Characterization of a Novel Isoform of Caspase-9 That Inhibits Apoptosis

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We have identified a novel isoform of rat caspase-9 in which the C terminus of full-length caspase-9 is replaced with an alternative peptide sequence. Casp-9-CTD (where CTD is carboxyl-terminal divergent) is expressed in multiple tissues, with the relative highest expression observed in ovary and heart. Casp-9-CTD was found primarily in the cytoplasm and was not detected in the nucleus. Structural predictions suggest that in contrast to full-length caspase-9, casp-9-CTD will not be processed. Our model is supported by reduced protease activity of casp-9-CTD preparations in vitro and by the lack of detectable processing of casp-9-CTD proenzyme or the induction of cell death following transfection into cells. Both neuronal and non-neuronal cell types transfected with casp-9-CTD were resistant to death evoked by trophic factor deprivation or DNA damage. In addition, cytosolic lysates prepared from cells permanently expressing exogenous caspase-9-CTD were resistant to caspase induction by cytochrome c in reconstitution assays. Taken together, our observations indicate that casp-9-CTD acts as a dominant-negative variant. Its expression in various tissues indicates a physiological role in regulating cell death.

Apoptosis is a physiological form of cell death that serves a number of functions, including tissue development and remodeling, cellular homeostasis, and removal of damaged cells. In regular animal development, cell death is involved not only in morphogenesis, but also in optimizing function of the nervous and immune systems (for review, see Refs. 1 and 2). Apoptosis requires regulated activation of members of a family of cysteine proteases (caspases) that cleave cellular substrates at the carboxyl side of Asp residues, thereby initiating and executing an apoptotic death program. Caspases are produced in cells as full-length zymogens in a poorly active or inactive form, but autocleavage and processing by other proteases yield active enzyme. The processing normally involves the removal of an N-terminal prodomain and cleavage of the remaining protein sequence to yield large and small subunits (for review, see Refs. 2 and 3). Proteolytic cleavage between these protein domains results in caspase activation by formation of heterotetramers composed of small and large subunits, but it has been shown for caspase-9 activation that caspase activity in vitro is observed also in the absence of caspase processing (4).

There are presently 14 known caspase family members, and each has distinguishable enzymatic properties and substrate specificities (for review, see Ref 2). Depending on the mode of activation or downstream effectors, caspase cascades have been identified that involve the orchestrated activation of downstream caspases by upstream initiator proteases. Of these, caspase-9 appears to be one of the major initiators of cell death cascades in diverse apoptotic paradigms, and transgenic as well as null animal models exhibit significant phenotypes that underscore its importance (for review, see Ref. 5). A wide array of apoptotic stimuli lead to release of cytochrome c (and in certain cell types, even caspase-9) from mitochondria, and its presence in the cytosol induces the formation of a multimeric complex that consists of caspase-9, the caspase-9/ cytochrome c-binding protein Apaf-1 (apoptotic protease-activating factor-1), and accessory proteins. Caspase-9 is activated during the formation of the complex and subsequently cleaves and activates downstream death-promoting executioner caspases such as caspase-3, -6, and -7 (6–9).

Considering the importance of self-association and complex formation with downstream substrates for the regulation and consequences of caspase-9 activity, it is not surprising that catalytically inactive forms inhibit the activation of downstream executioner caspases and inhibit cell death. Furthermore, endogenous forms of caspase-2, -8, and -9 that act as apoptosis inhibitors have been found (10–14).

In this work, we have identified and characterized a novel isoform of rat caspase-9 that we have designated casp-9-CTD (where CTD is carboxyl-terminal divergent). This form differs from previously described isoforms of caspase-9 in that the small caspase subunit found in full-length caspase-9 is replaced by an alternative sequence. Here we examined the consequences of the alternative C terminus on caspase activity, intracellular localization, and function.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF308469 and AY008275.

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1 The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; IPTG, isopropyl-β-D-thiogalacto-pyranoside; pNA, p-nitroanilide; AMC, 7-amino-4-methylcoumarin; T4 polynucleotide kinase; GST, glutathione-S-transferase; MEM, minimal essential medium; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein; UTR, untranslated region.
Cloning of Rat Full-length Caspase-9 and Casp-9-CTD

To clone rat full-length caspase-9 and casp-9-CTD, primers 5'-CT-TATGCACCAGATGATGCTCTCAGAGATGAAGC-3' and 5'-AGTACACATATGAC-3' were used for 3'-RACE and nested 3'-RACE amplification. For 5'-RACE, primer sets 5'-CTCCAGGGCTGGGCAGCGCCG-3' and 5'-CAGCCGGTCCATTCCTGAAGTTTTA-3' were used along with the vector, pCMS-EGFP-full-length caspase-9 and pCMS-EGFP-casp-9-CTD, respectively.

Constructs

The cysteine residue in the QACGG active-site motif of full-length caspase-9 was mutated to alanine by overlapping PCR using primers 5'-CATTCCAGGGCTGGGCAGCGCCG-3' and 5'-TTTCTGCTCACCACCCAGGCCCATGGAGTCA-3'. Primers 5'-GAATTCCGACATTCCAGGGCTGGGCAGCGCCG-3' and 5'-GAATTCCGACATTCCAGGGCTGGGCAGCGCCG-3' were used as the reverse primer for caspase-9-CTD. All PCR products were sequenced.

EXPERIMENTAL PROCEDURES

Materials and Cell Culture

RPML 1640 medium, DMEM, 7% FBS and DNA polymerase. The reverse transcriptase-pre-amplification kit, and LipofectAMINE 2000 were from Life Technologies, Inc. The Marathon cDNA amplification library kit was from CLONTECH, and PCR primers were obtained from Integrated DNA Technologies or Life Technologies, Inc. Tissue total RNA was purchased from Ambion Inc. Hoechst dye 33342 and anti-FLAG antibody were from Sigma. IPTG was from Life Technologies, Inc. Escherichia coli BL21(DE3) lysozyme was from Novagen. Anti-His antibody was from Invitrogen, and anti-caspase-9 antibody was from Medical and Biological Laboratories, Co., Ltd. DE-VD-pNA and benzylxycarbonyl-VAL-fluoromethyl ketone were from Enzyme Systems Inc., and AMC- and AFC-conjugated substrate peptides were from Pharmaqen or BIOMOL Research Labs Inc. Metal-chelating affinity chromatography was performed on Hitrap chelating columns from Amersham Pharmacia Biotech.

Cell Culture

PC12 cells were cultured as described (15) in collagen-coated dishes with RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum. Neuronal differentiation was induced with 100 ng/ml human recombinant NGF (a kind gift of Genentech). Rat-1a fibroblasts or NGF-overexpressing cells were transfected as described previously (16). The concentrations of proteins was measured by the Bio-Rad method. The activity of caspase-3-like enzymes in reconstituted cell lysates was determined as described previously (17). In brief, concentrated cytoplasmic protein lysates were obtained by resuspending PC12 control cells and polyclonal PC12 cell cultures expressing casp-9-CTD-FLAG at 4°C in hypotonic cell lysis buffer (20 mM Pipes, 10 mM KCl, 5 mM EDTA, 2 mM MgCl₂, and 1 mM dithiothreitol, pH 7.4), followed by cell lysis and centrifugation at 13,000 × g.

Transformations and Trophic Factor Deprivation

293 cells were transfected using calcium phosphate as described (19). For PC12 cells, transfection with 10 μg of plasmid/well was performed after 2 days of NGF treatment for NGF-differentiated PC12 cells or immediately after plating for naive cells. PC12 and Rat-1a cells were transfected using LipofectAMINE 2000. After 9 h for PC12 cells and 5 h for Rat-1a cells, medium with LipofectAMINE 2000 was exchanged for fresh medium with NGF or insulin or with serum containing DMEM for NGF-treated naive PC12 cells and Rat-1a fibroblasts. On day 3 after transfection for PC12 cells or on day 2 for Rat-1a fibroblasts, cultures were washed 5–10 times with RPMI 1640 medium for PC12 cells or with DMEM for Rat-1a cells. The cells were scraped from their dishes and replated onto collagen-coated 15-mm wells at 2 × 10⁵ cells/well. For PC12 cells, trophic factor-deprived cultures were maintained in RPMI 1640 medium, and the control non-deprived cells were grown with NGF or NGF/insulin for NGF-differentiated or naive cells. Rat-1a and Rat-1a c-myc-overexpressing fibroblasts were maintained in DMEM only.

Establishment of Permanent PC12 Cell Lines

PC12 cells (100-mm plates) were transfected with 28 μg of rat caspase-9-CTD-FLAG cloned into the pcDNA3.0 plasmid or with empty pcDNA3.0 plasmid. After 1 week, permanently transfected cells were selected for neomycin resistance using 500 μg/ml G418 (Cellogro). The surviving cells were expanded to establish clonal lines or polyclonal mass cultures. Cells were tested for expression of FLAG-tagged caspase-9-CTD by Western blot analysis with anti-FLAG and anti-caspase-9 antibodies. Survival in the absence of serum was determined by counting intact nuclei after 2 days (20).

Western Immunoblotting

293 cells or PC12 cells were harvested in Laemml Sample buffer, and concentration of proteins was measured by the Bio-Rad method. The proteins were resolved by SDS-polyacrylamide gel electrophoresis using a 4–20% gradient gel and transferred onto nitrocellulose according to standard procedures. The nitrocellulose membrane was stained with Ponceau S to locate proteins; cut into sections; and blocked with 5% milk in PBS, pH 7.4, at room temperature for 1 h. The blot was probed with anti-caspase-9 p20 antisemur (Bur73, a kind gift from Guy Salvesen, Burnham Institute, San Diego, CA) for 293 cells or with anti-FLAG antibody for PC12 cells, followed by ECL, according to the manufacturer’s instructions (Amersham Pharmacia Biotech).

In Vitro Transcription and Translation

pCMS-EGFP-full-length casp-9 and pCMS-EGFP-casp-9-CTD vectors were mixed with single-step T₇ promoter-driven TNT-coupled reagents (Promega) and with [35S]methionine (1000 Ci/mmol). The protocol was carried out as recommended by the supplier. 25% of the radiolabeled mixture of full-length caspase-9 and casp-9-CTD was resolved on 4–20% Novex gradient gels. The gel was then dried and exposed to X-ray film.

Purification of Recombinant Caspases and Protease Activity Assays

Following IPTG induction (caspase-3 at 0.2 mM IPTG for 45 min and caspase-9 isoforms at 0.4 mM IPTG for 1.5 h at 30°C), caspases were purified as described (17) and stored at −80°C. Purified caspase-9 activity was determined using benzyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethyl ketone as a substrate, while caspase-3 activity was determined using the 4-chloro-7-nitrobenzofurazan (NBD)-conjugated substrate peptide Ac-DEVD-pNA. The activity was determined by active-site titration using benzyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethyl ketone or by comparison with known amounts of caspase enzymes using Western blotting. Caspase activity assays were performed at 37°C in 50 μl reaction volume using 0.2 mM synthetic peptide or caspase-3 as a substrate. For comparison of different AMC-coupled substrate peptides, an end point analysis was performed in a Turner TD-70 fluorometer using an excitation wavelength of 365 nm and an emission wavelength filter of 455–500 nm after a 30-min incubation at 37°C. The reaction rates of hydrolysis of Ac-DEVD-pNA or Ac-DEVD-AFC substrate peptides were determined using a Beckman DU530 spectrophotometer equipped with a Peltier temperature module in kinetic rate mode. Liberated pNA or AFC were calculated using a standard concentration curve. The activity of caspase-3-like enzymes in reconstituted cell lysates was determined as described previously (18). In brief, concentrated cytoplasmic protein lysates were obtained by resuspending PC12 control cells and polyclonal PC12 cell cultures expressing caspase-9-CTD-FLAG at 4°C in hypotonic cell lysis buffer (20 mM Pipes, 10 mM KCl, 5 mM EDTA, 2 mM MgCl₂, and 1 mM dithiothreitol, pH 7.4), followed by cell lysis and centrifugation at 13,000 × g.

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Assessment of Cell Survival

Strip Counting—The numbers of healthy, non-apoptotic, EGFP-positive cells in a defined strip across the culture were assessed by phase and fluorescence microscopy. The same strip was scored each day during the time course, and the percentage of surviving cells was calculated relative to the number present in the same well at 1.5 h after plating (20, 21).

Apoptotic Nuclei—At various time points, PC12 or Rat-1a cells were fixed in 4% formaldehyde for 10 min and then, after washing with PBS, exposed to 10% nonimmunogen goat serum and 0.2% Triton X-100 in PBS for 30 min at room temperature (20). Staining for FLAG fusion protein was carried out for 1–2 h at room temperature with 20 μg/ml anti-FLAG antibody in PBS containing 10% nonimmunogen goat serum and 0.2% Triton X-100, followed by a 1-h exposure to rhodamine-labeled secondary antibody (1:100 dilution). To visualize nuclear DNA, the fixed cells were incubated with Hoechst dye 33342 at 1 μg/ml in PBS and 0.08% Triton X-100 for 15 min at room temperature. Cells possessing condensed nuclei and fragmented chromatin were scored as apoptotic. Data are presented as the proportion of either EGFP- or FLAG-expressing cells with apoptotic nuclei.

RESULTS

Cloning of a Rat Carboxyl-terminal Divergent Caspase-9 (Casp-9-CTD)—Efforts aimed at the cloning of a full-length cDNA of rat caspase-9 by 5′ and 3′RACE/PCR led to the discovery of a rat caspase-9 variant cDNA that encodes an alternative C-terminal sequence immediately following the QACGG active site (GenBank™/EBI accession number AY008275) (Fig. 1A, lower sequence). This alternative C terminus (amino acids 327–383) shows no homology to other reported sequences, including those previously reported for full-length caspase-9 or other short forms of caspase-9. From the N-terminal methionine through QACGG, the predicted protein sequence is identical to rat full-length caspase-9 (GenBank™/EBI accession number AF308469) (Fig. 1A, upper sequence). The variant was independently confirmed by 3′-RACE/PCR from rat PC12 cells and by PCR from rat brains, in each case using multiple alternative primer pairs (data not shown). RT-PCR and subsequent sequencing also detected the variant in several different rat tissues, including adult liver and ovary, and in whole rat embryos (see also Fig. 3). We have designated the novel variant as casp-9-CTD for caspase-9 carboxyl-terminal divergent. A portion of the casp-9-CTD 5′-UTR (16 base pairs) was sequenced, and this showed complete identity to the corresponding sequence of full-length caspase-9 (Fig. 1A). In contrast, the 3′-UTR of casp-9-CTD contains no similarity to the 3′-UTR of the full-length form, thus suggesting that the variant C terminus of casp-9-CTD is derived from an alternative exon of the rat caspase-9 gene. Furthermore, Southern blots of rat genomic DNA showed a single distinct band when hybridized with a probe shared by sequences of both forms of caspase-9 (Fig. 1A). The failure to detect any additional hybridizing bands using these enzymes and the fact that the 5′-UTR sequences of both cDNAs are identical suggest the presence of a single caspase-9 gene in the rat genome that gives rise to both full-length caspase-9 and casp-9-CTD by alternative splicing.

Structural Analysis of Casp-9-CTD Suggests an Inactive Isoform—Alternative short isoforms of caspase-9 are catalytically inactive and act as endogenous inhibitors of caspase-9 activation. These isoforms include a human isoform (caspase-9S, caspase-9b, CASP9, or caspase-9S; GenBank™/EBI accession numbers AF110376, AF093130, AB020979, and AB015653, respectively) that is most likely derived from human full-length caspase-9 by omission of exons 3–6 (Fig. 1B). The ability of the human short isoform to act as an endogenous inhibitor is not surprising considering that the omission of these exons will result in the deletion of its active site. A short variant of mouse caspase-9 has been reported (Fig. 1B) that appears to be generated by deletion of 107 nucleotides and a resulting frameshift within sequences encoding the small caspase subunit (GenBank™/EBI accession number AB019601). This mutant form, however, differs from the variant that we have identified in that a transcript is not normally seen in mouse embryos or tissues (11). In contrast, the variant of rat caspase-9 that we have observed was present at varying expression levels in various rat tissues and cell lines, and it was also detected at different stages in rat embryos (see also Fig. 3A).

A closer examination of the predicted amino acid sequence of rat casp-9-CTD reveals that the alternative carboxyl terminus does not contain the two aspartic acid residues that are present in the full-length forms of caspase-9 (Fig. 1A). These residues are required for the processing of the zymogen into large (p37) and small (p12) subunits that are necessary for the formation of a fully activated enzyme (4). Using the Smith-Waterman algorithm for sequence alignment (22), we detected similarities of 51.7 and 53.7% between the predicted protein sequence of human caspase-3 (GenBank™/EBI accession number U26493) and those of rat caspase-9 and casp-9-CTD, respectively. Using PrISM (protein informatics system for modeling) (23), we were able to unambiguously compute a model for the protease domain of both caspase-9 isoforms using the known structures of human caspase-3 (24, 25) as a template. A further analysis of casp-9-CTD using PrISM did not predict any alternative secondary structure for the divergent C-terminal domain in the context of the casp-9-CTD protein. Thus, it is unlikely that the alternative C terminus in casp-9-CTD protein will contribute to the formation of a small subunit or any other predictable secondary structure (Fig. 2).

RT-PCR Analysis of Casp-9-CTD Reveals Expression in Multiple Rat Tissues—To compare the tissue distributions of rat full-length caspase-9 and casp-9-CTD, we conducted semiquantitative RT-PCR using primers that span the open reading frame of each form using total RNA from various tissues (Fig. 3A). Products corresponding to the predicted size were detected in all tissues examined. The identity of the 1188-base pair casp-9-CTD product was confirmed by sequence analysis. A fast migrating DNA product was also detected using these primer pairs, but it encoded an unrelated DNA product. Expression of casp-9-CTD was consistently highest in ovary, whereas the relative ratio of casp-9-CTD to full-length caspase-9 was highest in heart and ovary. The presence of casp-9-CTD transcripts in PC12 cells was confirmed, but we did not find regulation by NGF (Fig. 3A). 5′- and 3′-RACE/PCR as well as Western blotting confirmed the presence of full-length caspase-9 in PC12 cells (data not shown).

Expression Analysis of Casp-9-CTD Reveals a Molecular Mass of 45 kDa—The molecular masses of full-length caspase-9 and casp-9-CTD were predicted to be 50.4 and 42.3 kDa, respectively, using PeptideMass (26). In vitro transcription and translation followed by SDS–polyacrylamide gel electrophoresis analysis revealed apparent molecular masses of ~45 kDa for rat casp-9-CTD and ~55 kDa for full-length caspase-9 (Fig. 3B). Similar results were obtained with protein expressed in E. coli or after transient transfection of casp-9-CTD into 293 cells (Fig. 3B). In transient transfections, however, we detected only cleavage of full-length caspase-9, possibly because of autoprocessing (data not shown).

Casp-9-CTD Expression Is Limited to the Cytoplasm—To examine the subcellular distribution of casp-9-CTD, a C-terminal FLAG tag was transiently transfected into 293 cells (Fig. 4A) and Rat-1a (data not shown) cells and visualized by staining with an anti-FLAG antibody. To facilitate comparison with full-length caspase-9, we used a full-length, FLAG-tagged form of rat caspase-9 that is rendered catalytically inactive by mutation of the cysteine residue within the QACGG active site.
(C327A) and that will not induce cell death (see also Figs. 6 and 7). Confocal fluorescence microscopy revealed that casp-9-CTD was localized to the cytoplasm and excluded from the nucleus, whereas full-length caspase-9(C327A) was detected in both the cytoplasm and the nucleus. These observations indicate that full-length caspase-9 and casp-9-CTD differ in their subcellular distributions and underscore the potential contribution of the C terminus in regulating nuclear localization. Similar results were obtained following subcellular fractionation of lysates from transiently transfected cells, followed by Western blot analysis (data not shown).

Overexpression of Casp-9-CTD Does Not Cause Cell Death—Our structural considerations suggested that casp-9-CTD is catalytically compromised, but possibly not inactive. Because expression of exogenous full-length caspase-9 leads to activation, processing, and consequent cell death in many cell types, we compared the effects of full-length caspase-9 and casp-9-CTD on cell survival. 293 cells were transiently transfected

Fig. 1. Sequence comparison of rat caspase-9 and casp-9-CTD. A, the nucleotide and predicted amino acid sequences of rat full-length caspase-9 are depicted. The predicted 5'-Met residue indicating the translation start site, the translation stop site, and Asp residues preceding potential cleavage sites between the N terminus and large and small subunits are indicated in boldface. The caspase active-site signature motif QACGG is shaded. Boldface lettering marks differences (at positions 322 and 754) from known sequences for rat caspase-9. The alternative C-terminal nucleotide sequences of rat casp-9-CTD are shown where different from full-length caspase-9, and an amino acid translation is provided.

B

Casp-9-CTD Is an Anti-apoptotic Form of Caspase-9
with either full-length caspase-9 or casp-9-CTD. Transfection of cells was confirmed by examining the expression of EGFP encoded by the same plasmid. Counts of healthy, EGFP-expressing cells at 1 and 2 days revealed nearly comparable numbers in cultures transfected with vector alone or with casp-9-CTD, but many fewer in cultures transfected with full-length caspase-9 (Fig. 4B). Furthermore, cultures transfected with full-length caspase-9 exhibited many fragmented and shrunken fluorescent cells, whereas few, if any, such cells were present in cultures transfected with vector alone or casp-9-CTD. Similar results were achieved in cultures of PC12 and Rat-1 cells (data not shown). These findings indicate that in contrast to full-length caspase-9, which evokes death, overexpression of casp-9-CTD does not compromise cell survival.

Casp-9-CTD Is a Poorly Active Protease Enzyme—To examine the activity of rat caspase-9 and casp-9-CTD in vitro, we expressed both isoforms and human caspase-3 in E. coli BL21(DE3) pLys using the pET/IPTG prokaryotic expression system (17). Recombinant proteins were purified using standard metal-chelating affinity chromatography, and the concentration of caspase-3 and full-length caspase-9 was determined by active-site titration using benzyloxycarbonyl-VAD-fluoromethyl ketone (17). For casp-9-CTD, protein concentrations were determined by quantitative Western blot analysis against known protein standards. Following IPTG induction, we were able to purify caspase-3 in its full-length,zymogen form. In contrast, both full-length caspase-9 and casp-9-CTD were partially processed, and the extent of cleavage was dependent upon the time of induction. For full-length caspase-9, two major bands (12 and 43 kDa) were observed, most likely as a result of cleavage at amino acids 167 and 367. A smaller proportion remained uncleaved or was fully processed into the p35/p12 enzyme (data not shown). In contrast, the majority of casp-9-CTD protein migrated at the expected size of 45 kDa, with smaller fragments at 35 kDa depending on the induction time (data not shown). These smaller fragments were likely to be caused by degradation and not by autoprocessing since they were also present in preparations of mutated casp-9-CTD (C327A).

We first examined whether purified full-length caspase-9 or casp-9-CTD preparations exerted any activity against the caspase-9-specific substrate Ac-LEHD-AMC or other synthetic peptide substrates that are used to detect caspase activity in vitro (27). For these experiments, we used the caspase-9 isoforms at 10 nM, a concentration that is similar to the concentration of rat caspase-9 that we have observed in cytosolic cell lysates from PC12 cells (concentration of total protein, ~12 mg/ml). This concentration resembles the concentration of human caspase-9 (20 nM) that has been observed in concentrated cytosolic lysates from 293 cells (4). Whereas 10 nM purified rat full-length caspase-9 efficiently cleaved the Ac-LEHD-AMC synthetic substrate peptide and, to a much reduced extent, also Ac-VEID-AMC synthetic substrate peptide, no protease activity above background was detected with any of these substrates when using 150 nM human caspase-9 (20 nM) that has been observed in concentrated cytosolic lysates from 293 cells (4). Whereas 10 nM caspase-9-CTD preparations exerted any activity against the QACGG are shown, and the catalytic Cys327 residue is colored in pink. The N terminus is marked, and α-helices 1–5 and β-sheets 1–6 are labeled where applicable. As indicated under “Results,” no unambiguous predictions could be made for the alternative C-terminal sequences in casp-9-CTD.
Casp-9-CTD is an Anti-apoptotic Form of Caspase-9

To characterize the underlying deficiency of apoptosis induction in cells overexpressing casp-9-CTD, we generated PC12 cells lines that permanently expressed FLAG epitope-tagged casp-9-CTD or control vector. Cells were selected for neomycin resistance and examined by Western blot analysis (Fig. 7). Densitometry performed on neuronally differentiated PC12 cells transfected with full-length caspase-9(C327A) (Fig. 6B). The data in Fig. 7 demonstrate that for both Rat-1a and Rat-1a-myc cells, casp-9-CTD provided protection from serum deprivation similar to or even greater than that observed after transient expression of inactive full-length caspase-9(C327A).

Cells with Permanent Overexpression of Casp-9-CTD Are Resistant to Apoptosis—To characterize the underlying deficiency of apoptosis induction in cells overexpressing casp-9-CTD, we generated PC12 cells lines that permanently expressed FLAG epitope-tagged casp-9-CTD or control vector. Cells were selected for neomycin resistance and examined by Western blot analysis using anti-FLAG and anti-caspase-9 antibodies. Several cell lines as well as polyclonal cultures were generated that expressed levels of casp-9-CTD below or equal to the levels of endogenous caspase-9 (data not shown). Following trophic substrate (Fig. 5B). The reduced activity of casp-9-CTD against caspase-3 that we established by Western blot analysis (Fig. 5B) was further confirmed after examining the kinetic rate of conversion of the caspase-3 peptide substrate Ac-DEVD-AFC in a coupled protease assay (8). In this assay, the activity of purified caspase-3 against substrate peptide is an indication of the ability of initiator protease to cleave caspase-3 and to induce caspase-3 activity. Using a ratio of 10 nM initiator peptide to 100 nM caspase-3, which resembles the ratio of caspase-9 to caspase-3 in cell lysates (4, 18), we observed significant caspase-3 activity following incubation with full-length caspase-9 (Fig. 5C). 10 nM granzyme B also activated caspase-3 in our experiments, but no significant caspase-3 activity was observed after incubation of caspase-3 with casp-9-CTD (Fig. 5C). The residual hydrolysis of the Ac-DEVD-AFC substrate that was observed when caspase-3 was incubated with casp-9-CTD was similar to that of Ac-DEVD-AFC hydrolysis by casp-9-CTD alone and possibly due to protease activity against this substrate (see also Table I).

Using a similar approach, we determined the reaction rates of Ac-DEVD-pNA hydrolysis using initiator proteases alone or with caspase-3 in a coupled caspase assay at a molar ratio of 200 nM caspase-9/casp-9-CTD to 100 nM caspase-3 (Table I). In these experiments, 200 nM full-length caspase-9 was able to induce a significant activation of 100 nM caspase-3 in a coupled enzyme assay. Casp-9-CTD at 200 nM was not able to induce significant Ac-DEVD-pNA hydrolysis through secondary acti-

Casp-9-CTD Protects Neuronal and Non-Neuronal Cells from Apoptosis—The findings that casp-9-CTD is catalytically compromised and does not induce death raised the possibility that it could, in turn, act to suppress apoptosis. To test this, several different cell lines were subjected to different death paradigms after transient transfection with casp-9-CTD (with or without a C-terminal FLAG tag) cloned into the pCMS-EGFP vector. A FLAG-tagged construct verified expression of casp-9-CTD. For comparison, cells were also transfected with the FLAG-tagged inactive construct of full-length caspase-9(C327A).

We first tested a paradigm in which naive PC12 cells undergo apoptotic death by serum deprivation. Cultures were fixed and stained with Hoechst dye number 33342 to visualize nuclei. Counterstaining with anti-FLAG antibody identified the cells expressing the desired proteins, and transfected cells were then scored for the presence of apoptotic nuclei. As shown in Fig. 6A, ~30% of the transfected cells deprived of support by serum had apoptotic (condensed) nuclei when vector alone was present compared with <3% of cells that expressed casp-9-CTD or inactive full-length caspase-9(C327A) or that were treated with NGF (a trophic factor for PC12 cells). A parallel experiment performed on neuronally differentiated PC12 cells evoked cell viability at 22 and 48 h after transfection. Values represent the numbers of non-apoptotic EGFP-positive cells in the same strip at each time point. Similar results were obtained independently.
factor deprivation, polyclonal cultures and cell lines with detectable amounts of casp-9-CTD (CTD1-3, -7, and -10) were resistant to apoptosis induction, whereas control or nonexpressing cells (CTD1-1) were not (Fig. 8). Examination of cytosolic cytochrome c levels using subcellular fractionation revealed that these cells responded comparably to control cells,
The hydrolysis of 0.2 mM Ac-DEVD-pNA peptide substrate was examined by continuous recording at A405 nm in kinetic rate mode in a Beckman DU530 spectrophotometer at 37 °C under linear rate conditions. Concentrations of liberated pNA were calculated using a standard concentration curve. Caspase-9 and casp-9-CTD were used either alone or in combination with caspase-3 in coupled protease assays at the indicated concentrations. The incubation with caspase-3 will hereby determine the ability of initiator proteases to induce caspase-3 activation. The reaction rates shown are representative of multiple experiments using different protein purifications and were obtained after subtracting substrate autoproteolysis in reaction buffer only. Substrate autoproteolysis in our experiments was determined to occur at a rate of 0.3 nM/s. Thus, the observed reaction rates are significantly higher than background hydrolysis. The activity of 200 nM casp-9-CTD against the Ac-DEVD-pNA substrate peptide is highlighted in boldface, but caspase-3 was not cleaved by casp-9-CTD under these experimental conditions as determined by Western blot analysis (data not shown; see also Fig. 5B).

Caspase(s) | Reaction rate for peptide hydrolysis (nM/s)
---|---
Caspase-3 (100 nM) | 10
Caspase-9 (200 nM) | 10
Caspase-9-CTD (200 nM) | 25
Caspase-3 (100 nM) + caspase-9 (200 nM) | 300
Caspase-3 (100 nM) + casp-9-CTD (200 nM) | 40

with release of mitochondrial cytochrome c during trophic factor deprivation and etoposide treatment (data not shown). These data indicate that the release of cytochrome c from mitochondria was not affected in cells expressing casp-9-CTD. **Exogenous Casp-9-CTD Inhibits Caspase-3 Activation by Cytochrome c**—To test the caspase activation cascade downstream of mitochondrial damage responses in *vitro*, we tested cytosolic extracts from control cells or polyclonal cultures expressing caspase-9-CTD protein for caspase-3-like activity following cytochrome c addition. The activity of endogenous caspase-3-like enzymes was determined using the fluorogenic substrate Ac-DEVD-AFC under continuous reading conditions. Cytosolic lysates from these cells did not exert any caspase-3-like activity before addition of cytochrome c (Fig. 9). Treatment of cytosolic lysates prepared from control and casp-9-CTD cells with 20 nm purified granzyme B resulted in hydrolysis of 6.7 μM Ac-DEVD-AFC substrate peptide-s⁻¹·mg⁻¹ of total protein for both types of lysates (data not shown). These results suggested that the levels of activable caspase-3 were not altered in cells expressing casp-9-CTD. We then examined the ability of exogenous cytochrome c to induce caspase-3-like activity in our *in vitro* reconstitution model. At low concentrations of cytochrome c (0.1 μM), no significant caspase-3-like activity was detected in lysates prepared from either cell type, possibly because the rate of enzymatic conversion was below detectable levels (Fig. 9). However, cytosolic lysates prepared from control cells and cells expressing casp-9-CTD differed significantly in the extent of caspase-3 activation when 1 μM cytochrome c was added (Fig. 9). Significant activity was present with control cell lysates, whereas no detectable peptide proteolysis was observed in cytosolic lysates from cells expressing casp-9-CTD (Fig. 9). These findings indicate that the presence of casp-9-CTD in cell lysates inhibits the cytochrome c-dependent induction of caspase-3-like enzyme activity.

**DISCUSSION**

Our findings indicate that both casp-9-CTD and full-length caspase-9 are derived from the same rat gene. The caspase recruitment and p20 domains of both forms are identical, and their identity continues through the active-site sequence QACGG. The 5’-UTR sequence thus far determined for the carboxyl-terminal divergent form is also identical to that of the full-length form. It is only 3’ of the active site that the two forms diverge. In addition, results from Southern blot experiments are consistent with the assumption of only a single gene that gives rise to both caspase-9 isoforms. These observations are similar to those reported for the short form of caspase-2 (Ich-1 short) that is an alternative splice product diverging from the full-length form beginning with sequences after the catalytic core and that acts as a dominant-negative inhibitor of apoptosis (10).

The isoform casp-9-CTD that we have identified in rat is distinct from other reported variant forms of caspase-9. A human form lacks a caspase catalytic domain, and its overexpression in cells inhibits apoptosis, at least in part, by means of caspase recruitment domain-mediated interaction with Apaf-1 (12, 13). A short variant of mouse caspase-9 has also been reported that is not processed under conditions that lead to cleavage of the full-length enzyme and that does not significantly increase overall caspase-9 activity when overexpressed (11). However, transcripts encoding this mutant were not detected in mouse embryos or tissues, suggesting a cell linespecific expression that resembles that of mutant forms of caspase-3 in the human breast cancer cell line MCF-7 (30). In contrast, casp-9-CTD was detected in various rat tissues, cell lines, and whole rat embryos, indicating a potential role in apoptosis regulation.

The lack of a defined secondary structure prediction for the alternative C-terminal sequence of casp-9-CTD could be relevant when considering the contribution of the α-helices 4 and 5 encoded by the small subunit to the overall structural integrity of full-length caspases (24, 25). The importance of these helices is further underscored by their presence in other proteases, where they are thought to be involved in forming the backbone for substrate recognition and processing in the context of an antiparallel β-sheet conformation (24, 25). Residues in the small subunit of full-length caspase-9 that are present in the known structures of caspase-1 and caspase-3 and that form the binding sites P3–P5 and, to a lesser extent, are involved in the formation of P1 are also absent in casp-9-CTD. These residues are especially important in facilitating the correct relative orientation of the Asp residue N-terminal to the P4–P5 peptide bond. We have observed a residual proteolytic activity of caspase-9-CTD when using the peptide substrates Ac-DEVD-AFC and Ac-DEVD-pNA. Since Ac-DEVD-AFC is not efficiently cleaved by full-length caspase-9, our data also indicate that casp-9-CTD exerts either an altered or more promiscuous enzyme specificity compared with full-length caspase-9. Structural consideration led us to predict that casp-9-CTD does not exert significant protease activity against physiological caspase-9 substrates such as caspase-3. Our prediction was confirmed by examining the catalytic activity of casp-9-CTD in *vitro* and using cytosolic lysates. The observation that the overexpression of casp-9-CTD in different cell types does not induce apoptosis is consistent with the likelihood that this form is functionally inactive within living cells. Because casp-9-CTD shares many sequences with full-length caspase-9, including caspase recruitment and catalytic core domains, we further postulated that this caspase-9 isoform should act as a dominant-negative for full-length caspase-9. There are several nonexclusive mechanisms by which casp-9-CTD might function as a dominant-negative mutant. One is that it binds to the Apaf-1 apoptosome complex via its caspase recruitment domain and thereby competitively prevents binding and activation of full-length caspase-9. A second is that it forms oligomers with full-length caspase-9, thereby interfering with the formation of an active heterodimeric caspase-9 enzyme. A third is that it

**TABLE I**

| Caspase(s) | Reaction rate for peptide hydrolysis (nM/s) |
|---|---|
| Caspase-3 (100 nM) | 10 |
| Caspase-9 (200 nM) | 10 |
| Caspase-9-CTD (200 nM) | 25 |
| Caspase-3 (100 nM) + caspase-9 (200 nM) | 300 |
| Caspase-3 (100 nM) + casp-9-CTD (200 nM) | 40 |
competitively binds to downstream protein complexes that include caspase-3. In line with its in vitro anti-apoptotic activity, we have shown that transiently expressed casp-9-CTD effectively protects PC12 cells and Rat-1a fibroblasts from trophic deprivation and DNA damage and that cell lines with permanent expression of this isoform are protected from cell death evoked by serum deprivation. Past studies have implicated caspase-2 as a required element in the mechanism by which trophic factor-deprived PC12 cells undergo apoptosis (21). Our findings that casp-9-CTD and caspase-9(C327A) overexpression protects PC12 cells from death evoked by serum and NGF deprivation implicate caspase-9 in the apoptotic process.

We observed that casp-9-CTD was primarily cytoplasmic and excluded from the nucleus, whereas inactive full-length caspase-9 was present in both the cytoplasm and nucleus. The intracellular localization of caspase-9-CTD clearly contrasts with that of full-length caspase-9, which was previously found in the nuclei of cultured mammary epithelial cells, where it is activated in response to apoptotic signals (31). Faleiro and Lazebnik (32) have recently presented additional evidence that caspase-9 re-localization to the nucleus is important in nuclear disassembly. Thus, it will be important to determine the role of nuclear exclusion of casp-9-CTD in the induction of nuclear breakdown. At this point, our observations indicate that in the paradigms we studied, the step(s) in the death pathway suppressed by casp-9-CTD occur outside the nucleus and that any independent nuclear events involving full-length caspase-9 are not sufficient to evoke death. An additional point raised by our findings is that the C-terminal sequences regulate the nuclear localization of caspase-9 isoforms. It will be of interest to determine whether the alternative C-terminal domain of the caspase-9-CTD form promotes specific interactions with other proteins and/or subcellular compartments.

Our work has shown that casp-9-CTD mRNA transcripts are expressed in a variety of tissues. This suggests that this form plays a role in regulating apoptotic death in the intact orga-
nism. Two types of roles can be foreseen. One is that the casp-9-CTD form acts as a constitutive inhibitor to protect cells from low-level accidental activation of full-length caspase-9 following mitochondrial cytochrome c release. The second is that casp-9-CTD protects cells by interfering with activation of full-length caspase-9 in the presence of otherwise apoptotic stimuli. As such, casp-9-CTD is among a growing number of isoforms of apoptosis-related proteins that have the potential to regulate cell death and that result from alternative splicing. As reviewed above, several such alternative forms of caspase-9 have been recognized. Catalytically compromised forms of caspase-2 and caspase-8 have also been found (10, 33, 34). It is of note that these caspases share the potential to be initiators of apoptosis.

FIG. 7. Casp-9-CTD protects fibroblasts from apoptosis following serum withdrawal. Rat-1a and Rat-1a-Myc cells were transfected with pCMS-EGFP (Vector), pCMS-EGFP-caspase-9-CTD-FLAG (Casp-9-CTD), or pCMS-EGFP-caspase-9(C327A)-FLAG (Caspase-9(C327A)). After 2 days (time 0), serum was withdrawn, and cultures were scored for the percentage of transfected cells that expressed either EGFP- or FLAG-tagged constructs and exhibited apoptotic nuclei. For Rat-1a cultures, 300–500 cells were scored per time point; for Rat-1a-Myc cultures, 60–100 cells were scored per time point. Comparable results were obtained in an independent experiment.

FIG. 8. Cells with constitutive overexpression of casp-9-CTD are resistant to apoptosis. Neomycin-resistant PC12 cells transfected with the pcDNA3.0 plasmid expressing casp-9-CTD-FLAG (clones CTD1-1, -3, -10, -7, and polyclonal mass cultures) or the pcDNA3.0 empty vector (Control) were washed four times and then maintained in serum-free RPMI 1640 medium for 2 days. Expression of casp-9-CTD-FLAG was verified by Western blotting of cell lysates with anti-FLAG and anti-caspase-9 antibodies. The control and CTD1-1 lines had no detectable expression of casp-9-CTD. The percentages of apoptotic PC12 cells were determined as described under “Experimental Procedures.” Values represent means ± S.E. (n = 3).

FIG. 9. Cell lysates from cells overexpressing casp-9-CTD are resistant to cytochrome c. Cytosolic extracts were prepared from PC12 control cells and polyclonal mass cultures expressing casp-9-CTD. dATP (1 mM final concentration) and buffer or cytochrome c (CytoC; 0.1 or 1 μM final concentration) were added to the lysates (10 mg/ml of protein) at 37 °C. Proteolysis of Ac-DEVD-AFC was recorded at A_{380 nm} in 5-s intervals. The depicted data were repeated in multiple independent experiments. NT indicates nontreated.
of apoptotic cascades that catalyze activation of downstream caspases. Thus, cells may have evolved alternative forms with the potential to delay or suppress the untimely onset of apoptotic caspase cascades. It is intriguing that the ratios of caspase-9-CTD to caspase-9 are high in ovary, brain, and heart. In the case of the ovary, one may speculate that caspase-9-CTD plays a protective role during the portion of the ovarian cycle not associated with cell death. Likewise, the high levels of caspase-9-CTD in heart and brain may serve to protect non-replaceable cells.

It is also remarkable that for caspase-9, multiple complementary isoforms have been reported in humans and rodents. To date, we have not found sequences homologous to the alternative C terminus of rat caspase-9-CTD in a database. It remains to be determined if the existence of distinct isoforms of inhibitory variants of caspase-9 in humans, or the incomplete state of presently available data bases. It is intriguing that the ratios of caspase-9-CTD to caspase-9 are high in ovary, brain, and heart. In the case of the ovary, one may speculate that caspase-9-CTD plays a protective role during the portion of the ovarian cycle not associated with cell death. Likewise, the high levels of caspase-9-CTD in heart and brain may serve to protect non-replaceable cells.

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