Molecular Cloning and Characterization of Two Novel Pro-apoptotic Isoforms of Caspase-10*

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Patrick W. P. Ng, Alan G. Porter‡, and Reiner U. Jänicke

From the Institute of Molecular and Cell Biology, National University of Singapore, Singapore 117609, Republic of Singapore

Caspase-10/a (Mch4) and caspase-10/b (FLICE2) are related death effector domain-containing cysteine aspartases presumed to be at or near the apex of apoptotic signaling pathways. We report the cloning and characterization of two novel proteins that are splice isoforms of the caspase-10 family. Caspase-10/c is a truncated protein that is essentially a prodomain-only form of the caspase that lacks proteolytic activity in vitro but efficiently induces the formation of perinuclear filamentous structures and cell death in vivo. Caspase-10/c mRNA is specifically up-regulated upon TNF stimulation, suggesting a potential role of this isoform in amplifying the apoptotic response to extracellular stimuli such as cytokines. Caspase-10/d is a hybrid of the known caspases Mch4 and FLICE2, as it is identical to FLICE2 except for the small (p12) catalytic subunit, which is identical to Mch4. Caspase-10/d is proteolytically active in vitro and also induces cell death in vitro, although it is less active than Mch4. The mRNAs for all known isoforms of caspase-10 are abundantly expressed in fetal lung, kidney, and skeletal muscle but are very poorly expressed or absent in these tissues in the adult, implying a possible role for the caspase-10 family in fetal development.

Apoptosis is a process of regulated cell suicide crucial for the development and homeostasis of multicellular organisms that is characterized by chromatin condensation, DNA fragmentation, cell shrinkage, and plasma membrane blebbing (1). The gene products of ced-3 and ced-4 regulate the apoptotic death of cells during normal development of the nematode Caenorhabditis elegans (2). Subsequently, CED-3 was shown to have a mammalian homologue named interleukin 1β-converting enzyme (ICE; caspase-1 (3), which was the first of a series of proteases to be identified with an active-site cysteine and a unique aspartate-Xaa substrate specificity. In recognition of these properties, this family of apoptotic proteases was renamed the caspases, for cysteine-aspartic acid proteases (4). With the recent discovery of MICE (5), the number of caspases now stands at 14, including murine proteases.

Caspases are present as inactive proenzymes comprising a prodomain and a catalytic protease domain that can be further processed to give a large and a small subunit. Crystallographic data (6, 7) has revealed for caspases 1 and 3 and by extrapolation to the other caspase family members that the activated caspase exists as a heterodimeric tetramer of two large and two small subunits, with each subunit binding pocket spanning both subunits. Evidence for the sequential activation of caspases has led to the concept of a caspase cascade (8–11), with initiator caspases at the apex, transmitting signals to executor caspases that cleave a wide variety of substrates. In this scheme, caspases 2, 5, and 10 (12–16) have been demonstrated to associate with the death receptors Fas and TNF receptor 1 and are presumably initiator caspases.

In terms of their substrate specificities, the caspases can be classified into three groups (17, 18), all of which have an absolute requirement for an aspartate residue in the P1 position but otherwise show varying degrees of flexibility. Group I caspases (caspases 1, 4, and 5) prefer the tetrapeptide sequence WEHD; group II caspases (caspases 2, 3, and 7) have a DED specificity, whereas group III caspases (caspases 6, 8, 9, and 10) cleave after (I/L/V)ED. It should be noted, however, that these are only the preferred substrate motifs and are not absolute, being dependent on factors such as concentration, reaction time, and accessibility. Multiple redundancies are common among the caspases, with many caspases having the ability to cleave the same substrates, at least in vitro (17, 18). There is an additional level of complexity with the discovery that multiple isoforms are present for many caspases, including caspases 1, 2, 6, and 8 (19). These isoforms are most likely splice variants (20) or variants derived from post-translational modifications, but the biological significance of these variants is mostly unclear.

In this paper we describe the identification and characterization of two novel isoforms of caspase-10 (caspase-10/c and caspase-10/d) that incorporate features of both Mch4 (caspase-10/a) (14) and FLICE2 (caspase-10/b) (15). We describe their tissue expression and activity both in vitro and in vivo and examine the effect of exogenous stimulation by TNF on their mRNA expression.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—Expression vectors coding for PARP or caspase-3/Yama and the 293T cell line were generous gifts from Dr. V. Dixit. Antibodies against the large and small catalytic subunits of caspase-10 were obtained from Research Diagnostics, Inc. The polyclonal serum directed against a caspase-10/c-specific peptide sequence (EGSCVCQDEEPQRPL) was produced by Genosys, Inc. Chemiluminescent Western blots were developed using the Immun-Star kit from...
Bio-Rad. Unless otherwise mentioned, all other reagents were purchased from Sigma.

Cloning and Identification of Caspase-10 Variants—PCR was performed on human spleen, thymus, and peripheral blood leukocyte cDNA libraries (CLONTECH) using primers 5MCH4 (AAA GAA TCA GCT AGC ATG AAA TCT CAA GGT CAA CAT TGG TAT TCC) and 3MCH4 (AAA GAA TTC CTA TAT TGA AAG TGC ATC CAG GGG CAC AGG) to obtain the coding regions of Mch4, caspase-10/c, and caspase-10/d. Advantage-HF polymerase (CLONTECH) was used with cycling conditions recommended by the manufacturer. The final clones were from spleen (caspase-10/c) and thymus (caspase-10/d). The PCR products were inserted into pCITE-4a (Novagen) for in vitro expression as S-Tag fusion proteins or pCI-neo (Promega) for in vivo eukaryotic expression.

mRNA Expression Analysis—The primers CB14 (CGG AGT ATC ATG GAG GAG AAG AAC) and 3BRIDGE (TAT ATG CAC TGT ATC AAG GAG AGG AAG AAC) were used in PCR and RT-PCR experiments to obtain short DNA fragments corresponding to Mch4, caspase-10/c, and caspase-10/d. RNA from various cell lines was purified using the Qiagen RNaseasy kit, and reverse-transcription was performed using SuperScript II enzyme with oligo(dT) primers from Life Technologies, Inc. The primers were then performed for 30 cycles on the resulting cDNA at an annealing/extension temperature of 68 °C using Advantage-HF polymerase or on cDNA from adult and fetal multiple tissue cDNA panels (CLONTECH), and the products were analyzed in ethidium bromide-stained 2.5% agarose gels.

Transfections, Killing Assays, and Solubility Studies—For the killing assays, MCF7 cells were seeded at a density of 1 × 10^6 cells/cm^2 in 60-mm dishes in 5 ml of RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. At 6 h post-transfection, the cells were stained for 2 min in propidium iodide for nuclear visualization, mounted in Vectashield (Vector Laboratories), and visualized under confocal laser scanning microscopy (Bio-Rad MRC 1024).

TNF Stimulation and mRNA Expression—MCF7 cells were seeded at a density of 5 × 10^5 cells/100-mm dish in 10 ml of growth medium, then grown to half confluence, usually 48 h post-seeding. Recombinant human TNF-a (BIOSOURCE) was added directly to the conditioned medium at a final concentration of 50 ng/ml where required. Treated cells were harvested, and RT-PCR was performed essentially as described above.

Cytotoxicity Assay—MCF7 cells were seeded at a density of 3 × 10^4 cells/well in 96-well plates. Approximately 48 h post-seeding, recombinant human TNF-a was added at a final concentration of 50 ng/ml. At various time points, the medium was aspirated, and the cells were washed with 100 μl of PBS, fixed in 5% formaldehyde, PBS for 5 min at 37 °C with 100 μg/ml DNase-free RNase in 2× SSC. After rinsing with 2× SSC, the plates were stained for 2 min in propidium iodide for nuclear visualization, mounted in Vectashield (Vector Laboratories), and visualized under confocal laser scanning microscopy (Bio-Rad MRC 1024).

In Vitro Cleavage Analysis—The coding regions of Mch4, caspase-10/c, and caspase-10/d starting from Ile (194) (14) were each inserted into the pGEX-2TK bacterial expression plasmid (Amersham Pharmacia Biotech), enabling their expression as GST fusion proteins in E. coli JM109. The pellets from 200 ml of 1 mM isopropyl-β-D-thiogalactopyranoside-induced culture were resuspended in 20 ml of ice-cold lysis buffer (25 mM Hepes, pH 7.4, 5 mM EDTA, 2 mM dithiothreitol, 0.1% CHAPS) and sonicated for 3 x 20 s. After centrifugation for 30 min at 12,000 × g, the supernatants were collected and used as the source of active caspase. Between 2 and 5 μl of induced sonicate (depending on the amount of protein p12 small subunit, as described under “Results”) was added to 3 μl of [35S]methionine-labeled substrates expressed in vitro using the T7 Quick Tnt system (Promega). The reaction was then added to a final volume of 20 μl. Where used, the tetrapeptide inhibitors Ac-YVAD-CHO and Ac-DEVD-CHO were added to a final concentration of 1 μM. In some experiments, the radiolabeled in vitro translated proteins were first affinity-purified by mixing the entire translation product with 200 μl of S-protein agarose slurry (Novagen) and using 10 μl of the washed, protein-bound matrix as substrates.
RESULTS

Cloning and Identification of Caspase-10 Variants—Previous reports had indicated the possible existence of multiple isoforms of caspase-10 (15, 21). Our initial cloning by PCR of what appeared to be a single band of approximately 1500 bp emerged on sequencing to consist of three individual DNA fragments, wild type Mch4 (1440 bp), caspase-10/c (1477 bp), and caspase-10/d (1569 bp). It was possible to distinguish electrophoretically between these three DNAs by designing PCR primers (5MCH4/3BRIDGE) spanning a mid-insert region (comprising exons 6 and 7) common to caspase-10/d and FLICE2 (Fig. 1). Bands of 230, 260, and 350 bp in size correspond to Mch4, caspase-10/c, and caspase-10/d, respectively (see below).

In the recent paper on the characterization of Usurpin (22), the authors described the sequencing of the death effector domain (DED)-caspase gene cluster on human chromosome 2 band q33–34, which contains the genes for Usurpin, caspase-10/a/Mch4, and caspase-8/a/FLICE. Based on the genomic map, the mid-insert region mentioned above consists of exons 6 and 7 (Fig. 1). Hence Mch4 lacks exons 6, 7, and 11. In contrast, FLICE2 contains exons 6 and 7 but skips exon 10 and contains part of exon 11. Caspase-10/c contains exon 6 but not 7, whereas caspase-10/d contains both exons 6 and 7 (summarized in Fig. 1).

The predicted amino acid sequences show that caspase-10/d is a hybrid of FLICE2 and Mch4 (Fig. 2). Interestingly, the insertion of exon 6 in caspase-10/c leads to a frameshift at the protein level, resulting in a protein that is truncated shortly after the DED-containing prodomain. Thus, caspase-10/c is essentially a prodomain-only protein (Fig. 2). We confirmed the authenticity of the predicted protein sequences by translation in vitro and by performing immunoblotting on the products. As predicted, in vitro translated caspase-10/d was slightly larger in size than Mch4 and was recognized by antibodies against both the large and small protease subunits of Mch4 (Fig. 3). In contrast, in vitro translated caspase-10/c was a much smaller protein of approximately 40 kDa on SDS-polyacrylamide gel electrophoresis (Fig. 3, upper panel) that was not recognized by either of the anti-protease subunit antibodies (Fig. 3, lower panel).

Tissue- and Cell Line-specific mRNA Expression of Caspase-10 Isoforms—To determine the constitutive mRNA expression profiles of the caspase-10 variants, PCR and RT-PCR was performed on RNA extracted from various cell lines and either autoradiographed (A) or immunoblotted (Western blot) (B) with antibodies directed against the p12 subunit of Mch4 (lanes 1–4) or the p17 subunit of Mch4 (lanes 5–8). −, vector only; WT, Mch4; 10/c, caspase-10/c; 10/d, caspase-10/d.

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pheral blood leukocytes cDNA libraries. Although this method did not distinguish between FLICE2 and caspase-10/d expression (because they both contain the same exon 6 and 7 insert), it is clear that expression levels of caspase-10-related mRNAs are generally higher in fetal than in adult tissues (Fig. 4A). In particular, Mch4, caspase-10/c, and FLICE2/caspase-10/d are highly expressed in fetal skeletal muscle, lung, and kidney, whereas their expression in these tissues is virtually undetectable in the adult.

Expression of the caspase-10 isoforms was highly variable among various cell lines (Fig. 4B). There was no obvious pattern in their distribution. The 350-bp FLICE2/caspase-10/d band was consistently highly expressed in all cell lines tested, whereas caspase-10/c and Mch4 were present at very low levels in the MCF7 breast carcinoma and SW480T adenocarcinoma cell lines (Fig. 4B). Caspase-10/c was not absent, as increasing the number of PCR cycles resulted in easier visualization (not shown).

FIG. 4. Tissue and cell line distribution of Mch4, caspase-10/c, and caspase-10/d/FLICE2 mRNAs. A, first-strand cDNA (CLONTECH multiple tissue cDNA (MTC)) prepared from various human adult or fetal tissues was amplified by PCR using the primer pair C614/3BRIDGE to distinguish between Mch4, caspase-10/c, and caspase-10/d/FLICE2 transcripts, as described in the text. The 200-bp fragment seen in the adult pancreas lane is a spurious product not reproducible in other experiments. The slight variation in glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels is because of the multiple housekeeping gene normalization method employed by the supplier. SK MUSC., skeletal muscle; SMALL INT., small intestine; PBL, peripheral blood leukocytes. B, RT-PCR was performed on RNA prepared from various cell lines. WT, 10/c, 10/d, control PCR reactions using plasmids coding for Mch4, caspase-10/c, or caspase-10/d, respectively. CTRLs, controls; GAPDH, control RT-PCR using primers specific for glyceraldehyde-3-phosphate dehydrogenase.

FIG. 5. Mch4 and caspase-10/d, but not caspase-10/c, are autoprocessed and proteolytically active in vitro. This activity is inhibited by Ac-DEVD-CHO but not by Ac-YVAD-CHO. A, Mch4 and caspase-10/d undergo autoprocessing. 20 μl of crude bacterial sonicates from 200-ml bacterial cultures expressing GST fusion Mch4, caspase-10/c, or caspase-10/d were electrophoresed, transferred to nitrocellulose, and immunoblotted with antibody against the Mch4 p12 small subunit. B, sonicates of GST-Mch4 or GST-caspase-10/d cleave radiolabeled in vitro translated Mch4 and caspase-10/d. C and D, cleavage of PARP and caspase-3 by Mch4 or caspase-10/d is inhibitable by Ac-DEVD-CHO. 3 μl of radiolabeled in vitro translated PARP (C) or caspase-3 (D) were mixed with sonicates of GST-Mch4 (lanes 2, 6, and 10; 2 μl), caspase-10/c (lanes 3, 7, 11; 5 μl), or caspase-10/d (lanes 4, 8, 12; 5 μl). Lanes 1, 5, and 9 are controls with 5 μl of pGEX-2TK vector sonicate added; --, radiolabeled in vitro translated product only. Lanes 5–8, 1 μl Ac-DEVD-CHO inhibitor added; lanes 9–12, 1 μl Ac-YVAD-CHO inhibitor added. P1/P2, cleaved products of PARP; Y1/Y2, cleaved products of caspase-3; WT, 10/c, 10/d, Mch4, caspase-10/c, and caspase-10/d, respectively; GWT, G10/c, G10/d, GST-Ile/134-Mch4, GST-Ile/134-caspase-10/c, and GST-Ile/134-caspase-10/d, respectively. TnT, T7 in vitro transcription and translation system (Promega).
Caspase-10/d but Not Caspase-10/c Is Proteolytically Active in Vitro—Crude sonicated lysates of bacterial cultures expressing recombinant Mch4 and caspase-10/d were proteolytically active. Immunoblotting of the bacterial sonicates using antibodies against the Mch4 p12 small subunit revealed that autoprocessing had occurred in the case of Mch4 and caspase-10/d with the release of the p12 subunit but not with caspase-10/c (Fig. 5, panel A). The bacterial sonicates containing Mch4 and caspase-10/d cleaved in vitro translated full-length Mch4 and caspase-10/d proteins, demonstrating the possibility of intermolecular processing of caspase-10 isoforms (Fig. 5, panel B) and also cleaved in vitro translated PARP and caspase-3 (Fig. 5, panels C and D). Again, caspase-10/c was not proteolytically active in any of these experiments. The activity of Mch4 or caspase-10/d was completely abrogated by the addition of 1 μM specific caspase inhibitor Ac-DEVD-CHO but not Ac-YVAD-CHO (Fig. 5, panels C and D). The cleavage activity (and the inhibition thereof) is because of the expressed recombinant caspase and not to the activation of any other endogenous proteases because (i) bacteria do not have endogenous caspase activity (Ref. 23 and vector controls in Fig. 5, panels B–D), (ii) affinity-purified recombinant caspases are proteolytically active (14, 23), and (iii) the in vitro translation products used as substrates in some of our experiments were first purified from any contaminating reticulocyte components.

In addition, we consistently observed that caspase-10/d was less active than Mch4 after normalization of the starting quantities of bacterial sonicates used based on the amount of processed p12 subunit. We quantitated this activity using the caspase substrate DEVD-pNA and confirmed that Mch4 was approximately twice as active as caspase-10/d (not shown).

Caspases 10/c and 10/d Are Highly Toxic to MCF7 Cells—Despite the lack of a protease domain, caspase-10/c was highly efficient in causing apoptosis when transiently overexpressed in the MCF7 cell line. Caspase-10/d was also pro-apoptotic, as was Mch4 used as a control (Fig. 6). This killing by all three isoforms was caspase-dependent, as shown first by the dose-dependent inhibitory effect of the tetrapeptide inhibitor Ac-DEVD-CHO (Fig. 6, panel A) and second by the observation that PARP was cleaved in cells transfected with Mch4, caspase-10/c, and caspase-10/d (Fig. 6, panel B, lanes 3–5). Furthermore, the fact that Ac-DEVD-CHO but not Ac-YVAD-CHO inhibited killing indicated that apoptosis was transduced by downstream caspase-3-like enzymes, and caspase-1-like proteases were probably not involved (17, 18).

2 P. W. P. Ng, A. G. Porter, and R. U. Janicke, unpublished observations.

**Fig. 6.** Apoptosis induced by the transient overexpression of Mch4, caspase-10/c, or caspase-10/d in MCF7 cells involves PARP cleavage and is inhibited by Ac-DEVD-CHO but not by Ac-YVAD-CHO. A, solid black bars represent killing in the absence of inhibitors. Gray shaded bars represent killing in the presence of Ac-DEVD-CHO. The expression levels of the transfected proteins are shown in Fig. 7, panel B. B, immunoblot showing PARP cleavage in transfected MCF7 cells. NIL, no DNA added. CI (—), vector only; WT, 10/c, 10/d, Mch4, caspase-10/c, and caspase-10/d, respectively.

**Fig. 7.** Mch4 and caspase-10/d are soluble proteins, whereas caspase-10/c is present mainly in the insoluble fraction of cell lysates. 293T (A) or MCF7 (B) cells transiently overexpressing Mch4, caspase-10/c, or caspase-10/d (WT, 10/c, 10/d, respectively) were lysed and separated into detergent-soluble and -insoluble fractions as described in the text. Lysates were immunoblotted with anti-Mch4 p12 polyclonal antibody to detect Mch4 or caspase-10/d protein (open arrows, upper blots) or with custom antiserum against caspase-10/c protein (open arrows, lower blots). +, total protein lysates of cells overexpressing caspase-10/c; —, vector-only transfection.

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Mch4 and Caspase-10/d Are Soluble Proteins, Whereas Caspase-10/c Is Insoluble and Localizes to Filamentous Perinuclear Structures—Lysates of mammalian cells transiently overexpressing Mch4, caspase-10/c, or caspase-10/d were prepared as detergent-soluble and -insoluble fractions, and immunoblotting was performed using either anti-Mch4 p12 antibodies to detect Mch4 and caspase-10/d or custom anti-caspase-10/c polyclonal antiserum. In both MCF7 and 293T cell lines, Mch4 and caspase-10/d were completely soluble, whereas caspase-10/c was predominantly insoluble (Fig. 7). These results are consistent with recent evidence that DED-only proteins might form insoluble filamentous structures (death effector filaments, DEF) either on their own or in association with as yet unidentified cytoskeletal elements (24). To determine whether DEFs were formed by caspase-10 isoforms, Mch4, caspase-10/c, and caspase-10/d were ectopically expressed as EGFP fusion proteins in MCF7 and 293T cells and visualized using confocal laser scanning microscopy. Distinct perinuclear filamentous structures were seen in all cells expressing caspase-10/c-EGFP (Fig. 8, panel A). In most cases these filaments were closely bundled together, but more haphazard arrangements were sometimes also observed. EGFP alone was present in both the nucleus and diffused in the cytoplasm (Fig. 8, panels N1 and N2). Mch4-EGFP and caspase-10/d-EGFP were mostly diffused throughout the cytoplasm (Fig. 8, panels WT1 and WT2), although these were never as distinct as those seen in caspase-10/c-transfected cells. Killing assays performed on MCF7 cells transiently overexpressing EGFP-caspase-10 fusion proteins confirmed that these EGFP fusion proteins were functionally active and induced apoptosis (data not shown).

Caspase-10/c mRNA Is Up-regulated by TNF—It has been shown that Mch4 and FLICE2 can associate with TNF receptor 1 via homotypic DED interactions with Fas-associated death domain protein (FADD) (15). It was of interest to know whether the expression of the mRNAs of any of the caspase-10 isoforms was altered by TNF stimulation. The addition of TNF resulted in a specific up-regulation of caspase-10/c mRNA. This up-regulation was transient and typically observed at 18 h after TNF stimulation (Fig. 9, panel A), although it was sometimes observed at other times between 12 and 24 h post-stimulation (data not shown). The peak of mRNA induction occurred before or during the majority of apoptotic cell death (Fig. 9, panel B) and was not observed in parallel control experiments in which TNF was omitted (Fig. 9, panel A).

**DISCUSSION**

Previous work on the DED-containing protein MRIT (c-FLIP/CASH/CLARP/I-FLICE/CASPER/FLAME-1/Usurpin) (22, 25–

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**FIG. 8. Caspase-10/c localizes to distinct perinuclear filaments.** MCF7 cells transiently overexpressing EGFP (N1 and N2), Mch4-EGFP (WT1 and WT2), caspase-10/c-EGFP (A), or caspase-10/d-EGFP (B) were stained with propidium iodide to allow nuclear visualization then observed under confocal laser scanning microscopy. N2 is a single-channel output of N1 and shows the nuclear and cytoplasmic distribution of EGFP.

**FIG. 9. Caspase-10/c mRNA is specifically up-regulated by TNF and correlates with TNF-induced cell death.** A, MCF7 cells were stimulated with 50 ng/ml TNF (lanes 4–7) and harvested at various times as indicated. RT-PCR was performed on RNA prepared from these cells as detailed in the text, and the products were electrophoresed in 2.5% agarose gels. Lanes 8–11, parallel experiment without TNF. The figure shows one set of results that are representative of at least three similar experiments. WT, 10/c, 10/d, control PCR reactions using plasmids coding for Mch4, caspase-10/c, or caspase-10/d, respectively. GAPDH, control RT-PCR using primers specific for glyceraldehyde-3-phosphate dehydrogenase. B, TNF was added to MCF7 cells grown in 96-well plates, and the extent of killing at various time points was measured as described under “Experimental Procedures.”

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31) has been controversial, because in some studies, MRIT had an inhibitory effect on apoptosis, whereas in others, MRIT induced cell death. Interestingly, this protein was reported to exist as two splice-isofoms, with the shorter variant essentially comprising just two DED domains, analogous in structure to the pro-apoptotic FLICE isoform MACH (12) and our caspase-10/c. The recent work on DEF (24) sheds some light on the reason for this controversy, suggesting that apoptotic activity is a concentration-dependent phenomenon. Expression of only the DED of FLICE (but not the full-length protein) above a certain concentration threshold resulted in the formation of an insoluble filamentous perinuclear structure that apparently acted as a scaffold to recruit and facilitate the autoactivation of cytoplasmic DED-containing pro-caspases (24). We also showed that pro-apoptotic caspase-10/c is present mainly in the insoluble fraction of cell lysates (in contrast to the predominantly soluble full-length Mch4 and caspase-10/d proteins) and that ectopically expressed caspase-10/c-EGFP fusion protein localizes intracellularly as distinct perinuclear structures identical to the DEFs described previously. These observations provide a plausible explanation for the mechanism of action of caspase variants containing only the DED and suggest that caspase-10/c and the β-isofoms of FLICE and MRIT may play a role in amplifying the apoptotic response through the formation of similar “activating scaffolds.”

How might caspase-10/c induce apoptosis after the possible formation of the DEF? We showed that cell death induced by caspase-10/c was inhibitable by Ac-DEVD-CHO, suggesting the requirement for caspase-3-like proteases. Yet, in MCF7 cells, caspase-3 is absent (32), and we were unable to detect specific caspase-10/c-induced activation of the two other known DEVD-inhibitable caspases 2 and 7,2 which is similar to the situation in TNF-induced apoptosis (33). Thus, the caspases that are activated by the overexpression of caspase-10/c in MCF7 cells remain to be identified.

It is known that caspase transcripts are constitutively present in many cells, albeit at different levels of expression, and because apoptosis can occur in the presence of protein synthesis inhibitors such as cycloheximide (21), pro-caspases must already be present at sufficient levels to allow the apoptotic response to proceed. Nonetheless, it has been shown that caspase transcripts are up- and down-regulated in development. For example, caspase-2 (Ich-1/Nedd2) mRNA is more highly expressed in the mouse during embryonic development than in the adult, particularly in the brain, liver, lung and kidney (34). Our observations that the expression of Mch4 and caspases-10/c and -10/d/FLICE2 is considerably higher in fetal than in adult tissues, particularly in skeletal muscle, lung, and kidney, suggests a role for the isoforms of caspase-10 during development.

Caspase mRNAs may also be up-regulated by extracellular stimuli as well as in a genetic program during development. Caspase-1, -3, -4, -7, -8, and -10 transcripts are up-regulated within 16 h of interferon-γ stimulation (35, 36), and the CASP-2 and -3 genes are also up-regulated in response to etoposide treatment (37). We showed TNF induces the expression of caspase-10/c mRNA but not caspase-10/d or Mch4 transcripts, the first demonstration that the mRNA of a protein encoding only the DED is up-regulated by a specific stimulus. Overall, these studies suggest that cytokine-induced cytotoxicity could potentially involve the up-regulation of specific caspase isoforms.

Accordingly, we propose a hypothetical model (Fig. 10) wherein the binding of TNF to its receptors (most likely TNF receptor 1 (15)) results in the recruitment and activation of caspase-8 and/or -10 at the plasma membrane, and simulta-
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