Apoptotic Cleavage of Scaffold Attachment Factor A (SAF-A) by Caspase-3 Occurs at a Noncanonical Cleavage Site*

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Members of the caspase family of cysteine proteases play essential roles in the disintegration of cellular architecture during apoptosis. Caspases have been grouped into subfamilies according to their preferred cleavage sites, with the “apoptotic executioner” caspase-3 as the prototype of DEXD-dependent proteases. We show here that caspase-3 is more tolerant to variations of the cleavage site than previously anticipated and present an example of a noncanonical recognition site that is efficiently cleaved by caspase-3 

in vitro and in vivo. The new cleavage site was identified in human scaffold attachment factor A, one of the major scaffold attachment region DNA-binding proteins of human cells thought to be involved in nuclear architecture by fastening chromatin loops to a proteinaceous nuclear scaffold, the so-called nuclear matrix or scaffold. Using an amino-terminal recombinant construct of scaffold attachment factor A and recombinant caspase-3, we have mapped the cleavage site by matrix-assisted laser desorption ionization/time of flight mass spectrometry and Edman sequencing. We find that cleavage occurs after Asp-100 in a sequence context (SALD) that does not conform to the hitherto accepted DEXD consensus sequence of caspase-3. A point mutation, D100A, abrogates cleavage by recombinant caspase-3 in vitro and during apoptosis in vivo, confirming SALD as a novel caspase-3 cleavage site.

Apoptosis, or programmed cell death, is an active process of cellular self-destruction involved in normal development as well as in the maintenance of tissue homeostasis (reviewed in Ref. 1). Cells undergoing apoptosis show distinct morphological changes, including membrane blebbing, cytoplasmic and nuclear condensation, chromatin aggregation, and internucleosomal cleavage of DNA. In the final stages, the dying cells become fragmented into “apoptotic bodies,” which are rapidly eliminated by phagocytic cells without eliciting significant inflammatory damage to surrounding cells. Many of the typical changes observed in cells undergoing apoptosis have been attributed to the limited proteolysis of a number of key substrates by a family of well conserved proteases, the caspases (reviewed in Refs. 2 and 3). Caspases are present in almost all eukaryotic cells as inactive zymogens that become activated after exposure to a variety of stimuli that lead to apoptosis. Activation occurs by one of two mechanisms, autocatalytic processing induced by their aggregation and/or cleavage by other caspases (2). Based on their substrate specificity, caspases have been grouped into three subfamilies (4). Group I caspases (e.g. caspase 1, 4, and 5) prefer the tetrapeptide sequence WEHD and have been implicated mainly in inflammatory processes rather than in apoptosis. Group II (e.g. caspases 2, 3, and 7) and group III (e.g. caspases 6, 8, 9, and 10) caspases have the recognition consensus sequence DEXD and (I/L/V)EXD, respectively, and are involved in apoptosis. The different caspases have also been grouped by function into apoptotic initiators (caspase-2, 8, 9, and 10), apoptotic executioners (caspase-3, 6, and 7), and cytokine processors (caspases 1, 4, 5, 11, 12, 13, 14). Apoptotic initiators such as caspase-8 directly interact with death receptors through their amino-terminal death effector domains or caspase recruitment domains, resulting in an activation of the apoptotic pathway. Apoptotic executioners like caspase-3 are considered the downstream effector proteases responsible for the proteolysis of key substrates later in apoptosis. Caspase-3 has been shown to be required for morphological changes associated with apoptosis as well as for DNA fragmentation by the caspase-activated DNase (also known as DNA fragmentation factor) (5, 6). In vivo, caspase 3 appears to be essential for brain development but dispensable for apoptosis in other organs (7). In cell culture, the natural caspase-3 “knock-out” cell line MCF-7 (6, 8) or caspase-3-deficient lymphocytes (9) are able to die upon apoptotic stimuli, but do not display DNA fragmentation, nuclear breakdown, or membrane blebbing. To understand the molecular basis of the apoptotic process, it is therefore necessary to identify the key substrates that must be cleaved to commit the cell to die and to elucidate the substrate specificities of caspases in vivo.

We have focused on morphological and biochemical changes that are associated with one of the most dramatic reactions in apoptosis, the decomposition of the internal architecture of the cell nucleus. During this work, we have recently found that scaffold attachment factor A (SAF-A) is a target in apoptotic nuclear breakdown (10, 11). SAF-A is an abundant component of the nuclear scaffold (nuclear matrix) and also occurs in heterogeneous nuclear ribonucleoprotein complexes. Our previous findings have implicated the protein in nuclear organization due to its high binding specificity toward architectural DNA elements in the genome, the so-called scaffold or matrix attachment regions (12). Indeed, we and others have shown that SAF-A is one of the major scaffold attachment region binding proteins in human cells that appears to fasten the base of chromatin loops to the nuclear scaffold (13–17). During apoptosis, but not necrosis, SAF-A is cleaved by a single cut in the amino-terminal third of the protein, resulting in a loss of DNA

†The abbreviations used are: SAF-A, scaffold attachment factor A; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight; MS, mass spectrometry; CHO, Chinese hamster ovary.
binding activity and a concomitant detachment of the protein from the nuclear scaffold (10). Thus, cleavage of SAF-A might be linked to critical alterations in nuclear chromatin that occur before caspase-activated DNase-catalyzed cleavage of DNA into oligonucleosomal fragments. Cleavage of SAF-A occurs after the formation of high molecular weight DNA fragments but before DNA laddering and can be blocked by peptide inhibitors of caspases (10). These results suggested that cleavage of SAF-A might be catalyzed by a downstream caspase, although no caspase recognition consensus sequence is evident in the primary structure of the protein. To clarify this point, we have decided to identify the protease responsible for SAF-A cleavage and to precisely map the cleavage site on the protein.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfection, and Apoptosis Induction—**Jurkat cells were grown in very low endotoxin-RPMI 1640 medium (Biochrom, Berlin, Germany) containing 10% fetal calf serum and COS7 and MCF-7 cells in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 0.6 μg/ml insulin (for MCF-7 cells only). All cells were kept at 37 °C in a humidified atmosphere and passaged every 3 days by 5-fold dilution into fresh medium. Cells were transfected with expression vectors using the SuperFect reagent (Qiagen) as recommended by the manufacturer.

For induction of apoptosis, Jurkat cells were pelleted for 5 min at 190 × g and washed twice in serum-free medium before the addition of anti-CD95 antibody (CH-11, Immunotech, final concentration 100 ng/ml). Apoptotic cells were harvested 4 h later. COS7 and MCF-7 cells were induced to die apoptotically by incubation with staurosporine at 1 μM for 3 h by centrifugation (60,000 × g for 3 h). The resin was washed with 0.1 M Tris (pH 8.0, 0.2% Triton X-100, 150 μg/ml lysozyme), frozen overnight, and lysed by thawing slowly. NaCl and imidazole were added to 500 and 5 mM, respectively, the bacterial DNA was sheared by sonication, and the lysate was cleared by centrifugation (10,000 × g, 30 min). The supernatant, containing active caspase-3, was mixed with 1 μl of nickel nitrotriacyclicarate (Agose) and incubated on a rocking plate at 4 °C for 1 h. The resin was washed with 10 volumes of buffer A (50 mM Tris (pH 8.0), 10 mM mercaptoethanol, and 0.25 M potassium phosphate (pH 7.4) and bound protein was eluted with a linear gradient from 0 to 10 M imidazole in buffer B. Active fractions were made 40% in glycerol and 50% in diethiothreitol and stored at −20 °C. The yield was approximately 0.5 mg from 500 ml of bacterial culture, with an average specific activity of 3.6 × 10^6 unit/mg as determined by the fluorometric caspase-3 assay described below. Note that caspase-3 was cloned as a the 32-kDa precursor but was recovered from expressing bacteria as the processed, active form, presumably because of autocatalytic proteolysis.

**Fluorometric Caspase-3 Activity Assay—**Caspase-3 activity was assayed by DEVD-7-amido-4-trifluoro-methylcoumarin cleavage as described (19). The assay was carried out in microtiter plates with a substrate concentration of 40 μM and 3 μg of recombinant caspase-3. Cleavage of DEVD-7-amido-4-trifluoro-methylcoumarin was followed in caspase-3 reaction buffer (50 mM HEPES, 10 mM diethiothreitol, 1% sucrose, 0.1% CHAPS) over a period of 30 min at 37 °C with λ390 nm and λex = 390 nm, and the activity was calibrated with 7-amido-4-trifluoro-methylcoumarin standard solutions. One unit was defined as formation of 1 pmol of 7-amido-4-trifluoro-methylcoumarin/min.

**Cleavage Site Mapping—**For cleavage site mapping by MALDI-TOF mass spectrometry (20), 10 μg of recombinant ZZ-N247 protein (10) was incubated with 50 ng (180 units) of purified caspase-3 for 2 h at 37 °C in caspase-3 reaction buffer (see above) in a total volume of 10 μl. One-tenth of the cleavage reaction was mixed with 1 μl of c-eyano-4-hydroxycinnamic acid (Alrich; dissolved in acetonitrile:water 2:1, 0.1% trifluoroacetic acid) and allowed to dry on a MALDI-TOF target plate. The target was washed with 0.1% trifluoroacetic acid three times before an additional 1 μl of c-eyano-4-hydroxycinnamic acid was applied and allowed to dry. MALDI-MS analysis was performed with a Bruker-Biflex linear mass spectrometer (Bruker-Franzen) equipped with an OSM VSL 337 nitrogen laser (λ = 337 nm, bandwidth 0.1 nm) and pulses of 3 ns. Instrumental conditions of data acquisition and evaluation were as described previously (21).

For cleavage site mapping by amino acid sequencing, 50 μg of purified recombinant ZZ-N247 was incubated with 200 ng (720 units) of purified caspase-3 for 2 h at 37 °C. Cleavage products were separated on a 15% SDS-polyacrylamide gel (22) and transferred to a polyvinylidenefluoride membrane using the buffer system described by Mat-sudaïra (23). The membrane was stained with Coomassie Brilliant Blue, and the two fragments were cut out for sequencing in an automated amino acid sequencer (Applied Biosystems, Model 477A).

**RESULTS**

We have previously described SAF-A as cleaved during apoptosis in Jurkat leukemic T-lymphocytes exposed to anti-CD95 (Fas, Apo-1) antibodies (10). In this well established model system for apoptosis, caspase-3 is known to be the major effector protease responsible for cleavage of most “death substrate” proteins (24). Indeed, cleavage of SAF-A can be blocked by DEVD-CHO (10), a peptide inhibitor of caspase-3 and caspase-3 like activities (25). Our findings suggested that...
caspase-3 might also be responsible for cleavage of SAF-A, even though an amino acid motif conforming to the accepted caspase-3 consensus DEXD (or the weaker DXXD) sequence is not present in the primary structure of SAF-A. To investigate this point, we have cloned, expressed, and purified human recombinant caspase-3 (Fig. 1A, right panel) and performed in vitro cleavage assays with purified native SAF-A from human cells. Indeed, incubation of SAF-A with small amounts of recombinant caspase-3 resulted in cleavage of SAF-A to yield a fragment indistinguishable by SDS-polyacrylamide gel electrophoresis from that found in apoptotic cells (Fig. 1B, upper panel). However, this cleavage was completely blocked in the alanine-containing mutated protein (Fig. 1B, lower panel). Thus, SAF-A can be cleaved by caspase-3 in vitro. To confirm this cleavage, we repeated the in vitro cleavage assays with a recombinant fusion protein from the amino terminus of SAF-A (ZZ-N247, see Ref. 10 for details). We found that this model substrate could be cleaved by caspase-3 (Fig. 1B, left panel) and that cleavage could be counteracted by the caspase-3 inhibitor DEVD-CHO (Fig. 1B, right panel), both in a concentration-dependent manner. These titration experiments show that SAF-A is cleaved by recombinant pure caspase-3 at concentrations comparable with estimated cellular concentrations, reflecting very efficient proteolysis in vitro. Thus, cleavage of SAF-A does not result from unphysiological high concentrations of caspase-3. We concluded that SAF-A is cleaved by caspase-3 at a hitherto unknown cleavage site and that the amino-terminal sequences AQHDEAVD (8 cycles) and ARIKQMLEENGAAAG (14 cycles), respectively. These results unequivocally identified the caspase-3 cleavage site in SAF-A to Asp-100 of the tetrapeptide sequence SALD. This cleavage site has not been observed in other caspase-3 substrates before nor does it conform to the presumed DEXD consensus. In addition, searches of the SWISSPROT and TrEMBL data bases gave no indication of a preferred occurrence of the SALD sequence in other known protein substrates of caspase-3, suggesting that it is not possible to deduce caspase cleavage sites from protein sequence information alone.

Precise mapping of the cleavage site enabled us to introduce a point mutation to confirm that Asp-100 is indeed the site cleaved by caspase-3 in vitro and possibly also in vivo. To this end, we constructed a clone encoding full-length SAF-A with a single amino acid exchange D100A. The corresponding protein as well as wild type controls in two different vectors were produced and radioactively labeled by coupled in vitro transcription/translation and subjected to recombinant caspase-3. Fig. 3 demonstrates that, at least in vitro, cleavage of SAF-A was completely blocked in the alanine-containing mutated protein but not in wild type SAF-A.

To investigate the in vivo relevance of our cleavage site mapping, we transfected a wild type and a mutated form of carboxyl-terminal myc-tagged SAF-A into COS7 cells. These cells are quite resistant to many apoptotic stimuli including 0.5 μM staurosporine, which is sufficient to induce apoptosis in many other cell types. However, staurosporine at 10 μM was effective in producing >80% apoptotic cells after 24 h (Fig. 4A). When applied to cells transfected with SAF-A expression clones, we found that the D100A mutant, but not the transfected wild-type, was completely resistant to cleavage (Fig. 4B, upper panel). In the same samples, endogenous SAF-A was

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cleaved equally in all cells transfected with either enhanced green fluorescent protein (a control to estimate transfection efficiency and to rule out general transfection-related problems) or wild type or mutated SAF-A. Thus, the cleavage site mapped by using a recombinant model substrate and recombinant caspase-3 in vitro is identical to the cleavage site used in vivo.

The experiments described above clearly show that SAF-A is cleaved by caspase-3 at aspartate 100 both in vitro and in vivo using a cleavage site (SALD) that does not conform to the caspase-3 consensus DEXD.

FIG. 3. The point mutation D100A abrogates cleavage by recombinant caspase-3 in vitro. Wild type SAF-A (wt) and point mutated (D100A) SAF-A were synthesized and radioactively labeled by in vitro transcription/translation from the SAF-A cDNA cloned in pBluescript or pCMV-Tag1 (control) and incubated with 10 ng (30 units) of recombinant caspase-3 for 1 h at 37 °C (+ caspase 3). Samples were resolved on a 10% SDS-polyacrylamide gel, and labeled proteins were visualized by fluorography. Full-length SAF-A and the cleavage product SAF-A* are indicated.

DISCUSSION

In the present study, we have identified the protease responsible for cleavage of SAF-A during apoptosis and precisely mapped the cleavage site on the protein. We demonstrate that caspase-3 cleaves SAF-A at aspartate 100 both in vitro and in vivo using a cleavage site (SALD) that does not conform to the caspase-3 consensus DEXD.

Apoptotic cell death is associated with a variety of morphological and biochemical changes that are independent from the initiating death signal (27). One of the hallmarks of apoptosis is the decomposition of the cell nucleus induced by proteolytic cleavage of key nuclear proteins by caspases and degradation of DNA to oligonucleosomal pieces (28). As in the case of structural changes in the cytoplasm, the identification of nuclear caspase substrates has provided first insights into how a limited number of cuts in critical target proteins might cause these changes (for a recent review, see Ref. 2). Proteolysis of lamins (29, 30), NuMa (31–33), and SAF-A (10) appears to promote the destruction of the peripheral nuclear lamina as well as the internal nuclear scaffold that is thought to organize the genome. Investigation of these proteins sheds light on their role during apoptotic nuclear breakdown and, in addition, more clearly defines the function of these proteins in intact cells. An interesting example is the mapping of the scaffold attachment region-specific DNA binding domain of SAF-A that was facilitated by the observation that SAF-A loses its DNA binding activity upon cleavage in apoptosis (10). Our original findings suggested that SAF-A might be cleaved by a caspase but neither identified the responsible protease nor the cleavage site on the protein. We can now show that recombinant caspase-3 is
cells were induced for apoptosis by the addition of staurosporine to 10 μM for 24 h in complete medium, harvested, and analyzed by immunodetection with polyclonal SAF-A antibodies. COS7 and MCF-7 cells were treated with staurosporine at 5 μM final concentration in complete medium for 24 h. Cells were harvested and analyzed for cleavage of SAF-A by immunodetection with polyclonal anti-SAF-A antibodies.

Fig. 4. The point mutation D100A abrogates cleavage of SAF-A during apoptosis in vivo. A, titration of COS7 cells with increasing amounts of staurosporine to find optimal conditions for the induction of apoptosis. COS7 cells were incubated with the indicated concentrations of staurosporine for 24 h in complete medium, harvested, and analyzed for SAF-A cleavage by SDS-polyacrylamide gel electrophoresis and immunodetection with polyclonal SAF-A antibodies. B, COS7 cells were transfected with expression vectors encoding myc-tagged wild type (wt) SAF-A and D100A point-mutated SAF-A, respectively, and enhanced green fluorescent protein (EGFP) as a control. At 36 h post-transfection, cells were induced for apoptosis by the addition of staurosporine to 10 μM for 24 h. Cells were harvested and analyzed for the cleavage of transfected SAF-A by immunodetection with anti-myc-tag antibodies (upper panel) or cleavage of endogenous SAF-A by immunodetection with polyclonal anti-SAF-A antibodies (lower panel). Controls: untreated cells (neither transfected nor induced for apoptosis) and cells transfected with enhanced green fluorescent protein or SAF-A clones, but not induced.

able to cleave SAF-A in vitro, although no caspase-3 consensus sequence is present on the primary structure of SAF-A. The cleavage site was mapped by MALDI-TOF mass spectrometry (20) and amino acid sequencing of cleavage products derived from a recombinant SAF-A fusion protein. Interestingly, SAF-A is cleaved by caspase-3 at a noncanonical cleavage site, SALD, that has neither been described earlier in other caspase-3 substrates nor identified as a possible caspase-3 cleavage site in a combinatorial approach (4). In that paper, Thornberry et al. (4) used a positional scanning synthetic combinatorial library and fluorometric activity assays to determine the optimal cleavage sequence for granzyme B and all caspases known at that time. Interestingly, the optimal recognition motif for group II caspases (caspase-3 and the related caspases 2 and 7) found in these experiments, DEXD, was similar or identical to the cleavage sites in many proteins that are pro
teolytically cleaved during apoptosis, including poly(ADP-ribose) polymerase (34), the catalytic subunit of DNA-dependent protein kinase (35, 36), the 70-kDa subunit of the U1 small ribonucleoprotein (37), and protein kinase Cδ (38). In addition, these studies revealed that the peptide preference within the separate subfamilies of caspases are remarkably similar. Most striking was the similarity between caspase-3 and caspase-7, with virtually indistinguishable specificity profiles. It was concluded that, aside from the stringent requirement for aspartate in the P1 position common to all caspases, group II caspases have an almost absolute requirement for aspartate in the P4 position and that this aspartate is needed for efficient catalysis. The structural basis for the distinct specificities of different caspases can be inferred from the crystal structures of caspase-3 and caspase-1 in complex with the tetrapeptide inhibitors DVAD fluoromethyl ketone or YVAD chloromethyl ketone, respectively (3, 39, 40). In the case of caspase-3, the crystal structure clearly shows that the P4 side chain (Asp) is buried in a narrow pocket that can accommodate small acidic or hydrophilic side chains but rejects large aromatic residues. The residue at P3 (Val) is recognized only by main chain interactions with the protease, with no specific side chains that discriminate between different residues at this site, whereas the P2 residue (Ala) points into a shallow depression that can accommodate small hydrophobic side chains. From these studies, it appears possible that caspase-3 is more tolerant to variations in the cleavage site than suggested by the combinatorial peptide approach. Indeed, we show in this paper that the amino acid sequence SALD is an efficient cleavage site for caspase-3 both in vitro and in vivo. This is at variance with the results from the combinatorial approach, which indicated less than 5% cleavage of peptides with a serine in P4 instead of the aspartate (set to 100%). However, the SALD sequence of SAF-A might easily be accommodated into the active site of caspase-3 as judged from the crystal structure of the enzyme (Fig. 6) and most likely interacts with the amino acid residues that are also involved in binding of the tetrapeptide inhibitor DVAD fluoromethyl ketone (39). In addition, our modeling suggests a hydrogen bond between the P4 serine hydroxyl group and the oxygen atom in the peptide bond of phenylalanine 256 of caspase-3. Our data clearly show that the proposed requirement of caspase-3 for aspartate in the P4 position is not absolute, and cleavage site recognition in native proteins is more...
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In agreement with our in vitro data, we find that SAF-A is not cleaved in vivo in the human breast carcinoma cell line MCF-7, which is devoid of caspase-3 (6) but has all other components necessary to perform apoptosis (5). In particular, caspase-7 that has a specificity indistinguishable from that of caspase-3 in vitro is far more abundant in MCF-7 cells than e.g. in HeLa cells (8). It is conceivable that caspase-7 can substitute caspase-3 with regard to the cleavage of some substrate proteins in MCF-7 cells. Indeed, many proteins normally cleaved by caspase-3 during apoptosis are also cleaved in MCF-7 cells; examples are poly(ADP-ribose) polymerase, p53, PAK2, and the catalytic subunit of DNA-dependent protein kinase (8). Other substrates such as α-fodrin, gelsolin, and SAF-A are not cleaved in absence of caspase-3 (Ref. 8 and this publication), and e.g. ICAD (DNA fragmentation factor 45) appears to be processed inappropriately by a yet unknown caspase (5). These experiments suggest that, although specificities of caspase-3 and caspase-7 are virtually identical when tested on simple peptide substrates, they may differ markedly on natural proteins. As to the biological relevance of these results, it is interesting to recall that MCF-7 cells die without displaying some of the distinct morphological features typical of other apoptotic cells, like cell shrinkage, membrane blebbing, and nuclear breakdown. Apparently, the absence of these morphological changes is a consequence of the inability of MCF-7 cells to cleave a certain group of key substrates, thereby leaving several elements of cellular architecture intact. For cell shrinkage and membrane blebbing, these critical targets could be the cytoskeletal proteins α-fodrin and gelsolin, whereas for nuclear breakdown, the endonuclease caspase-activated DNase (DNA fragmentation factor) and the scaffold attachment region binding nuclear scaffold protein SAF-A are likely candidates. Future experiments using cells stably transfected with the non-cleavable SAF-A construct characterized in this paper will reveal if cleavage of SAF-A is necessary to facilitate nuclear breakdown in apoptotic cells.

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