only 20% occurred in the presence of caspase-3 (lane 2). The lower molecular mass band apparently represents the APP 31-residue carboxyl-terminal fragment resulting from caspase cleavage. The rate of the caspase-8-mediated conversion is in the same order of magnitude as that described for the in vitro cleavage of Bid, a proximal substrate of caspase-8 in the Fas and TNF apoptotic signaling pathway (28, 29). This result proves formally that activated caspases, especially caspase-8, are capable of cleaving the APP cytoplasmic domain.

Characterization of the APP Caspase Cleavage Site by Site-directed Mutagenesis—Examination of the cytoplasmic domain of APP revealed the presence of a consensus sequence for caspases: the sequence $\text{VEVD}$ (numbered according to APP$^{695}$; Ref. 1) matches the sequence known for group III caspase-6, -8, and -9 ((IVL)ExD) (30–32). To test whether the observed proteolytic processing of the APP cytoplasmic domain occurs at residue Asp$^{664}$, two constructs were generated by polymerase chain reaction: in one clone, Asp$^{664}$ was mutated into Ala (SPA4CT$^{D664A}$), whereas in the other clone, a stop codon was introduced after Asp 664, thereby terminating translation after this residue (SPA4CT$^{del664}$). Both constructs were expressed in cells, followed by analysis with anti-β4 antibody (Fig. 4A). In contrast to nonmutated SPA4CT (lanes 1 and 2), a single band was observed for the D664A mutant (lane 3) even

Proteolytic Conversion of SPA4CT upon Treatment by Apoptosis Inducers Is Time Dependent and Is Inhibited by the Caspase Inhibitors IETD-CHO and zVAD-fmk—To further characterize the proteolytic processing of the APP cytoplasmic domain, pulse-chase experiments were performed in the presence of staurosporine and doxorubicin (Fig. 3A). Before the induction of cell death, almost all SPA4CT was detected as a 12-kDa species (lanes 1 and 4). After induction with staurosporine for 9 h, about 50% of SPA4CT was converted into the 8-kDa fragment (lane 2). Incubation for 30 h caused a decrease in the intensities of both SPA4CT and the 8-kDa fragment, a result attributed to the lower synthesis of SPA4CT in dying cells (lane 3). A similar precursor-product relationship between SPA4CT and its 8-kDa fragment was observed in doxorubicin-treated cells (lanes 4–6).

In contrast, cells cotransfected with low amounts of wild-type PS2 showed a pattern indistinguishable from that of the cells expressing solely SPA4CT (lane 4). This finding is consistent with reports that cells that overexpress mutant PS2-I are more prone to undergo apoptosis than cells expressing wild-type PS2 (22, 23).

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after the induction of apoptosis with staurosporine (lane 4), indicating that the single point mutation completely inhibited proteolytic conversion. The faster migration of SPA4CTD/A on SDS gels compared with SPA4CT is most likely due to the removal of the negatively charged Asp664 residue, resulting in an altered mobility on SDS-PAGE. A similar increase in mobility has been also observed for other proteins in which the critical Asp residue was mutated into Ala (33). The electrophoretic mobility of the deletion mutant SPA4CTdel664 was identical to the 8-kDa fragment generated from wild-type SPA4CT (lane 5). The faster electrophoretic mobility of SPA4CTD/A (lanes 3 and 4) is likely to be due to the removal of the negatively charged Asp from SPA4CT. Analysis of the corresponding APP derivatives. Cells were stably transfected with APP695 (lanes 1–4), APP695D/A (lanes 5–8), and APPdel664 (lanes 9–12) and treated with either staurosporine (lanes 3, 7, and 11) or doxorubicin (lanes 4, 8, and 12). Total cellular homogenates were subjected to SDS-PAGE, followed by immunoblotting with mAb 22C11. The N-glycosylated moiety of APPdel664 (lanes 9–12) exhibits about the same electrophoretic mobility as the caspase-derived fragments at about 90 kDa, as detected in lanes 3 and 4. The additional signals of about 95 kDa in APPdel664-expressing cells represent posttranslationally higher glycosylated APP species.

**Fig. 4. APP and SPA4CT derivatives mutated at Asp664.** 
A. cells were transfected with SPA4CT (lanes 1 and 2), SPA4CTD/A (lanes 3 and 4), and SPA4CTdel664 (lane 5). The deletion construct SPA4CTdel664 (lane 5) displays the same molecular mass on SDS-PAGE as the 8-kDa proteolytic fragment generated from SPA4CT (lane 2). No additional fragment was observed for SPA4CTD/A, even in the presence of staurosporine (lane 4). The faster electrophoretic mobility of SPA4CTD/A (lanes 3 and 4) is likely to be due to the removal of the negatively charged Asp from SPA4CT. B. Analysis of the corresponding APP derivatives. Cells were stably transfected with APP695 (lanes 1–4), APP695D/A (lanes 5–8), and APPdel664 (lanes 9–12) and treated with either staurosporine (lanes 3, 7, and 11) or doxorubicin (lanes 4, 8, and 12). Total cellular homogenates were subjected to SDS-PAGE, followed by immunoblotting with mAb 22C11. The N-glycosylated moiety of APPdel664 (lanes 9–12) exhibits about the same electrophoretic mobility as the caspase-derived fragments at about 90 kDa, as detected in lanes 3 and 4. The additional signals of about 95 kDa in APPdel664-expressing cells represent posttranslationally higher glycosylated APP species.

**Fig. 3. Proteolytic conversion of SPA4CT in apoptotic stimulated cells: time course and inhibition by caspase inhibitors.** A, SPA4CT-transfected COS-7 cells were treated with either staurosporine (2 μM, lanes 1–3) or doxorubicin (100 ng/ml; lanes 4–6) for the time periods indicated. Total cellular proteins were fractionated by SDS-PAGE and analyzed by immunoblotting with mAb W02. B, inhibition of apoptotic conversion by different caspase inhibitors. Transfected cells were incubated for 8 h in the presence of staurosporine (2 μM) together with various caspase inhibitors (100 μM). Only IETD-CHO and zVAD-fmk displayed an inhibitory effect on the conversion of SPA4CT. C, inhibition of proteolytic conversion by zVAD-fmk is concentration dependent. Cells were treated with either 100 ng/ml doxorubicin (lanes 1–5) or 2 μM staurosporine (lanes 6–10) for 8 h in the presence of different zVAD-fmk concentrations, as indicated. The relative conversion of SPA4CT was calculated after densitometric analysis and is indicated for each lane. D, in vitro cleavage of SPA4CT by recombinant caspases. SPA4CT was immunoprecipitated from transfected, 35S-labeled COS-7 cells and incubated for 30 min either without caspases (lane 1) or with recombinant caspase-3 (lane 2) or caspase-8 (lane 3). The reaction products were separated by Tris-Bis-Tris PAGE and detected by phosphorimaging. The rate of the conversion was calculated after correction for the [35S]methionine residues encoded by SPA4CT (4 residues) and the proteolytic fragments (respective 2 residues).
COS-7 cells, displayed different populations in flow cytometric analysis, making a clear assertion difficult, we used Jurkat T cells that are well established for studying apoptotic mechanisms. Jurkat cells endogenously express several alternatively spliced isoforms of APP (including APP695 and APP-KPI, lane 1). The identity of the detected bands as APP isoforms was confirmed not only by immunoprecipitation or immunoblotting with different anti-APP antisera, but also by electroporation of APP-encoding plasmids, which resulted in an increase of the band intensities (data not shown). To investigate the time course of APP cleavage in relation to the cell apoptotic status, Jurkat cells were treated with staurosporine for 3, 6, and 9 h, followed by APP immunoblotting and parallel Annexin V-based flow cytometry (Fig. 5A, C and B, respectively). After 3 h of induction, 30% of total APP695 and APP-KPI were detected as carboxyl-terminal-truncated derivatives as deduced from the small shift in molecular mass observed, which is consistent with a loss of a 6-kDa carboxyl-terminal fragment (Fig. 5A, lane 2). After 6 and 9 h, most of the APP was detected as caspase-cleaved species (lanes 3 and 4). The corresponding flow cytometric analysis revealed that 3 h after the induction of apoptosis, about 66% of the cells were stained with Annexin V (Fig. 5B). Because cleavage of APP parallels the flipping of phosphatidylserine, we conclude that proteolysis of APP occurs before gross changes in cell morphology become detectable.

**DISCUSSION**

To date, evidence is accumulating that links apoptotic pathways to neurodegeneration: (i) apoptotic cell death has been reported to be a pathological feature of Alzheimer’s disease, as determined by histochemical studies (34–37). (ii) Similarly, Guo et al. (38) have recently shown that the levels of apoptotic mediator proteins like Par-4 are increased in the neurons of Alzheimer’s disease brains, suggesting the involvement of apoptotic processes in neurodegeneration in vivo. (iii) Expression of mutant APP carrying FAD mutations has been reported to induce apoptosis, indicating that apoptosis may contribute to the neuronal loss in FAD (6–8). (iv) Overexpression of PS2 in neuronal and non-neuronal cells was found to enhance apoptosis, whereas transfection with a PS2 antisense construct rescued cells from apoptosis (22, 23, 39). Inhibitory effects on apoptosis were also observed when a carboxyl-terminal portion of PS2 was expressed in cells (39, 40). Additionally, the PS2 N141I FAD mutation was reported to confer enhanced basal activity for apoptosis (22, 41) similarly to the L286V mutation in PS1 (42). (v) Both PS1 and PS2 have been shown to be targets for caspase-mediated proteolytic conversion (19–21). The mutant PS2-I might be even converted to a higher extent as compared with the wild-type PS2 (20). The corresponding PS fragments derived by caspase-mediated proteolysis were detected in all tissues and stages of animal development (19, 21), suggesting a role of PS processing in the cellular response to apoptotic signals.

In this study, we present evidence that APP can be proteolytically processed within its cytoplasmic domain by a caspase-like protease. The involvement of caspase activity in APP processing was supported by the following findings: (i) the induction of cellular apoptosis by chemical agents such as staurosporine and doxorubicin, which are known to activate the caspase cascade, or by the expression of PS2 and PS2-L resulted in an increased proteolytic conversion (Fig. 2). (ii) APP matches the consensus sequence for group III caspases (Fig. 1). (iii) Mutation at the cleavage site of this canonical sequence completely abolished proteolytic conversion (Fig. 4). (iv) Proteolysis is inhibited by a caspase-specific inhibitor in a concentration-dependent manner (Fig. 3, B and C). (v) Activated caspase-8 recognizes APP in vitro as a target molecule (Fig. 3D). Taken together, the data strongly suggest a participation of caspase-like proteases in the conversion of the APP cytoplasmic domain.

The caspase inhibitor profile shown here differs from that observed for presenilins; the peptide inhibitor DEVD-CHO blocked the proteolytic conversion of PS1 and PS2, suggesting the participation of a caspase-3-like protease (19, 20). As shown in Fig. 3B, neither this inhibitor nor VVAD-CHO, which is specific for caspase-1-like proteases, was effective at blocking the proteolytic conversion of APP, making the involvement of group I and group II caspases unlikely. However, zVAD-fmk was able to completely inhibit the conversion of APP. zVAD-fmk is a broad range inhibitor that blocks the activation of all caspases including caspase-6, caspase-8, and caspase-9 belonging to group III that recognize the consensus sequence (D-Val-Leu-Asp-D-Val-Asp) (30–32). Such a motif is encoded within the APP carboxyl terminus at residues 661–664 (VEVD; Fig. 1), making APP a likely substrate for group III caspases. The latter con...
clusion is also supported by two findings: (i) IETD-CHO, a more specific inhibitor for group III caspases, was effective in blocking the conversion of APP (Fig. 3B, lane 5); and (ii) the APP carboxyl-terminal domain was more efficiently cleaved by a group III caspase (caspase-8) than a group II caspase (caspase-3) in vitro (Fig. 3D). However, it seems likely that other group III caspases, for example, effector caspses such as caspase-6, contribute to the in vivo conversion of the APP carboxy terminus in cells.

Recent findings suggest that presenilins are targets for caspases and that PS2 is capable of inducing programmed cell death. To study the PS2-induced activation of caspases, both SPα4CT and PS2 were coexpressed (Fig. 2C). The observed difference between PS2- and PS2-1-transfected cells on the proteolytic conversion of SPα4CT is consistent with the observation that cells overexpressing mutant PS2-I are more prone to undergo apoptosis than wild-type PS2-expressing cells (20, 22, 23, 41). Because APP and PS2 are known to interact and form stable complexes (24, 43, 44), an alternative interpretation could be that the carboxyl-terminal fragment of APP displays a different binding capacity to PS2 versus PS2-1, causing the observed dissimilarities. Such a difference was not observed; both PS2 and PS2-1 were equally able to form stable complexes with SPα4CT (data not shown). However, it remains to be tested whether PS2 exerts its effects on the apoptotic processing of APP as a APP/PS2 complex and whether the PS mutations alter cellular routing or processing of APP in a pathological manner. This could be accomplished by an analysis of cells that do not express endogenously encoded PS.

Two functional motifs have been described within the cytoplasmic domain of APP: (i) a consensus sequence for internalization, and (ii) a site for binding G proteins (Fig. 1). Removal of the internalization signal from the APP carboxyl terminus results in an elevated secretion of APP into the extracellular space (45). In agreement with these reports, increased levels of secretory APP are observed in the conditioned media of APPΔ664-transfected cells (data not shown).

As mentioned above, FAD mutations in the APP gene were found to induce cellular apoptosis. Recently, this was shown to be dependent on a short stretch within the cystolic domain of APP: deletion of residues His657-Lys676 of APPΔ664 abolished the apoptotic cell death provoked by FAD mutations. The same site has been shown to be required for the induction of apoptosis by G-proteins (7, 46). Correspondingly, a synthetic peptide comprising His657-Lys676 activates G0 in vivo (47). The ability of APP to activate G0 is dependent on the continuous primary structure: synthetic peptides lacking the carboxyl-terminal part Arg672-Lys676 were several times less potent in activating G0 than peptides spanning the whole region from His657-Lys676 (46). Because the proteolytic conversion described here takes place at position Arg664 the carboxyl-terminal part of the activating domain becomes cleaved in apoptotic cells, which then results in an inability of APP to further activate G0 proteins. Such a regulatory mechanism would permit interruption of apoptotic stimuli and control of apoptosis. The observation that cleavage of APP occurs very early in the course of apoptosis (Fig. 5) may support such a hypothesis. Indeed, several mechanisms have been discovered in which an initial apoptotic stimulus does not cause cellular death but is controlled by antiapoptotic proteins including members of the Bcl family, activation of nuclear factor κB, or XIAP (reviewed recently in Ref. 48). The same mechanism has also been proposed for PS2, which may either induce or increase apoptosis but simultaneously serves as a target for caspases too (19, 20, 39, 40). Thus, apoptosis might be closely linked with neurodegeneration not only in neuronal diseases such as amyotrophic lateral sclerosis (49) or Huntington’s disease (50) but also in Alzheimer’s disease.

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