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Substrate Specificities of Caspase Family Proteases*

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The caspase family represents a new class of intracellular cysteine proteases with known or suspected roles in cytokine maturation and apoptosis. These enzymes display a preference for Asp in the P1 position of substrates. To clarify differences in the biological roles of the interleukin-1β converting enzyme (ICE) family proteases, we have examined in detail the specificities beyond the P1 position of caspase-1, -2, -3, -4, -6, and -7 toward minimal length peptide substrates *in vitro*. We find differences and similarities between the enzymes that suggest a functional subgrouping of the family different from that based on overall sequence alignment. The primary specificities of ICE homologs explain many observed enzyme preferences for macromolecular substrates and can be used to support predictions of their natural function(s). The results also suggest the design of optimal peptidic substrates and inhibitors.

A growing body of evidence supports important roles for the interleukin-1β converting enzyme (ICE)[1] (1, 2) and its homologs (recently renamed caspases (3)) in cytokine maturation and apoptosis. The caspase gene family, defined by protein sequence homology but also characterized by conservation of key catalytic and substrate-recognition amino acids, includes caspase-2 (4), caspase-3 (5–7), caspase-4 (8–10), caspase-5 (10), caspase-6 (11), caspase-7 (12–14), caspase-8 (15–17), caspase-9 (18, 19), and caspase-10 (17). Each is an intracellular cysteine protease that shares with the serine protease granzyme B specificity for Asp in the P1 position of substrates. The specific biological roles and interrelationships of these enzymes are for the most part unknown and are areas of active investigation in many laboratories.

A role for caspase-1 in inflammation is supported by several lines of evidence. Caspase-1-deficient mice, and cells derived from those animals, are deficient in IL-1β maturation and are resistant to endotoxic shock (20, 21). Peptidic inhibitors of caspase-1 can be effective in blocking maturation and release of IL-1β by cultured cells (1) and in whole animals (22, 23) and of inflammation in animal models (24, 25). The selectivity of the inhibitors employed in these studies among the caspases has not been demonstrated, and so the precise role of each caspase in inflammation is uncertain. Nevertheless the results uphold the promise of caspase-1 and/or its homologs as targets for anti-inflammatory drug discovery.

Caspases play important roles in apoptosis signaling and effector mechanisms. Sequence alignments reveal homology with Ced-3 (26), a nematode cysteine protease (27, 28) that is required for cell death. The viral proteins CrmA and p35 are antiapoptotic and act by inhibition of caspases (29, 30). A bacterial invasin induces apoptosis by binding to and activating caspase-1 specifically (31). Caspase-3 is necessary and sufficient for apoptosis in one acellular model (6); however, in mice the essential function of this enzyme is limited to apoptosis in the brain (32). A hallmark of apoptosis is the proteolytic inactivation of poly(ADP-ribose) polymerase (33), and several caspases can catalyze that cleavage (7, 11–13, 18, 34, 35). Lamin A cleavage during apoptosis is catalyzed by caspase-6 (34, 36). Cleavage of other proteins at Asp residues, including DNA-protein kinases C, protein kinase C-δ, and Gas2, also accompanies apoptosis (37–41), although in most cases the enzyme(s) responsible for those reactions have not been identified.

Caspases can transduce or amplify signals by mutual activation. The "death domain" motifs of caspase-8 (15–17, 19) and -10 (17, 19) and their association with Fas or tumor necrosis factor receptors via interaction with FADD, suggests that they are upstream activators that proteolytically mature other caspases. Fas-induced apoptosis is characterized by an early, transient "caspase-1-like" protease activity followed by a "caspase-3-like" activity (42), suggesting an ordered activation cascade. Reconstitution experiments suggest that both caspase-3 and -7 are activated by caspase-6 (43) and caspase-10 (17). Little is known regarding caspase regulation or inactivation. Caspase-1 contains a site that is rapidly autodegraded *in vitro*, and cleavage at this site may represent a physiological mechanism of down-regulation (44).

To facilitate studies of the roles of caspase family proteases in inflammation and apoptosis, and to help elucidate protease-mediated mechanisms of signal transduction and regulation, we have characterized in detail the substrate preferences of caspase-1 and five of its homologs. We present here our findings of the specificities of each enzyme, which suggest the design of novel specific peptidic substrates and inhibitors, and help to predict the biological roles of these enzymes.

MATERIALS AND METHODS

Peptides—Underivatized peptides (Quality Controlled Biochemicals, Hopkinton, MA) were prepared by standard solid-phase methods, purified to ≥95% by HPLC, and confirmed by low resolution mass spectrometry. Concentrations of stock solutions in Me_2SO were determined in duplicate as described (45). Chromogenic and fluorogenic peptides were from California Peptide Research (Napa, CA). Concentrations of chromogenic peptide stock solutions were determined in duplicate as described (46). Concentrations of fluorogenic peptide stocks were determined in duplicate by UV absorbance using ϵ_{325} = 15,990 in aqueous buffer at pH 7.5.

Enzyme Expression and Purification—Caspase-1, -2, -3, and -4, containing N-terminal His tags to facilitate purification, were prepared as described (9, 30, 47). Caspase-1 contained the stability-enhancing point mutation D381E (47). Caspase-6 (Mch2α) (11) beginning at Ala-2, or

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[1] The abbreviations used are: ICE, interleukin-1β converting enzyme; IL-1β, interleukin-1β; HPLC, high performance liquid chromatography; pNA, *p*-nitroanilide; Amc, aminomethylcoumarin; Ac, acetyl; Am, amide; CHO, aldehyde.

caspase-7 beginning at Ala-23, with amino-terminal polyhistidine tags (MGHHHHHHGSG), were subcloned into a pBluescript II KS(+) (Stratagene, La Jolla, CA) derivative under control of the Lambda P_L promoter. Amino-terminal coding sequences were modified to reflect *Escherichia coli* codon preferences. To increase soluble expression the proteases were coexpressed with the bacterial chaperones groES and groEL (48). The *gro*ESL operon, under control of the isopropyl-1-thio-β-D-galactopyranoside-inducible P_{tac} promoter, was cloned into a pACYC177 (49) derivative encoding *Lac*I and the temperature-sensitive repressor cI^{857} inserted into the β-lactamase gene. Both plasmids were stably maintained in the same bacterial cell. Simultaneous expression of the chaperones and proteases were induced by addition of isopropyl-1-thio-β-D-galactopyranoside to 2 mM and temperature shift from 30 °C to 40 °C at A_{600} = 0.6. The bacteria were harvested at t = 5 h, and the proteases were purified as described (47).

Enzyme Assays—Peptide cleavage assays were modeled on those of Howard *et al.* (50). Substrate concentration depletion during the reaction is described by Equation 1.

$$\frac{d[S]}{dt} = \frac{V_{max}[S]}{[S] + K_m} \quad \text{(Eq. 1)}$$

which solves to the general case.

$$\frac{[S]_t}{[S]_0} e^{([S]_t - [S]_0)/K_m} = e^{-V_{max}t/K_m} \quad \text{(Eq. 2)}$$

Assuming $[S] \ll K_m$, Equation 2 reduces to the following equation.

$$\frac{[S]_t}{[S]_0} = e^{-kt} \quad \text{(Eq. 3)}$$

where k is the apparent first order substrate cleavage rate constant equal to V_{max}/K_m. Assays contained in 810 μl: 100 mM sodium acetate at pH 6.2 (for caspase-2) or 100 mM HEPES at pH 7.5 (all others), 20% (v/v) glycerol, 5 mM dithiothreitol, 0.5 mM EDTA, and 0.3–10 μg of purified enzymes. After a 30-min preincubation at 30 °C, substrates were added to 10 μM (except as noted). At 10-min intervals for 60 min, 110-μl aliquots were stopped with 11 μl of 3 M HCl. Samples were analyzed by HPLC using a 250 × 4.6 mm C_{18} reverse phase column (Vydak, Hesperia, CA) and a linear gradient of MeCN/H_2O with 0.1% (v/v) trifluoroacetic acid, monitoring at 214 nm. The identical elution times of one of the two product peaks for each peptide in a series confirmed cleavage at the same site despite amino acid variations. The area under the substrate peaks (arbitrary units) was plotted *versus* time and fitted to Equation 3. Relative V_{max}/K_m values (unitless) were obtained by normalizing apparent V_{max}/K_m values to 1.00 for a chosen peptide. K_m values were determined for selected substrates and enzymes by fitting initial rates of peptide cleavage at various substrate concentrations from 10 to 250 μM to the Michaelis-Menten equation.

Assays of chromogenic or fluorogenic substrate cleavage contained in 100 μl: 100 mM HEPES (pH 7.5), 20% (v/v) glycerol, 5 mM dithiothreitol, 0.5 mM EDTA, 0.1% (w/v) bovine serum albumin, and 0.3 (fluorogenic substrates) or 3.0–200 (chromogenic substrates) ng of purified enzymes. After a 30-min preincubation at 30 °C, substrates in Me_2SO were added to various concentrations. For chromogenic substrates, enzyme-catalyzed release of *p*-nitroanilide was monitored at 405 nm in a microtiter plate reader (Molecular Devices, Menlo Park, CA). For fluorogenic substrates, Amc release was monitored at 460 nm using 385 nm excitation in a Labsystems (Needham Heights, MA) Fluoroskan Ascent fluorescence plate reader. K_m and k_{cat} values were determined from plots of activity *versus* substrate concentration. Absolute k_{cat} values for chromogenic substrates were calculated using a standard curve determined with *p*-nitroanilide and are uncorrected for enzyme purity and percent activity.

Assays of inhibitor potency were performed with fluorogenic substrates using nominal enzyme concentrations of 0.1 nM or less. Apparent K_i values were calculated by dividing IC_{50} values by $(1 + [S]/K_m)$.

RESULTS

Assays of ICE Homolog Specificity—Building on previous work (1, 50, 51) we studied the substrate specificity of several caspases by measuring relative V_{max}/K_m values, a measure of enzymatic specificity (52), toward a series of defined peptide

TABLE I
K_m values of caspases for preferred peptide substrates
K_m values were obtained as described under "Materials and Methods."

Enzyme	Substrate	K_m
		μM
Caspase-1	Ac-YEVDGW-Am	105
Caspase-4	Ac-LEVDGW-Am	430
Caspase-3	Ac-VDQMDGW-Am	200
Caspase-2	Ac-VDVADGW-Am	150
Caspase-6	Ac-VQVDGW-Am	130
Caspase-7	Ac-VDQVDGW-Am	125

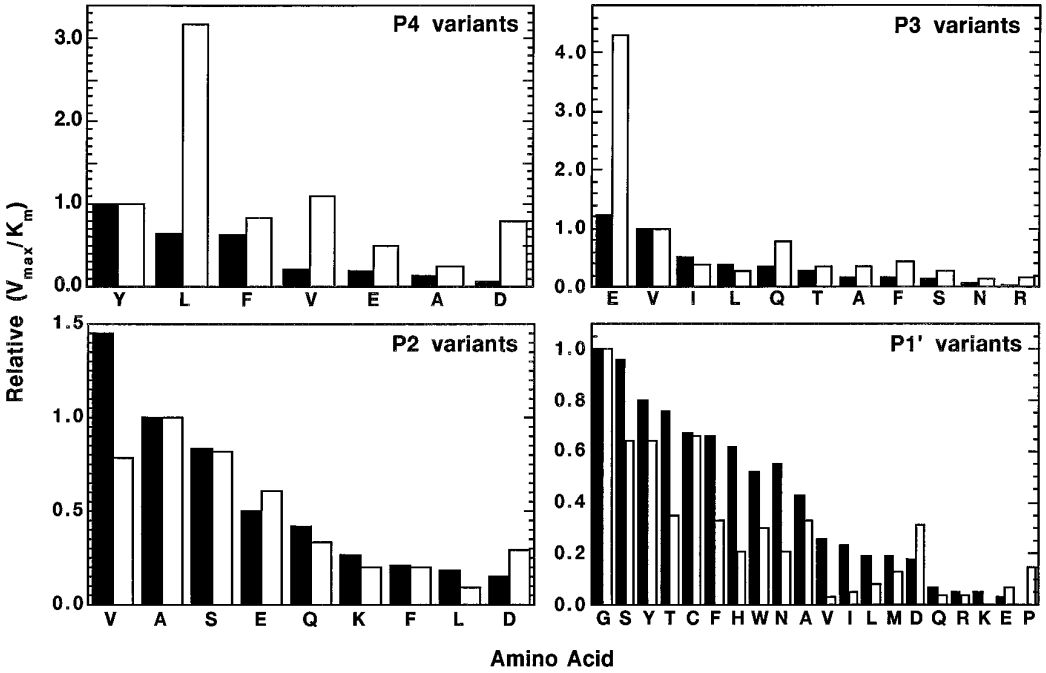

FIG. 1. **Peptide substrate preferences of caspase-1 and caspase-4.** Peptide substrate cleavage was measured for caspase-1 (*black bars*) and caspase-4 (*white bars*) using sequence variants of Ac-YVADGW-Am. Each panel displays the results for variants at a single position in the peptide, with substitutions shown on the *x axis*. Relative (V_{max}/K_m) values (*y axis*) for both enzymes are normalized to 1.00 for the results obtained with Ac-YVADGW-Am.

TABLE II
Kinetic constants for chromogenic peptide substrates

Enzymes were assayed and kinetic constants were calculated as described under "Materials and Methods." k_{cat} values are uncorrected for enzyme purity and fractional activity and so permit comparisons only between different substrates for a single enzyme. NC, no cleavage up to 1 mM substrate; ND, not done.

Substrate	K_m (μM), k_{cat} (M^{-1} s^{-1}), k_{cat}/K_m (s^{-1})					
	Caspase-1	Caspase-4	Caspase-2	Caspase-3	Caspase-7	Caspase-6
Ac-YVAD-pNA	23, 1.5, 63,000	874, 0.24, 280	NC	29,000, 3.0, 100	NC	NC
Ac-YEVD-pNA	7.3, 0.57, 79,000	31, 0.08, 2,600	ND	370, 14.1, 39,000	490, 6.9, 14,000	1200, 0.1, 90
Ac-LEVD-pNA	8.5, 0.40, 54,000	44, 0.14, 3,200	ND	ND	ND	160, 6.8, 42,000
Ac-DEVD-pNA	18, 0.50, 30,000	32, 0.05, 1,800	NC	11, 2.4, 218,000	12, 0.43, 37,000	180, 0.4, 2,000
Ac-DQMD-pNA	45, 0.94, 21,000	350, 0.09, 270	ND	44, 11, 262,000	130, 3.2, 25,000	1300, 0.3, 230
Ac-VDQQD-pNA	ND	ND	530, 8.0, 15,000	ND	3100, 4.8, 1,600	7000, 0.08, 11
Ac-VDVAD-pNA	ND	ND	53, 4.5, 84,000	67, 5.1, 76,000	200, 2.6, 13,000	ND
Ac-VEID-pNA	46, 0.56, 12,000	205, 0.15, 750	NC	250, 16, 61,000	570, 3.74, 6,600	30, 5.0, 168,000
Ac-VQVD-pNA	120, 0.33, 2,800	720, 0.12, 170	NC	510, 15, 29,000	2100, 6.2, 3,000	580, 7.1, 12,000

sequence variants. We chose this rather than a combinatorial approach to obtain quantitative data on both optimal and suboptimal substrate sequences. Peptides were generally of minimal length, contained C-terminal Trp residues to facilitate detection and concentration determination, and were acetylated and amidated to avoid introduction of nonnatural charges. Sequence variants were selected to test hypotheses of substrate recognition and to explore the structural range of natural amino acids. Application of Equation 3 requires that $[S]_0 \ll K_m$. K_m values of each enzyme toward preferred peptide substrates were determined from the initial rates of peptide hydrolysis as a function of peptide concentration (Table I). All observed values were at least 10-fold greater than the peptide concentration utilized in the specificity studies (10 μM), confirming that the use of Equation 3 is appropriate.

Caspase-1 and -4 Specificity—Caspase-1 and -4 specificities were tested using variants of Ac-YVADGW-Am, a preferred peptide substrate (1) based on the caspase-1 cleavage site within IL-1β (YVHD^{117}A) (53, 54). In P4, caspase-1 and -4 prefer hydrophobic amino acids (Fig. 1), with the former preferring aromatics (consistent with IL-1β cleavage), and the latter, aliphatics. In P3, both enzymes prefer Glu. The crystal structure of caspase-3 in complex with Ac-DEVD-CHO (55) shows that the P3 Glu of the peptidic ligand makes an ionic interaction with the side chain of the conserved residue Arg-207. Ligands containing P3 Glu probably make analogous favorable contacts with caspase-1 and -4. In P2, a broad range of amino acids are tolerated by both enzymes, consistent with the observation from the caspase-1 and -3 crystal structures that side chains of amino acids in this position are solvent-exposed. As expected (1, 51), caspase-1 and also caspase-4 prefer Gly in the P1′ position of substrates. In addition, we find that all of the aromatic and the thiol- and hydroxyl-containing amino acids were also efficiently cleaved.

As predicted from the peptide cleavage results, the chromogenic tetrapeptide *p*-nitroanilides Ac-YEVD-pNA and Ac-LEVD-pNA were preferentially cleaved by caspase-1 and -4, respectively, with k_{cat}/K_m values considerably higher than related peptides, including in the former case the widely used substrate Ac-YVAD-pNA (Table II).

Caspase-2 Specificity—In a survey of peptide substrates spanning known or suspected cleavage sites within caspases and their substrates, we found that only peptides containing VDQQD (caspase-2 residues 312–316) and LDVVD (caspase-6 residues 175–179) were cleaved by caspase-2, but peptides Ac-DQQDGW-Am and Ac-DVVDGW-Am were not. We examined the length dependence of peptide cleavage by caspase-2 (Table III) and found that, in contrast to caspase-1 (1), caspase-2 requires a P5 residue in peptide substrates for efficient cleavage. Like caspase-1 (1), efficient cleavage of peptides by caspase-2 did not require prime-side substrate residues (Table III). We therefore examined the substrate specificity of caspase-2 using sequence variants of Ac-VDQQDGW-Am (Fig. 2). Hydrophobic residues were preferred in the P5 position. Like caspase-3 and -7 (see below), caspase-2 displayed a strong preference for Asp in P4. In P3 and P2, Val and Ala, respectively, were preferred over Gln, but like caspase-1 and -4, Glu was a favored P3 substrate residue and in P2 a structurally wide range of amino acids were tolerated.

TABLE III
Length dependence of peptide cleavage by caspase-2

Peptide cleavage assays were performed, and relative (V_{max}/K_m) values were calculated as described under "Materials and Methods." Peptides are aligned with respect to the P1 residue, which is underlined for the first peptide.

Peptide	Relative (V_{max}/K_m)
Ac-RGVDQQD GKNHW-Am	1.00
Ac-GVDQQD GKNW-Am	0.65
Ac-VDQQD GKNW-Am	0.74
Ac-DQQD GKNW-Am	0.07
Ac-QQD GKNW-Am	0.02
Ac-VDQQD GKW-Am	0.75
Ac-VDQQD GW-Am	0.73
Ac-VDQQD W-Am	0.69

The results suggested that optimal caspase-2 peptidic substrates and inhibitors would contain the sequence VDVAD, and consistent with that, Ac-VDVADGW-Am was cleaved at a correspondingly greater efficiency than either of the singly substituted peptides (not shown). We tested Ac-VDVAD-pNA as a caspase-2 substrate and found it was preferred by caspase-2, cleaved with a k_{cat}/K_m value similar to those of other enzymes and their preferred substrates (Table II). Ac-DEVD-pNA, widely used as a caspase-3 substrate, was poorly cleaved by caspase-2. Using the fluorogenic substrate Ac-VDVAD-Amc (K_m = 80 μM), the pentapeptide aldehyde Ac-VDVAD-CHO

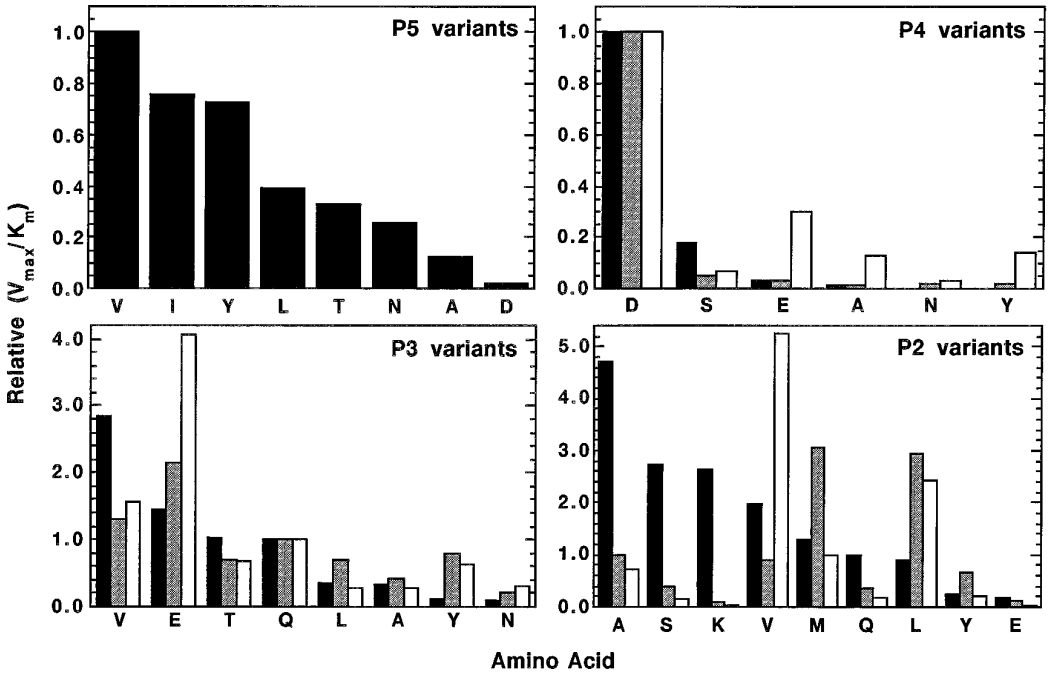

FIG. 2. **Peptide substrate preferences of caspase-2, caspase-3, and caspase-7.** Peptide substrate cleavage was measured for caspase-2 (*black bars*), caspase-3 (*gray bars*), and caspase-7 (*white bars*) using sequence variants of Ac-VDQQDGW-Am. Relative (V_{max}/K_m) values are normalized to 1.00 for the results obtained with Ac-VDQQDGW-Am, with the exception of P2, where the caspase-3 results are normalized to Ac-VDQADGW-Am and the caspase-7 results to Ac-VDQMDGW-Am.

inhibited caspase-2 with a K_i value of 3.5 nM. In contrast, the K_i of Ac-DEVD-CHO was 1.75 μM, again supporting the observed *in vitro* requirement of caspase-2 for a P5 peptide residue.

Caspase-3 and -7 Specificity—Caspase-3 and -7 were probed in P4-P2 using the peptide variants designed for caspase-2. Length dependence experiments showed that for these enzymes a P5 residue was unnecessary for efficient cleavage, and the presence of the P5 Val residue in particular did not significantly affect cleavage rates.[2] The two enzymes displayed many similarities in their primary specificities. The P4 Asp preference of caspase-3 was almost absolute (Fig. 2), with peptides containing charge-conserving (Glu) or isosteric (Asn) substitutions cleaved poorly. Caspase-7 also displayed a strong preference for Asp in P4. Like the other caspases, both enzymes tolerated a broad range of amino acids in P3 but preferred Glu, again consistent with the structure of caspase-3 bound to Ac-DEVD-CHO (55). In P2, caspase-3 and -7 were also similar to other caspases in their preferences for hydrophobic residues, but for these enzymes the preference was stronger. The favorable interaction of caspase-3 for Met in P2 probably contributes to its potent inhibition by p35, which inactivates caspase-3 by binding at a site containing DQMD (30, 56).

Consistent with the peptide cleavage assay results, the preferred chromogenic substrate for caspase-7 was Ac-DEVD-pNA (Table II). Caspase-3 also efficiently cleaved this peptide, and, as expected, cleaved the p35-based substrate Ac-DQMD-pNA with greater efficiency. The corresponding fluorogenic peptide Ac-DEVD-Amc displayed K_m values of 10 and 11 μM for caspase-3 and -7, respectively. With this substrate, Ac-DEVD-CHO inhibited both enzymes potently, with apparent K_i values of 0.2 and 0.3 nM, respectively. The caspase-2-designed inhibitor Ac-VDVAD-CHO also potently inhibited caspase-3 and -7, with apparent K_i values of 1.0 and 7.5 nM, respectively.

Caspase-6 Specificity—Sequence variants of a peptide based on the caspase-6 cleavage site in lamin A (34, 36) were used in a limited study of the substrate preference of caspase-6 (Fig. 3). Supporting its proposed role in cleavage of lamin A at VEID^{230}N during apoptosis, the results show a favorable interaction of caspase-6 with peptides containing Val in P4. The similar preference for Thr in P4 suggests that, for this enzyme, it is not necessarily hydrophobics that are preferred in P4, but rather the β-branched amino acids. Unexpectedly, the observed preference in peptide substrates for Gln and Val in P3 and P2, respectively (Fig. 3), was not reflected in the corresponding chromogenic peptides, where Ac-VEID-pNA displayed a k_{cat}/K_m value more than 10 times that of Ac-VQVD-pNA (Table II).

DISCUSSION

We are interested in the development of caspase-1 inhibitors as anti-inflammatory drugs. We are studying the biological roles of caspases to predict the consequences of cross-inhibition by our compounds and to reveal new promising drug discovery targets. The present study resulted in new research tools with which to probe the structure and function of caspases, explanations for observed activities toward specific natural substrates, and information that can be used to predict activation pathways and which enzyme(s) are likely or not to cleave newly discovered substrates.

Consistent with previous studies, we find the most significant differences in caspase specificities at the P4 positions. P3 specificities are similar between these enzymes, and in P2 a wide range of amino acids is tolerated. These observations are consistent with the structures of caspase-1 and -3 (55, 57, 58), which show that the P3 and P2 side chains of peptidic inhibitors are relatively solvent-exposed, and that the P4 side chains occupy defined pockets that vary significantly between those enzymes. The P4 preferences can be categorized as hydrophobic (caspase-1, -4, and -6) or Asp (caspase-2, -3, and -7). This categorization is at odds with sequence alignment (3), which predicts that caspase-6 is more closely related to caspase-3 and -7 than to caspase-1, and that caspase-2 is more distantly related. The caspase-3 structure (55) shows that the P4 Asp side chain of a cocrystallized peptidic inhibitor makes polar

[2] R. V. Talanian and C. Quinlan, unpublished results.

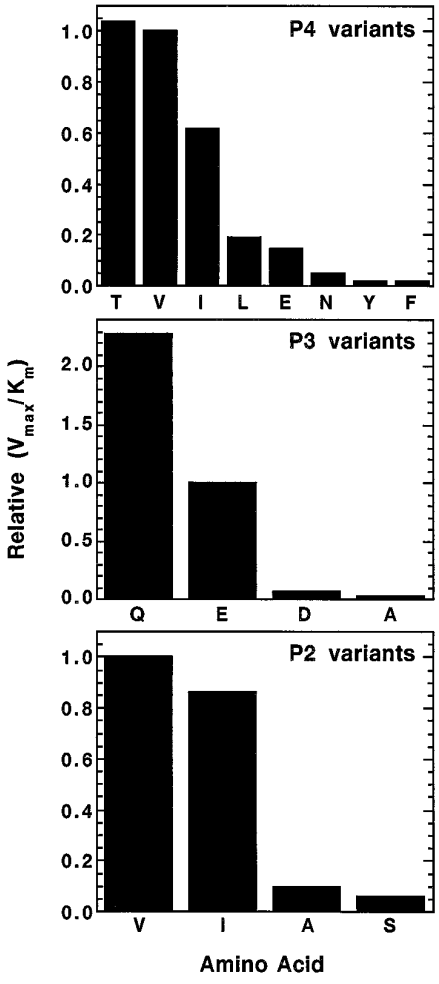

FIG. 3. **Peptide substrate preferences of caspase-6.** Substrate cleavage by caspase-6 was measured using variants of Ac-LEVDGW-Am. Relative (V_{max}/K_m) values are normalized to 1.00 for the results obtained with Ac-VEVDGW-Am.

contacts with the backbone amide nitrogen of Phe-250 (referred to as Phe-381B in Ref. 55 by alignment with caspase-1) and the Nδ2 of Asn-208 (referred to as Asn-342). By alignment, the analogous residue in caspase-6 is Glu-221, which might make an unfavorable ionic interaction with Asp in P4 of a substrate.

Caspase-1 and -4 display very similar specificities in each substrate position including P1′, and preliminary results suggest that caspase-5 is also similar.[2] The P4 specificity of caspase-1 suits it to activation of pro-IL-1β, and the defect in pro-IL-1β maturation in caspase-1-deficient mice (20, 21) suggests that little or no pro-IL-1β is matured by other proteases. This does not rule out a requirement for a second protease in IL-1β maturation, for example by regulating maturation of caspase-1. Murine Ich-3 stimulates pro-IL-1β maturation by ICE (59), and caspase-4 and -5, for which clear roles have not been established, may function analogously. The P4 hydrophobic preference of caspase-4 is consistent with activation of caspase-1, where cleavages occur at AVQD^{119}N, WFKD^{297}S, and FEDD^{316}A. Although caspase-1 is capable of autoactivation *in vitro* (60), it exists largely as its inactive precursor *in vivo* (61) and therefore may require activation by another enzyme in cells. Since caspase-1 knockout mice display normal apoptosis, the transient caspase-1-like activity (defined as cleavage activity toward a P4 Tyr-containing peptide substrate), that precedes caspase-3-like activity and is required for Fas-induced apoptosis in mouse W4 cells (42) might include caspase-4 or -5.

The primary specificity of caspase-6 suits it well to cleavage of one known natural substrate, lamin A (34, 36). The caspase-6 preference for the β-branched amino acids in P4 distinguishes it from caspase-1 and -4 and suggests cleavage of other substrates with sites containing β-branched P4 residues, including maturation sites within caspase-6 (19) (TETD^{23}A and TEVD^{193}A) and caspase-3 (IETD^{175}S). The latter is consistent with proposals based on cotransfection (43) and *in vitro* maturation experiments (19, 43). Intolerance for P4 Tyr (Fig. 3) would prevent caspase-6 maturation of IL-1β.

Caspase-2, -3, and -7 each display similar specificities, which suggests that their roles in cells, if not completely redundant, are at least overlapping. They share a strong requirement for Asp in P4, which qualifies them for cleavage of several known P4 Asp-containing apoptosis substrates. The relationships between these enzymes are largely unknown. In one model system, caspase-3 is required for apoptosis (6), but other enzymes may participate in other tissues or after different apoptotic stimuli. Despite the *in vitro* cleavage of a peptide spanning a known maturation site of caspase-6, the intact protein is not a caspase-2 substrate,[3] demonstrating that the structural context of a protein sequence also contributes to recognition as a caspase substrate. Notably, the caspase-3-like proteolytic activity often measured in apoptotic cells is usually defined by cleavage of Ac-DEVD-pNA or similar substrates. Caspase-2, if active, would not be observed using that substrate, but could be observed along with caspase-3 and -7 by using Ac-VDVAD-pNA.

The design of efficiently recognized novel peptidic substrates and inhibitors based on *in vitro* specificity studies was straightforward and successful, and several such compounds are described here. However, optimized peptides were recognized similarly by enzymes with similar specificities, limiting their usefulness as specific *in vivo* probes of these enzymes. The requirement of caspase-2 for a P5 residue allows for inhibitors and substrates (such as Ac-DEVD-CHO and Ac-DEVD-pNA) that are not well recognized by caspase-2. Unfortunately the presence of the P5 residue, while not required, was not detrimental to recognition by other enzymes such as caspase-3 and -7. It may be possible to design new peptidic probes that display greater specificity by using residues that are suboptimal for target enzymes and not tolerated by others.

The caspase proteases have overlapping substrate specificities that suggest at least partially overlapping functions. Despite evidence for a central role for caspase-3 in apoptosis, mice deficient in this enzyme display a defect in apoptosis that is limited to the brain (32), suggesting that in most tissues other enzymes such as caspase-1 and -7 function redundantly with it. Partial functional overlap may allow cells to modulate the degree or nature of the response to different proinflammatory or proapoptotic stimuli by differential regulation of the caspases. Alternatively, functional overlap might simply provide backup mechanisms for critical processes such as viral or tumor clearance. Some caspase functions suggested by *in vitro* studies may not occur because of tissue or subcellular compartmentalization or regulation of activation or expression during cell cycle or development. It should not be surprising that the systems that execute key steps in the processes of cytokine maturation and apoptosis are capable of both fine regulation and redundant function.

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