# A Combinatorial Approach Defines Specificities of Members of the Caspase Family and Granzyme B

FUNCTIONAL RELATIONSHIPS ESTABLISHED FOR KEY MEDIATORS OF APOPTOSIS\*

(Received for publication, March 6, 1997, and in revised form, May 1, 1997)

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There is compelling evidence that members of the caspase (interleukin-1ß converting enzyme/CED-3) family of cysteine proteases and the cytotoxic lymphocytederived serine protease granzyme B play essential roles in mammalian apoptosis. Here we use a novel method employing a positional scanning substrate combinatorial library to rigorously define their individual specificities. The results divide these proteases into three distinct groups and suggest that several have redundant functions. The specificity of caspases 2, 3, and 7 and Caenorhabditis elegans CED-3 (DEXD) suggests that all of these enzymes function to incapacitate essential homeostatic pathways during the effector phase of apoptosis. In contrast, the optimal sequence for caspases 6, 8, and 9 and granzyme B ((I/L/V)EXD) resembles activation sites in effector caspase proenzymes, consistent with a role for these enzymes as upstream components in a proteolytic cascade that amplifies the death signal.

Apoptosis is a form of cell death that is essential for morphogenesis, tissue homeostasis, and host defense (for review see Ref. 1). There is accumulating evidence that defects in apoptosis may lead to several pathologies including some neurodegenerative disorders, ischemic injury, and cancer (2). The discovery that CED-3, the product of a gene necessary for programmed cell death in the nematode Caenorhabditis elegans, is related to the cysteine protease interleukin-1 $\beta$  converting enzyme (ICE, caspase-1)<sup>1</sup> established proteases to be key mediators in this process (3). Although the precise biochemical pathways involved in mammalian cell death remain ill-defined, it is now clear that proteases play an essential role both in the initial signaling events and in the downstream processes that result in the apoptotic phenotype. Those that are known to be involved include members of the ICE/CED-3 or caspase (4) family of cysteine proteases and the cytotoxic lymphocyte-derived serine protease, granzyme B. The identification of potential endogenous substrates has provided important clues to their molecular role(s); however, the identities of the enzymes involved and their relationships to each other remain obscure. To better understand the roles of proteases in apoptosis, to identify appropriate fluorogenic substrates, and to facilitate inhibitor design, peptide substrate specificities for the caspases and granzyme B were determined using a positional scanning synthetic combinatorial library (PS-SCL) (5). The results from this study establish new functional relationships between these important biological mediators.

## EXPERIMENTAL PROCEDURES

Preparation of Recombinant Caspases-The method used for production of caspases 1, 2, 3, 4, 5, 7, 8, and 9 involves folding of active enzymes from their constituent large and small subunits, which are expressed separately in Escherichia coli. The details of the methods used for preparation of caspases 1 and 3 are described elsewhere (6, 7). The other homologs were engineered in a similar manner by polymerase chain reaction-directed template modification to generate the following constructs: caspase-2 as a MetAsn<sup>150</sup>-Asp<sup>316</sup> large subunit with a MetSerGly<sup>332</sup>-Thr<sup>435</sup> small subunit; caspase-4 as a MetSerGly<sup>95</sup>-Asp<sup>270</sup> large subunit with a MetSerVal<sup>291</sup>-Asn<sup>377</sup> small subunit; caspase-5 as a MetSerGly<sup>136</sup>-Asp<sup>311</sup> large subunit with a MetSer<sup>331</sup>-Asn<sup>418</sup> small subunit; caspase-6 as a MetSerAla<sup>34</sup>-Asp<sup>179</sup> large subunit with a MetSerGln<sup>181</sup>-Asn<sup>293</sup> small subunit; caspase-7 as a MetSerLys<sup>25</sup>-Asp<sup>198</sup> large subunit with a MetSer<sup>199</sup>-Gln<sup>303</sup> small subunit; caspase-8 as a MetSer<sup>217</sup>-Asp<sup>374</sup> large subunit with a MetSer<sup>375</sup>-Asp<sup>479</sup> small subunit; and caspase-9 as a MetSerGly<sup>140</sup>-Asp<sup>305</sup> large subunit with a MetSerIle<sup>332</sup>-Ser<sup>416</sup> small subunit. To obtain active enzyme, the individual subunits from purified inclusion bodies were solubilized in 6 M guanidine HCl and then rapidly diluted to a final concentration of 100  $\mu$ g/ml at room temperature under conditions determined to be optimal for each enzyme. Caspase-2 was folded in 100 mM Hepes, 10 mM DTT, pH 7.5. Caspase-4 and caspase-5 were folded in 100 mM Hepes, 10% sucrose, 1% Triton X-100, 10 mM DTT, pH 7.5. Caspase-7 and caspase-8 were folded in 100 mM Hepes, 10% sucrose, 10 mM DTT, pH 7.5. Caspase-9 was folded in 100 mm Hepes, 20% sucrose, 1% Triton X-100, 10 mM DTT, pH 7.5. Active, recombinant CED-3 and caspase-6 were prepared by expressing a construct encoding the entire proenzyme, minus the N-terminal peptide, in E. coli, under conditions where a portion of the protein produced is cytosolic and undergoes self-maturation (C. elegans CED-3 was engineered for expression as a MetAla<sup>222</sup>-Val<sup>503</sup> prodomainless construct and caspase-6 as a MetSerPhe<sup>25</sup>-Asn<sup>293</sup> prodomainless construct).

Preparation of Human Granzyme B—To prepare homogeneous, human granzyme B, granules were isolated from cultured human natural killer leukemia YT cells and extracted with NaCl as described previously (8, 9) except that calcium was omitted. The resulting extract was diluted then loaded onto a Mono-S cation exchange column  $(0.5 \times 5 \text{ cm};$  Pharmacia Biotech Inc.) that had been pre-equilibrated in 50 mM MES, pH 6.1, 25 mM NaCl. After washing with 12 volumes of equilibration buffer, proteins were eluted with a linear gradient up to 1 M NaCl (in 50 mM MES, pH 6.1) over 40 column volumes. Granzyme B eluted at approximately 0.6 M NaCl and was homogeneous as judged by SDS-polyacrylamide gel electrophoresis.

Determination of Protease Specificity-The preparation of the PS-

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<sup>&</sup>lt;sup>1</sup> The abbreviations used are: ICE, interleukin-1 $\beta$  converting enzyme; PS-SCL, positional scanning synthetic combinatorial library; DTT, dithiothreitol; MES, 4-morpholineethanesulfonic acid; AMC, aminomethylcoumarin.



SCHEME 1.

SCL is described elsewhere (6). Each of the 60 samples (20 amino acids  $\times$  3 sublibraries) of the PS-SCL was prepared as a stock of approximately 10 mM in Me<sub>2</sub>SO. To determine protease specificity, enzyme was added to reaction mixtures containing 100  $\mu \rm M$  substrate mix, 100 mM Hepes, 10 mM DTT, pH 7.5, in a total volume of 100  $\mu \rm L$ . Under these conditions the final concentration of each individual compound is approximately 0.25  $\mu \rm M$ . Production of AMC was monitored continuously at room temperature in a Tecan Fluostar 96-well plate reader using an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

### RESULTS AND DISCUSSION

A PS-SCL with the general structure Ac-X-X-Asp-AMC was synthesized to determine the specificities of members of the caspase family and granzyme B (Scheme 1). The design of this library was based on several catalytic properties of the caspases, including their near absolute specificity for cleavage after aspartic acid and their ability to utilize tetrapeptides terminating in Asp-AMC as efficient fluorogenic substrates. Reports that granzyme B has a similar preference for aspartic acid in  $P_1$  (10, 11) suggested that this library would also be useful for defining the specificity of this enzyme. This PS-SCL is composed of three separate sublibraries of 8,000 compounds each. In each sublibrary, one position is defined with one of 20 amino acids (Scheme 1, O) (excluding cysteine), whereas the remaining two positions (Scheme 1, X) contain a mixture of amino acids present in approximately equimolar concentrations. Using this strategy, analysis of the three sublibraries (20 samples each) affords a complete understanding of the amino acid preferences across  $S_2$ ,  $S_3$ , and  $S_4$  subsites.

To validate this approach as providing a reliable measure of specificity, we have used it to determine the amino acid preferences of caspase-1/ICE (6). The results of these studies indicated that the preferred recognition motif for ICE is WEHD and led to the synthesis of a fluorogenic substrate, Ac-WEHD-AMC, that is cleaved 50-fold more efficiently than that previously believed to be optimal for this enzyme, with a second order rate constant ( $k_{cat}/K_m$ ) of  $3.3 \times 10^6$  M<sup>-1</sup> s<sup>-1</sup>. The corresponding tetrapeptide aldehyde, Ac-WEHD-CHO, was found to have a  $K_i$  for inhibition of caspase-1 of 56 pM, making it the most potent reversible, small molecule inhibitor described for any caspases. These results clearly demonstrated that this novel method furnishes an accurate identification of primary specificity that can be exploited to produce efficient substrates and potent inhibitors.

We have now extended this approach to determine the substrate specificities for *C. elegans* CED-3, nine of the ten known human caspase family members and granzyme B (Fig. 1). The results obtained with the caspases divide these enzymes into three major groups. Surprisingly, the peptide preferences within each group are remarkably similar. Members of Group I (caspases 1, 4, and 5) all prefer the tetrapeptide sequence WEHD. In contrast, the optimal peptide recognition motif for Group II caspases (2, 3, and 7 and CED-3) is DEXD. The similarity between caspase-3 and caspase-7 is particularly striking; their specificity profiles are virtually indistinguishable. The caspases in Group III (6, 8, and 9) prefer the sequence (L/V)EXD.

A comparison of the specificities of these enzymes reveals the following. First, aside from their stringent requirement for Asp in  $P_1$ ,  $P_4$  is the most critical determinant of specificity. Members of Group I can accommodate large aromatic/hydrophobic amino acids in this position, whereas those in Group II require Asp for efficient catalysis. The Group III caspases tolerate many different amino acids in  $P_4$  but prefer those with larger aliphatic side chains. Second, all of these enzymes prefer, to varying degrees, Glu in  $P_3$ . Finally, in general, liberal substitutions are tolerated in  $P_2$ . One notable exception is caspase-9, which has a stringent specificity for His in  $P_2$ . The structural basis for the distinct specificities of these enzymes can be inferred from the crystal structures of caspases 1 and 3 in complex with tetrapeptide-based inhibitors (7, 12, 13).

The results obtained with granzyme B indicate that the preferred recognition motif for this enzyme is (I/V)EPD. Interestingly, there are several striking similarities between the specificities of granzyme B and the caspases beyond what appears to be a common requirement for Asp in the  $P_1$ , including the importance of  $P_4$  and a preference for Glu in  $P_3$ . These results suggest that the active sites in this serine protease and the cysteine proteases of the caspase family may be structurally similar, even though there is no significant overall sequence homology between these proteins.

Several lines of evidence illustrate that the specificity observed with tetrapeptide substrates extends to macromolecules. Most compelling is the observation that the optimal tetrapeptide recognition motifs for some of these enzymes are identical or closely related to the sequences found in known macromolecular substrates (Fig. 2). In addition, with caspases 1 and 3, we have found that tetrapeptide substrates are cleaved just as efficiently or better than protein substrates (data not shown). We have also found that incorporation of the optimal tetrapeptide sequence into proteins that are not normally good substrates results in a substantial improvement in the rate of catalysis (data not shown). Clearly, other factors also influence the hydrolysis rate of macromolecules, such as tertiary structure. However, it appears that with these proteases primary sequence recognition is a necessary requirement for catalysis and that the results obtained with the PS-SCL approach accurately reflect macromolecular specificity.

Accordingly, an intimate understanding of their specificities provides important insights into their biological functions. First, the observation that closely related caspases have similar and in some cases identical specificities suggests either that these enzymes represent tissue-specific isoforms or that they have related or redundant functions within the same cell type. The similarities between the specificities of granzyme B and the caspases suggest an intriguing potential evolutionary link between these enzymes.

Second, the data presented here secure a functional relationship between the nematode death enzyme CED-3 and its closely related Group II human homologs. The optimal recognition motif for these enzymes (DEXD) is similar or identical to the cleavage sites in several cell maintenance and/or repair proteins that are proteolytically cleaved during apoptosis, including sterol regulatory element-binding proteins (14), D4-GDI (15), poly(ADP-ribose) polymerase (16), the 70-kDa subunit of the U1 small ribonucleoprotein (17), the catalytic sub-



FIG. 1. Substrate specificities of the caspases and granzyme B. Specificities were determined for nine of the ten known human caspases and CED-3 and granzyme B. The y axis represents the rate of AMC production expressed as a percentage of the maximum rate observed in each experiment. The x axis shows the positionally defined amino acids. The results indicate that these proteases fall into three major groups and suggest that several have redundant functions. Aliases for the proteases are shown in parentheses.

## Specificities of Caspases and Granzyme B

Enzyme	P4	P3	P2	P1		Ρ4	P3	P2	P <sub>1</sub>	Substrate
					[	F	Е	A	D	pro-interleukin-1ß site I
caspase-1	w	Е	н	D		Y	v	н	D	pro-interleukin-1B site II
						L	Е	s	D	interferon-y inducing factor
caspase-3			v	D		D	E	v	D	– poly(ADP-ribose) polymerase
						D	E	v	D	DNA-dependent protein kinase <sub>cs</sub>
	_	E				D	G	Р	D	70kDa U1 small ribonucleoprotein
	D					D	Е	P	D	sterol regulatory element-binding protein
						D	Е	L	D	D4 G-protein dissociation inhibitor
						D	Е	Е	D	huntingtin
caspase-6	v	Е	н	D	_	v	Е	I	D	– Iamin A
granzyme B		E	Ρ	D	_	1	Е	т	D	– caspase-3 proenzyme
	I						Q	А	D	caspase-7 proenzyme

FIG. 2. Comparison of protease specificities with cleavage site sequences within known substrates. These substrate sequences have been published elsewhere (14–17, 19, 24, 33, 37, 41, 42). The optimal tetrapeptide recognition motifs for some of these enzymes are identical or closely related to the sequences found in known macromolecular substrates, indicating that the tetrapeptide specificity determined in this study extends to macromolecules.

N-termina.	l Peptide	Large Subunit	<b>.C</b> XG Small Subunit					
			/					
	Proenzy	me	ļ	Ρ4	P3	P2	Р <sub>1</sub>	1
caspase-5	(ICE <sub>rel</sub> -III,	TY)	_	W	v	R	D	
caspase-4	(ICE <sub>rel</sub> -II,	TX, ICH-2)		w	v	R	D	
caspase-1	(ICE)			w	F	к	D	
caspase-7	(Mch3, IC	E-LAP3, CMH-1)	_		Q	A	D	
caspase-3	(CPP32, a	ipopain, Yama)		1	E	т	D	
caspase-6	(Mch2)			т	Е	v	D	
caspase-8	(MACH, F	LICE, Mch5)		v	Е	т	D	
caspase-10	(Mch4)				Е	Α	D	
caspase-2	(ICH-1)			D	Q	Q	D	
caspase-9	(ICE-LAP6	6, Mch6)		Р	E	Ρ	D	
C. elegans	CED-3			D	s	v	D	

FIG. 3. Activation sites in caspases. Known and putative activation sites in caspases (24, 38) are shown. Previous studies have established that the first event in activation of these proteases is cleavage at the C terminus of the large subunit (39, 40).

unit of DNA-dependent protein kinase (17), and protein kinase  $C\delta$  (18). Several of these are known endogenous substrates for caspase-3 (17, 19). It now appears that all members of this group function to cripple or destroy essential homeostatic pathways during the effector phase of apoptosis. The conclusion that these caspases have redundant effector functions is sup-

ported by the phenotype of caspase-3-deficient mice, where cleavage of poly(ADP)-ribose polymerase is observed in apoptotic thymocytes (20).

Finally, the optimal recognition motif for Group III caspases and granzyme B resembles activation sites within several effector caspase proenzymes (Fig. 3), specifically caspases 3 and 7, implicating these enzymes as upstream components in a proteolytic cascade that serves to amplify the death signal. This conclusion is supported by the results from several independent lines of research (21–32). For example, caspase-8 appears to be physically associated with the signaling complex during Fas-mediated cell death, suggesting that it functions as a initiator, as opposed to an effector, of the death pathway (30, 31). Notwithstanding this role, several of these enzymes tolerate broad substitution in  $P_4$ , leaving open the possibility that they have multiple functions in apoptosis, consistent with the proposed role for caspase-6 in cleavage of lamin A (27, 33).

Regarding the mechanism of activation of family members other than caspases 3 and 7, it is interesting to note that all of those with relatively long N-terminal peptides (caspases 1, 2, 4, 5, 8, 9, and CED-3) have specificities that are similar to their own activation sequences (Fig. 3), suggesting that these enzymes may employ an autocatalytic mechanism of activation. This observation implies that the N-terminal peptide plays an essential role in autocatalysis, perhaps in mediating dimerization between two proenzyme molecules. The importance of the N-terminal peptide to caspase-1 activation has recently been demonstrated (34). The fact that CED-3 falls into this category suggests that this enzyme can both self-activate and perform effector functions, consistent with the observation that it is the only caspase known to be in nematodes.

The results presented here do not provide compelling evidence for a role for the Group I caspases in apoptosis, because hydrophobic amino acids are not observed in the  $P_4$  position of proteins so far known to be cleaved during cell death. In contrast, studies with caspase-1-deficient mice have defined an important role for this enzyme in inflammation, where it appears to process both pro-interleukin-1 $\beta$  and pro-interferon- $\gamma$ inducing factor (35–37). Interestingly, although the cleavage site sequences in these proteins are consistent with the tetrapeptide specificity of caspase-1, they are not optimal (Fig. 3). This observation leaves open the possibility that this enzyme may have additional biological functions, consistent with evidence linking it to IL-1 $\alpha$  production (35, 36).

In conclusion, the results presented here, which precisely define the specificities of the caspases and granzyme B, suggest previously unknown functional relationships between these important biological mediators. This information can now be exploited to produce selective, small molecule inhibitors that will not only help to further define the biological roles of these enzymes but may have clinical utility. Moreover, these specificity "fingerprints" can now be used to identify caspases in different cell types and in other species, leading to additional insights into the biochemical mechanisms that govern apoptosis.

Acknowledgments—We thank Robert W. Myers and Antony Rosen for numerous helpful discussions and for critical evaluations of this manuscript.

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