Alternative, Non-secretase Processing of Alzheimer’s β-Amyloid Precursor Protein during Apoptosis by Caspase-6 and -8*

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Alzheimer’s disease (AD), 1 a progressive neurodegenerative disorder of later life, is characterized by deposition of β-amyloid plaques, accumulation of intracellular neurofibrillary tangles, and neuronal cell loss (1). It is widely believed that this disease is caused by the extracellular accumulation of the aggregated amyloidogenic form of Aβ peptide (Aβ1–42). This peptide arises from the processing of β-amyloid precursor protein (APP) by still unknown proteases (secretases) (2). The recent disorder of later life, is characterized by deposition of APP by caspase cleavage of APP, respectively; Aβ, amyloidogenic form of APP, from which Aβ arises by proteolysis, are associated with some forms of familial AD (FAD) and result in increased Aβ production. Two other FAD genes, presenilin-1 and -2, have also been shown to regulate Aβ production; however, studies examining the biological role of these FAD genes suggest an alternative theory for the pathogenesis of AD. In fact, all three genes have been shown to regulate programmed cell death, hinting at the possibility that dysregulation of apoptosis plays a primary role in causing neuronal loss in AD. In an attempt to reconcile these two hypotheses, we investigated APP processing during apoptosis and found that APP is processed by the cell death proteases caspase-6 and -8. APP is cleaved by caspases in the intracellular portion of the protein, in a site distinct from those processed by secretases. Moreover, it represents a general effect of apoptosis, because it occurs during cell death induced by several stimuli both in T cells and in neuronal cells.

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‡ The abbreviations used are: AD, Alzheimer’s disease; FAD, familial Alzheimer’s disease; APP, β-amyloid precursor protein; PCD, programmed cell death; PS, presenilin; APPNcas and APPPcas: NH2-terminal and COOH-terminal fragments resulting from caspase cleavage of APP, respectively; Aβ, β-amyloid peptide; PARP, poly(ADP-ribose) polymerase; PIPES, 1,4-piperazineethanesulfonic acid; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.

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RESULTS

Alternative, Non-secretase APP Processing during Apoptosis—To determine whether increased APP processing and Aβ production was a general phenomenon associated with apoptosis, we studied APP processing during Fas-induced cell death in Jurkat T cells. To this end, Jurkat cells were transfected with a vector expressing APP and subjected to metabolic labeling with [35S]methionine. Two h later, cells were treated with a cytotoxic anti-Fas antibody (CH-11) for 3 h. Cell lysates and cell culture supernatants were immunoprecipitated with either the 22C11 monoclonal antibody or C7 antiserum, specific for an N-terminal epitope of APP and is commercially available (Roche Molecular Biochemicals). Anti-APP Antibodies—Regions of APP recognized by the antibodies used in this study are depicted in Fig. 1. R1155 and C7 are rabbit polyclonal antiserum raised to a synthetic polypeptide of amino acids 649–664 and 676–695 of APP (numbering according to Kang et al. (Ref. 25), respectively (described by Selkoe et al. (Ref. 26) and Podlisny et al. (Ref. 27)). The monoclonal 22C11 antibody recognizes an N-terminal epitope of APP and is commercially available (Roche Molecular Biochemicals).

In Vivo Protein Labeling—Twenty-four h after transfection, Jurkat cells were washed once with methionine-free RPMI medium supplemented with 10% dialyzed fetal bovine serum (Life Technologies, Inc.) and incubated in the same medium for 40 min at 37 °C. [35S]-Labeled methionine (Amersham Pharmacia Biotech) (200 μCi) was then added in each transfection, and labeling continued for 2 h before the addition, where necessary, of anti-Fas.

Apoptosis Studies—Apoptosis was triggered in Jurkat cells 24 h after transfection by direct stimulation of the Fas molecule. Cells were supplied with fresh medium containing a monoclonal human anti-Fas antibody CH-11 (Upstate Biotechnologies, Inc.) at 0.1 μg/ml, incubated for the indicated time at 37 °C, and harvested. Alternatively, in Jurkat and N2A cells, apoptosis was stimulated with 250 ng/ml staurosporine (Sigma) for 6 to 10 h. The block of caspase-3 activity in Jurkat cells was obtained by incubating cells for 30 min with 50 μM ZDEVD-fmk, an irreversible caspase inhibitor (Enzyme System, Livermore, CA) prior to anti-Fas addition. Fifty μM mock inhibitor ZFA-fmk (Enzyme System) was used as a control. Of note, caspase-3 activity is required for caspase-6 activation (28).

Immunoprecipitation and Immunoblot Analysis—Cells were harvested, washed once in ice-cold phosphate-buffered saline, and lysed in lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, pH 7.6) containing the protease inhibitors 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, aprotenin, pepstatin, and leupeptin (Sigma). Lysate was spun at 10,000 × g for 10 min, and the supernatant recovered. Antibody was added and the immunoprecipitation reaction incubated at 4 °C for 12 h with gentle rocking. Immunocomplexes were captured by addition of protein A/G-agarose beads (Pierce) and incubation for 2 h at room temperature. Beads were washed three times in wash buffer I (50 mM Tris, 500 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40, pH 7.6), washed three times in wash buffer II (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40, 0.1% SDS, pH 7.6), and resuspended in 100 μl of Laemmli loading buffer. Samples were heated at 95 °C for 2 min and separated on a polyacrylamide-SDS gel (Novex Experimental Technology). For detection of in vitro labeled proteins, gels were fixed, enhanced, vacuum-dried, and exposed to x-ray films at −80 °C using intensifying screens. Unlabeled proteins were blotted onto nitrocellulose membranes and probed with the specified antibodies. Immunoblots were developed using the SuperSignal system (Pierce).

In Vitro Cleavage Assay—[3H]Leucine- or [35S]methionine-labeled proteins were made using a TNT-coupled transcription and translation system (Promega, Madison, WI). Caspase cleavage reactions were performed at 37 °C in 25 μl of caspase buffer (20 mM PIPES, 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, pH 7.2) containing 0.5 μg of recombinant caspase (Pharmingen).

Fig. 1. APP undergoes an alternative, non-secretase processing during apoptosis. A, schematic representation of APP (numbering is based on the 695-amino acid isoform of human APP). The extracellular, transmembrane (TM, shown in black), and intracellular portions of the molecule are indicated. The cleavage sites for β-, α-, and γ-secretase and caspases (cas.) are indicated by arrows. The caspase consensus cleavage sequences present in the cytoplasmic tail (VEVD(A,95)), are marked. The regions recognized by the antibodies used in this study (22C11, R1155, and C7) are apo-specified. B, Jurkat cells were transfected with APP. Sixteen h after transfection, cells were metabolically labeled with [35S]methionine. Two h later, half of the cells were induced to die with 100 ng/ml anti-Fas monoclonal antibody CH-11. After another 3 h, cells were lysed and culture medium was recovered. In vivo metabolically labeled APP was immunoprecipitated from both cell lysates and culture medium (not shown), gel separated, and revealed by autoradiography. Full-length APP (f.L.) is precipitated by the C7 antiseraum only from the untreated sample (−). The 22C11 monoclonal specifically recognizes a smaller APP polypeptide (APPNcas) in the anti-Fas-stimulated sample (+). The multiple smaller bands observed in this immunoprecipitation with the 22C11 antibody are not recognized by this same reagent in western blot experiments (see Figs. 2A, 2B, and 3A), and are therefore likely to be aspecific. C, Jurkat cells were transfected with APP and treated the following day with anti-Fas antibody (CH-11). Cell lysates, prepared 6 h after the indicated treatments, were immunoprecipitated with either the C7 or R1155 antiseraum, gel separated, blotted, and probed with the 22C11 antibody. Western blot analysis shows that APPNcas is precipitated only by the R1155 antiseraum and possesses a lower molecular mass than APPf.L (68.9 kDa versus 83.2 kDa). Data shown in this figure were confirmed by three independent experiments. Bands of higher molecular mass than the full-length APP (see also Figs. 2, 3, and 5) represent posttranslationally modified APP species, derived from O-glycosylation, phosphorylation, and sulfation events occurring during APP trafficking in the cell (45).
left panel). On the other hand, the anti-NH₂-terminal monoclonal antibody 22C11 revealed the presence of an APP polypeptide of lower molecular weight, termed APPNCas (NCas), in lysates from cells undergoing apoptosis (Fig. 1B, right panel). In addition, the 22C11 monoclonal antibody did not precipitate any soluble APP proteins in the Fas-stimulated sample (data not shown). Together, these data indicate that the COOH-terminal region of APP containing the C7 epitope is cleaved off during apoptosis and that the NH₂-terminal APP fragment, APPNCas, remains associated with the cells.

The apoptotic processing of APP appears therefore to be distinct from that mediated by secretases, because the NH₂-terminal fragments resulting from these activities are usually released extracellularly. Alternatively, PCD could selectively enhance secretase processing of APP localized in intracellular compartments such as Golgi apparatus and endoplasmic reticulum. NH₂-terminal APP fragments generated by secretases in these compartments remain associated with the cells. To distinguish between these two possibilities, Jurkat cells were transfected with APP and treated with CH-11 the following day. After 6 h, cell lysates were immunoprecipitated with either C7 or R1155 antiserum. The latter is specific for an intracellular APP transcript. Immunoprecipitates were resolved by gel electrophoresis and probed with 22C11. As shown in Fig. 1C, the APPNCas molecule generated during PCD was immunoprecipitated by the R1155 but not by the C7 antiserum. Thus, APP is processed during PCD between the C7 epitope and the γ-secretase cleavage site.

APP Processing Is a General Phenomenon during Apoptosis and Requires Caspase Activity—Implementation of programmed cell death requires activation of caspases. These cysteine proteases are present in the cell as proenzymes and are activated by proteolysis. Active caspases execute cell death by cleaving intracellular substrates (29, 30). Thus, progression of apoptosis can be monitored by assessing the processing of caspases and their substrates. The 113-kDa protein PARP is one such substrate and is cleaved into two polypeptides of 89 and 24 kDa (31). Fas stimulation of APP-transfected cells induced apoptosis as shown by proteolytic activation of caspase-3 and cleavage of PARP (Fig. 2A, top two panels, second lane). Concomitantly, cleavage of APP and appearance of APPNCas was also detected (Fig. 2A, top panel). Thus, cleavage of APP correlates with activation of caspases.

To determine whether APP processing during apoptosis was dependent on caspase activity, transfected cells were treated with either an irreversible caspase inhibitor (ZDEVD-fmk) or with a control molecule (ZFA-fmk) prior to apoptosis induction. While ZFA-fmk had no effect, ZDEVD-fmk completely inhibited caspase-3 activation and, consequently, PARP cleavage (Fig. 2A, two bottom panels; compare third and fourth lanes). Consistently, inhibition of caspase activity resulted in suppression of the apoptotic processing of APP (Fig. 2A, top panel, fourth lane). Thus, cleavage of APP during PCD requires caspase activity.

To demonstrate that the apoptotic processing of APP is not specific to Fas-induced cell death but, rather, is common to apoptotic pathways initiated by several different stimuli, we treated Jurkat cells with staurosporine or ceramide, two other inducers of PCD. Both staurosporine (Fig. 2B) and ceramide (data not shown) resulted in the apoptotic processing of APP.

APP Is Cleaved by Caspases during Apoptosis between Asp664 and Ala665—In looking at the amino acid sequence of APP, we noticed a putative caspase consensus cleavage sequence (Val661-Glu-Val-Asp664) (32–34) (Fig. 1A). This sequence is present in the cytoplasmic tail of APP, and cleavage at the predicted Asp664-Ala665 site would give rise to an NH₂-terminal segment compatible in size with the APPNCas. Caspases have an absolute requirement for aspartic acid at position P1 of their substrates (32–34). To address whether APP was cleaved at this site by caspases during apoptosis, an APP mutant was made in which position P1 (Asp664) was substituted with an asparagine (mutant D664N). As a control, another aspartate present in the extracellular portion of APP was mutated (mutant D620H). Jurkat cells were transfected with these two mutants, and apoptosis was induced with the anti-Fas antibody CH-11. While mutation of Asp620 did not affect the processing of APP, mutant D664N was completely resistant to cleavage (Fig. 3A, top panel). Of note, caspases were normally activated in these samples, as demonstrated by caspase-3 and PARP processing (Fig. 3A, two bottom panels). Identical results were also observed using an alternative approach. Transfected Jurkat cells were metabolically labeled, and APP was immunoprecipitated with the C7 antiserum or the 22C11 monoclonal antibody. As shown in Fig. 3B, APPNCas was specifically immunoprecipitated with the C7 antiserum but not with the C7. Instead, mutation at the Asp664 completely abolished cleavage of APP by caspases. Together, these data indicate that APP is a direct substrate for caspases during PCD and that processing occurs at the Asp664-Ala665 site of its cytoplasmic domain. Of interest, the APPCAs fragment generated in apoptotic cells is probably short-lived, because it could not be detected even when lysates from 4 × 10⁷ cells taken at different time points after apoptosis triggering were analyzed by immunoprecipitation (data not shown).

APP Is Preferentially Cleaved by Caspase-6 and -8—Apoptosis progresses through a sequential activation of caspases. Triggering of Fas, for example, induces recruitment of caspase-8 to its intracytoplasmic tail followed by activation of this protease (35, 36). Active caspase-8 activates, either directly or indirectly, effector caspases such as caspase-3 and caspase-6. To test whether APP is directly cleaved by any of these caspases, we tested recombinant caspase-3, -8, and -6 in an in vitro cleavage assay using in vitro-translated recombinant APP protein as a substrate. As shown in Fig. 4A and
recently by others (37, 38), APP was cleaved by caspase-3 and -8. However, both caspase-6 and -8 appeared to be more efficient in processing APP, as observed by the amount of the 13 kDa fragment immunoprecipitated with the C7 antiserum (APPc7; Fig. 4A). Thus, unlike PS-1 and PS-2, which are specific substrates of caspase-3 (18–20), APP in vivo might be preferentially cleaved by caspase-6 and -8. This conclusion is further substantiated by dose titration assays. In these experiments, caspase-3 cleaved more efficiently its established target PARP than APP, while caspase-6 showed the inverse specificity (Fig. 4C).

The size of the APPc7 fragment is compatible with processing occurring at the Asp664-Ala665 site. This supposition was indirectly confirmed by the fact that in vitro transcription/translation of a construct encoding a polypeptide spanning from Ala665 to the COOH terminus of APP (APPc7c) generated an APP polypeptide that co-migrated with APPc7 (Fig. 4A, last lane). However, to directly confirm this possibility, we produced recombinant-labeled APP D664N and D620H proteins and analyzed their susceptibility to caspase cleavage. As shown in Fig. 4B, while mutant D620H maintained APP sensitivity to caspase-8 processing, mutant D664N was resistant to it.

Caspase Processing of APP during Neuronal Cell Death—APP plays a central role in the etiological pathogenesis of both sporadic and familial forms of AD. Because this disease is
characterized by extensive neuronal loss, it was important to determine whether apoptotic processing of APP also occurred during neuronal cell death. To address this question, we used the mouse neuronal cell line N2A and induced PCD by treating these cells with staurosporine. Triggering of apoptosis resulted in a molecular shift of endogenous APP (Fig. 5, compare first and second lanes), similar to that observed for transfected APP in apoptotic Jurkat cells. Moreover, APPNcas, which could not be immunoprecipitated with the C7 antiserum (Fig. 5, fourth lane), was pulled down by the R1155 antiserum (Fig. 5, sixth lane). Thus, endogenous APP is readily and completely processed by caspases during neuronal apoptosis.

**DISCUSSION**

In the present study, we have demonstrated that APP is processed during apoptosis. Apoptotic cleavage of APP is common to death pathways initiated in diverse cell types and by various stimuli. The proteolytic proteolysis of APP is distinct from that mediated by secretases. In fact, while secretases cleave APP either in the extracellular (β- and α-secretases) or in the transmembranous (γ-secretase) regions of the protein (2), APP processing in apoptotic cells occurs in the intracytoplasmic tail. The intracellular portion of APP contains a canonical cleavage site for caspases. Using specific caspase inhibitors together with mutagenesis studies and an in vitro cleavage assay, we have shown that APP is a direct and specific substrate of caspase-6 and, to a lesser extent, caspase-3.

Caspases are the mammalian homologues of the cell death Caenorhabditis elegans gene CED3. CED3 is essential for the execution of PCD in this nematode. Likewise, the proteolytic activities of caspases are required to complete the apoptotic program in mammalian cells. The proapoptotic function of these enzymes is mediated by processing of a number of intracellular substrates. Upon caspase-mediated cleavage, some endogenous proteins acquire proapoptotic activity, such as DFF/ICAD (39, 40), Bid (41), and procaspases themselves. Antinfective proteins, such as Bcl-2, can also be processed by caspases, and this cleavage inactivates their protective function and results in the generation of proapoptotic polypeptides (42). Caspase cleavage has also been shown to generate a negative feedback signal. Processing of PS-2 by caspase-3 inactivates the proapoptotic function of PS-2 and generates a dominant negative inhibitor of apoptosis, PS2Cas (21). Whether caspase-mediated cleavage of APP inactivates its proapoptotic role or, on the contrary, mediates the function of APP in cell death is currently under investigation in the laboratory.

Two neuronal proteins, FE65 and X11, have been shown to bind to the cytoplasmic region of APP (43, 44). Interestingly, the YENPTY motif of APP responsible for modulating this interaction is located COOH-terminal to the VEV6 caspase cleavage site (43). It should then be concluded that the APP-cas fragment that originates from caspase cleavage does not bind to these two proteins. Therefore, upon caspase cleavage of APP the functional role of the APP/X11 and APP/FE65 interaction is likely to be lost. Thus, one possible immediate effect of caspase cleavage of APP is to abrogate its binding to X11 and FE65 and the functional consequences of these interactions. Whether APPNcas retains the ability to bind to X11 and/or FE65 and whether this putative interaction affects the progression of the apoptotic process remains to be determined.

Because AD is characterized by neuronal cell loss, one possibility is that the disease might be caused by dysregulation of PCD in neuronal cells. Studies regarding the biological functions of the three FAD genes have generated data supporting this hypothesis and lead to a model where even a slight imbalance in favor of apoptosis could eventually progress to neuronal cell loss seen in FAD. In fact, all three genes have been shown to positively regulate cell death, and FAD mutations generate molecules that enhance the sensitivity of cells to apoptotic stimuli (12–18). Moreover, all three FAD genes are acted on by the cell death proteases. The data we present here, together with the findings that apoptosis can enhance amyloidogenic processing of APP (22), raise the important possibility that cleavage of APP by caspases could regulate Aβ production. This link between apoptosis and production of amyloidogenic forms of Aβ could be relevant to the pathogenesis of AD. A correct understanding of the causative processes of AD represents an extremely important step in the identification of effective and much-needed therapeutic agents.

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