A Caspase-9 Variant Missing the Catalytic Site Is an Endogenous Inhibitor of Apoptosis*

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It is likely that endogenous inhibitors of the apical caspases such as caspase-9 exist to prevent undesirable activation of caspase cascades. A naturally occurring variant of caspase-9 named caspase-9S was cloned from human liver. Caspase-9S is missing most of the large subunit of caspase-9, including the catalytic site, but has the intact prodomain and small subunit. Caspase-9S did not show apoptotic activity in transfection analysis. Overexpression of caspase-9S inhibited apoptosis induced by caspase-9, indicating that caspase-9S is an endogenous dominant-negative of caspase-9. Moreover, caspase-9S inhibited apoptosis induced by tumor necrosis factor(TNF)-α, TNF factor-related apoptosis-inducing ligand (TRAIL), Bax, or Fas-associated death domain-containing protein (FADD) as well as the combination of Apaf-1 and caspase-9. In vitro binding assays demonstrated that caspase-9S binds to Apaf-1 and blocks the binding of caspase-9 to Apaf-1. Coexpression of caspase-9 and caspase-9S mRNA was identified in various cell lines. Thus, caspase-9S acts as a dominant-negative inhibitor of caspase-9 activation, at least in part, by blocking Apaf-1-caspase-9 interaction.

Apoptosis, an evolutionarily conserved and genetically regulated biological process, plays an important role in the development and homeostasis of multicellular organisms (1, 2). Transmembrane receptor molecules including TNFα-R1, Fas, and TRAIL-R are known to activate apoptotic signaling pathways following ligand binding. TNF-α and Fas ligand (or agonistic Fas antibody) induce trimerization of the receptor molecules, eliciting interaction of the receptors with cellular adaptor proteins such as TRADD (3, 4) and FADD (5–7). FADD has been shown to directly interact with and activate procaspase-8 (8–10). This signaling event is followed by activation of caspase cascades leading to cleavage of cytosolic, cytoskeletal, as well as nuclear proteins and DNA. Although TRAIL-R is also known to induce cellular events similar to those activated by TNF-α and Fas, the molecular signaling events for this receptor are only partially characterized (11).

Most evidence implicates the activation of caspases as an essential step in apoptotic signaling (1, 2). All caspases, so far identified, are initially synthesized as inactive zymogens composed of the prodomain plus large and small subunits. Generation of the active caspase requires sequence-specific proteolytic cleavage to convert the zymogen to a corresponding active enzyme (1, 2). Several studies have shown that caspase-3 is pivotal in the execution of apoptosis (12, 13). Recently, caspase-3 was shown to liberate a DNase termed CAD (caspase-activated DNase) from an inhibitor of CAD (ICAD) by cleaving the ICAD protein (14–16). This process leads to DNA degradation, a hallmark event in apoptosis. Although many inducers of apoptosis lead to activation of caspase-3 activation and other terminal caspases, the pathways leading to this event are not well established. It is likely that numerous mechanisms exist to limit the activation of caspase-3 and other terminal caspases. For example, nitric oxide blocks caspase-3 (and potentially other caspases) activation and activity by modifying cysteine residue of the catalytic site (17–20). The anti-apoptotic p53 protein is enzymatically processed and forms a stable caspase-p53 complex, preventing autoproteolytic activation of caspases (21).

A mechanism for the activation of caspase-3 involving the release of cytochrome c from the mitochondria has been identified (22). TNF-α, Fas ligand (or agonistic Fas antibody), and TRAIL are known to induce the release of cytochrome c from the mitochondria during apoptosis (23). Recent studies have demonstrated that Bcl-2 family member Bid links receptor-mediated apoptotic signals to cytochrome c release from mitochondria (24, 25). The C-terminal portion of Bid generated by caspase-8 activated through Fas signaling pathway targets mitochondria and induces cytochrome c release (24, 25). However, the detailed biochemical mechanism by which cleaved Bid induces cytochrome c release from mitochondria remains to be resolved. Once released, cytochrome c is now known to bind to Apaf-1, a mammalian homologue of C. elegans Ced-4, inducing a conformational change of Apaf-1 (22). Apaf-1 associated with cytochrome c and dATP directly activates procaspase-9 (22) by interacting with the prodomain, termed CARD (caspase recruitment domain) of procaspase-9 (22, 26). Thus, caspase-9 is positioned at the apex in the apoptotic signaling cascades activated by released cytochrome c. The activated caspase-9 then is able to activate procaspase-3 (22). These findings suggest that caspase-9 may be a central regulator of caspase-3 in a cytochrome c-dependent signaling pathway involving Apaf-1. Thus, caspase-9 activation is a potential key site for the regulation of apoptosis. This hypothesis is supported not only by results obtained with caspase-9 knockout mice where caspase-3 activation is blocked (27, 28), but also by recent findings that a mutant caspase-9 generated by changing 287th cysteine to an alanine exhibits dominant-negative activity and inhibits apoptosis (26).
Previously, Northern blotting analyses suggested the possible existence of different forms of caspase-9 (29, 30). To date, only one form of caspase-9 has been cloned and characterized. Therefore, to better understand the functional role of caspase-9 in apoptosis, we cloned another form of caspase-9, named caspase-9S and characterized.

EXPERIMENTAL PROCEDURES

Expression Plasmids—The full-length caspase-9 and caspase-9S cDNAs obtained by PCR from human liver cDNA library (Invitrogen) were cloned into pcDNA3 (Invitrogen). The full-length caspase-3 cDNA was amplified by PCR from pKV-caspase-3 (from Dr. R. Talanian) and cloned into pcDNA3. The pCMV-GST was provided by Dr. E. White (Rutgers University, New Brunswick, NJ). The expression plasmids for Apaf-1 (22) and FADD (26) described previously were provided by Dr. X. Wang and Dr. V. Dixit, respectively.

Molecular Cloning of Caspase-9S cDNA—Human caspase-9S cDNA was cloned by PCR from a human liver cDNA library (Invitrogen) using two primers, 5′-ATGGACGAAGCGGATCGGCGGC-3′ (5′-primer) and 5′-TTATGATGTTTTAAAGAAAAGTTTTTTC-3′ (3′-primer). PCR was carried out for 60 s at 95 °C, 60 s at 55 °C, and 70 s at 72 °C for 35 cycles. The PCR products were run into a 1% agarose gel, transferred onto a nitrocellulose membrane, and subjected to Southern blotting as described (31). The DNA fragment encompassing the 294th amino acid to the stop codon of caspase-9 (Fig. 1B) was used as a probe for Southern blotting.

Western Blotting—Preparation of whole cell lysates and Western blotting was performed as described (20) using an anti-human caspase-9 monoclonal antibody (PharMingen) or polyclonal anti-human caspase-9 antibody (Dr. X. Wang).

Apoptosis Assays—MCF-7 cells were transiently cotransfected with β-galactosidase (pCMVβ-gal) and test expression plasmids using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. At the indicated time points after transfection, the number of round blue apoptotic cells out of the total number of blue cells was determined for each condition.

Production of Recombinant TRAIL and Caspase-9S—A human TRAIL cDNA fragment (amino acids 114–281) obtained by PCR was cloned into the pET-23d (Novagen) plasmid, and expressed protein was purified using the His-bind Resin and Buffer Kit (Novagen).

To purify recombinant caspase-9S protein, the full-length caspase-9S cDNA was cloned into pQE30 (Qiagen) bacterial expression vector. Expression and purification followed the procedures described to purify recombinant TRAIL.

Protein Binding Assay—The cDNA fragment corresponding to residues 1–97 of Apaf-1 was obtained by PCR and cloned into the mammalian expression vector pCMV-GST to generate the GST-Apaf-1(1–97) fusion protein. C-33A cells grown on 60-mm plates were transfected with RIP-lysis buffer (1% Nonidet P-40, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 1 mM sodium orthovanadate in phosphate-buffered saline) and incubated overnight at 4 °C with recombinant TRAIL carrying the beads were pelleted by centrifugation. Beads were washed five times with binding buffer and boiled for 3 min in 1× sample buffer. The resulting supernatants were loaded onto 15% SDS gel and visualized by autoradiography.

RESULTS AND DISCUSSION

Molecular Cloning of Caspase-9S cDNA—To clone a caspase-9 variant, we employed PCR. PCR using a set of primers encompassing the open reading frame of caspase-9 generated two products (1250 and 800 bp) from human liver cDNA library (data not shown). Nucleotide sequencing revealed that the 1250- and 800-bp PCR products coded for full-length caspase-9 and caspase-9S, respectively. The shorter variant of caspase-9 was named caspase-9S (caspase-9 short form, GenBankTM accession number U56390). The deduced amino acid sequence of caspase-9S contains the catalytic site (QACGG) and most of the large subunit (p17) of caspase-9, including the catalytic site (29, 30) are denoted by asterisks. The mismatched amino acid residue indicated by the arrowhead may result from sequencing error. Extensive nucleotide sequencing of other cDNA clones confirmed alanine (A) residues as the right amino acid. The nucleotide sequence of caspase-9S has been submitted to GenBankTM with accession number AF110376. Molecular structures of caspase-9 and caspase-9S are shown schematically. The full-length cDNA fragments encoding caspase-9 and caspase-9S identified in panel A were subcloned into pCDNA3 and in vitro translated using the TNT-coupled transcription/translation kit (Promega) in the presence of [35S]methionine. The translated products were fractionated on an 15% SDS gel and visualized by autoradiography.

FIG. 1. Molecular cloning and deduced amino acid sequence analysis of caspase-9S. A, the deduced amino acid sequence of caspase-9S was aligned with that of caspase-9 (GenBankTM accession number U56390). The box indicates the catalytic site of caspase-9. The dotted line describes missing amino acids. The putative sequence-specific cleavage sites (29, 30) are denoted by asterisks. The mismatched amino acid residue indicated by the arrowhead may result from sequencing error. Extensive nucleotide sequencing of other cDNA clones confirmed alanine (A) residues as the right amino acid. The nucleotide sequence of caspase-9S has been submitted to GenBankTM with accession number AF110376. B, molecular structures of caspase-9 and caspase-9S are shown schematically. The full-length cDNA fragments encoding caspase-9 and caspase-9S identified in panel A were subcloned into pCDNA3 and in vitro translated using the TNT-coupled transcription/translation kit (Promega) in the presence of [35S]methionine. The translated products were fractionated on a 15% SDS gel and visualized by autoradiography.
tected transcripts representing caspase-9 as well as caspase-9S in most cell lines (caspase-9 transcript in A549 cells was only detectable after overexposure) (Fig. 2A). RT-PCR/Southern blotting also revealed that the ratio of caspase-9 to caspase-9S varied in the different cell lines. In MCF-7, RL95–2 (Fig. 2A), HepG2, and MRC-5 cell lines (data not shown), the expression level of caspase-9 mRNA was higher than that of caspase-9S. In contrast, HeLa and SK-OV-3 cell lines, caspase-9S mRNA expression was detected to be higher than that of caspase-9 (Fig. 2). We have tested two antibodies (obtained from PharMingen and Dr. X. Wang) for their capacity to detect caspase-9 and caspase-9S proteins by Western blot analysis (Fig. 2B). Both antibodies detected only caspase-9 protein. MCF-7 and HeLa cells expressed more caspase-9 protein than A549 cells, correlating with the corresponding message levels.

**Dominant-negative Action and Inhibitory Role of Caspase-9S in Apoptosis**—To examine caspase-9S function by transient transfection, the MCF-7 cell line was chosen by two reasons: first, caspase-9S expression was detected to be lower than caspase-9 expression in the cell line; second, the cell line was

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**Fig. 2. Coexpression of caspase-9 and caspase-9S in various cell lines.** A, RT-PCR was carried out with RNA (lanes 5–9) isolated from various cell lines as described under “Experimental Procedures.” As positive controls, the expression constructs containing caspase-9 and caspase-9S cDNA (Fig. 1C) were used in the PCR step (lanes 3 and 4). The resulting RT-PCR or PCR products were resolved on 1% agarose gel and visualized by ethidium bromide staining. After transferred onto nitrocellulose membrane, RT-PCR/PCR products were also subjected to Southern blotting as described under “Experimental Procedures.” As an internal control for RT-PCR, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified by PCR at the same experimental condition and used for normalization. The lanes 1 and 2 are shorter exposed results of lanes 3 and 4. B, 80 μg of each whole cell lysate (lanes 3–5) was fractionated on 15% SDS-gel, and caspase-9 protein was detected by Western blotting using anti-human caspase-9 monoclonal antibody (PharMingen). Equal loading of the samples was monitored by Coomassie staining (data not shown). As positive controls, in vitro translated caspase-9 and caspase-9S proteins (Fig. 1C) were loaded onto lane 1 and 2, respectively. Tested caspase-9 antibody failed to detect caspase-9S protein as shown in lane 2. The arrowhead indicates a nonspecific band.

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**Fig. 3. Dominant-negative action and inhibitory role of caspase-9S in apoptosis induced by various molecules.** A, MCF-7 cells plated at 1.0 × 10^5/12-well were transiently transfected with 0.2 μg of pCMVβ-gal plus 0.5 μg of vector, caspase-9, caspase-9S, or caspase-3 expression plasmid using Lipofectin (Life Technologies, Inc.) according to the manufacturer’s instructions. Fifteen hours after transfection, cells were stained for β-galactosidase expression and examined for morphological evidence of apoptosis as described previously (Kumar et al. (38)). The experiments were carried out at triplicate. The bar indicates standard error. B, 0.2 μg of pCMVβ-gal plus 1.5 μg of vector or caspase-9S expression plasmid were transiently transfected with 0.5 μg of caspase-9, Bax, or FADD expression plasmids or vector into MCF-7 cells plated at 3.0 × 10^5/6-well. Twenty hours after transfection, apoptosis assays were performed as described in the legend to A. C, MCF-7 cells plated at 1.0 × 10^5/12-well were transiently transfected with 0.3 μg of pCMVβ-gal plus 0.5 μg of vector or caspase-9S expression plasmid as described in the legend to A. Fifteen hours after transfection, cells were treated without or with TNF-α (200 ng/ml) or TRAIL (200 ng/ml) for 5 h followed by apoptosis assays as described in the legend to A.
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easily killed by apoptosis stimuli such as TNF-α (without transcription or translation blocker) and TRAIL.

Overexpression of caspase-9 or caspase-3-induced apoptosis (Fig. 3A). In contrast, overexpression of caspase-9S did not lead to apoptosis (Fig. 3A), indicating that the deleted region of caspase-9, including the catalytic site, is crucial for its normal function. The cysteine residue of the catalytic site is well conserved throughout the caspase family members and required for catalytic function. Loss of the catalytic site in caspase-9S most likely accounts for the null activity. Previously, an artificial mutant caspase-9 (C287A), where the catalytic site cysteine residue (Cys, 287th amino acid) was mutated to alanine (Ala), demonstrated dominant-negative activity against wild-type caspase-9 (22, 26). Thus, we next examined whether caspase-9S also exhibits dominant-negative activity against wild-type caspase-9. Overexpression of caspase-9S protected cells from apoptosis induced by caspase-9 (Fig. 3B). This indicated that caspase-9S functions as a dominant-negative inhibitor of caspase-9. Overexpression of caspase-9S also inhibited apoptosis induced by Bax and FADD (Fig. 3B) as well as by TNF-α and TRAIL (Fig. 3C), suggesting that caspase-9S blocks a common signaling pathway activated by Bax, FADD, TNF-α, and TRAIL.

Overexpression of Caspase-9S Inhibits Apaf-1/Caspase-9-mediated Apoptosis—Previously, Apaf-1 was shown to directly interact with and activate procaspase-9 during apoptosis (22). To examine the functional role of caspase-9S in this process, MCF-7 cells were transiently transfected with plasmid constructs expressing caspase-9, caspase-9S, or Apaf-1, individually or in combination (Fig. 4A). Transfection of the vector did not significantly affect cell viability (column 1). Cells overexpressing caspase-9 exhibited moderate cell death (column 2), whereas cells overexpressing caspase-9S were mostly viable (column 3). Overexpression of Apaf-1 alone (column 4) or coexpression of Apaf-1 and caspase-9S (column 5) did not induce apoptosis. However, when coexpressed with caspase-9, Apaf-1 significantly increased apoptosis (column 2 versus column 6). This result is in agreement with the previous findings that showed direct activation of caspase-9 by Apaf-1 (22). Coexpression of caspase-9S inhibited apoptotic cell death induced by the combination of Apaf-1 and caspase-9 (column 7).

In order to activate caspase-9, Apaf-1 must interact with the prodomain of caspase-9 (22). Our data (Fig. 1, B and C, and Fig. 3) suggest that caspase-9S may bind to Apaf-1 through its prodomain as does caspase-9 and compete with caspase-9 for binding to Apaf-1 preventing caspase-9 activation. Therefore, we performed in vitro binding assays to examine this possibility directly (Fig. 4B). Since the N-terminal 97 amino acids of Apaf-1 has been shown to be responsible for interacting with the prodomain of caspase-9 (22), we used GST-Apaf-1(1–97) for our binding assays. As shown previously (22), in vitro translated caspase-9 bound to GST-Apaf-1(1–97) (lane 5). Under the same experimental conditions, caspase-9S also efficiently bound to GST-Apaf-1(1–97) (lane 6). However, neither caspase-9 nor caspase-9S bound to GST alone (lanes 3 and 4), indicating that specific interactions occur between Apaf-1(1–97) and caspase-9S. Importantly, the binding of caspase-9 to Apaf-1 was abrogated by caspase-9S (lane 7). These functional and binding assay results indicate that caspase-9S plays as a dominant-negative inhibitor, at least in part, by blocking Apaf-1-caspase-9 interaction through an interaction with Apaf-1.

Our data (Figs. 3 and 4) indicate that caspase-9S is an endogenous inhibitor of apoptosis. TNF-α, Fas ligand, TRAIL, and Bax are known to induce the cytochrome c release from mitochondria (23). This suggests that apoptotic signals initiated by such molecules involve Apaf-1 and converge on a biochemical event eliciting caspase-9 activation. This may explain why apoptosis induced by such molecules is blocked by caspase-9S (Figs. 3 and 4) as well as dominant-negative caspase-9 (C287A) (22, 26). Furthermore, recent studies demonstrated that apoptosis is significantly blocked in caspase-9 knockout mice, leading to embryonic lethality (27, 28). Caspase-3 activation was also blocked in caspase-9 knockout mice. These in vitro and in vivo results indicate that caspase-9 plays a critical role in apoptosis. Our functional and binding assays demonstrated that caspase-9S acts as an anti-apoptotic molecule by specifically blocking caspase-9 activation (Fig. 4). However, our data do not rule out the possibility that caspase-9S may directly inhibit enzymatic activity of caspase-9. Caspase-1 and 3 are homodimeric proteins (2Xp17/p12) containing two active sites (32–34). Therefore, caspase-9S could form an inactive heterodimeric complex with caspase-9.

It has been shown that the MCF-7 cell line does not express caspase-3 (35). We have observed this as well (data not shown). However, the cell line is easily killed by various apoptosis-inducing molecules that promote the cytochrome c release. This suggests that Apaf-1/caspase-9 system also functions to activate a downstream executioner caspase(s) other than caspase-3 in this cell line. Caspase-3-like caspases including caspase-6...
and caspase-7 have been identified, and it has been shown that caspase-9 also activates caspase-7 (36). The capacity of caspase-9 to cleave/activate multiple terminal caspases may explain why the defects observed in caspase-9 knockout mice (27, 28) are more serious than those observed in caspase-3 knockout mice (12). Inappropriate activation of apical caspases such as caspase-9 may cause undesirable activation of downstream caspases, which will be detrimental to cells. Thus, caspase-9S may guard against unwanted caspase-9 activation. FLIP (FLICE-inhibitory protein) acts through a similar mechanism to block procaspase-8, another apical caspase, which interacts with the transmembrane death receptor molecules including TNF-R1 and Fas (37).

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