When ectopically expressed in animal cells, cytokine response modifier A (CrmA), a product of the cowpox virus, prevents programmed cell death initiated by a variety of stimuli. Since CrmA is a protease inhibitor, its target is probably a protease that promotes cell death. The identification of this target is crucial in delineating essential regulation points that modulate the apoptotic program. We have compared the kinetics of interaction of CrmA with five proteases that may play a role in apoptosis. Four of the proteases, all members of the caspase family, are inhibited with widely different rates and affinities ranging over 5 orders of magnitude. One is not inhibited at all under the experimental conditions. CrmA is quite selective in its ability to inhibit caspases, showing the highest affinity for interleukin-1β-converting enzyme and the second highest for the caspase FLICE (Kᵢ = 0.95 nM), identified as a component of the intracellular signaling complex recruited by ligation of the death receptor Fas. On the basis of comparative inhibitor kinetics, we propose that CrmA is unlikely to inhibit the caspases Yama, Mch2, or LAP3 in vivo but that its inhibition of FLICE is of a magnitude for this protease to be a key target of CrmA during Fas-mediated apoptosis. Therefore, our results support the hypothesis that FLICE catalyzes a crucial step in the promotion of cell death.

The cowpox viral serpin known as cytokine response modifier A (CrmA) is a strong inhibitor of the cysteine protease interleukin-1β-converting enzyme (ICE) (1, 2), which is responsible for the proteolytic activation of interleukin-1β precursor (3, 4). ICE is a homolog of the ced-3 gene product (5), which plays a key role in the precisely controlled programmed cell death during development of the nematode Caenorhabditis elegans. On the basis that the mammalian Ced-3 counterpart should be an ICE-like protease, several groups have used CrmA as a tool to define promoters of cell death. Thus, CrmA is able to prevent apoptosis induced by tumor necrosis factor or Fas ligand (6–8), serum withdrawal (9), nerve growth factor withdrawal (10), as well as detachment from the extracellular matrix (11). This implies that CrmA has an additional function to the one ascribed to it in limiting the generation of mature interleukin-1β by inhibiting ICE and suggests that a CrmA-inhibitable protease is a key component of programmed cell death in several distinct systems.

Although ICE was at first thought to be the natural candidate for the results of gene ablation experiments question its role in apoptosis (12, 13). However, a family of ICE-like proteases has been identified by homology screening of nucleic acid databases using ICE and Ced-3 sequences, and some have been implicated as key components of the suicide machinery. At present the family, now referred to as “caspases” (14), consists of Ced-3 and the following 10 human proteins: ICE, Ich1, Yama, Mch2, LAP3, ICE-Erol-II, ICE-Erol-III, ICELAP6, Mch4, and FLICE. Other names for the family members are compiled in Ref. 14. Each caspase contains characteristic conserved sequences important for proteolytic activity and is able to cause apoptosis when overexpressed ectopically in recipient cells. However, which, if any, are the targets of CrmA is not clear, as studies to date are insufficient to settle the question. For example, there is some evidence that caspases other than ICE can be inhibited by CrmA, but the inhibition is rather weak. Presumably, the target(s) should be rapidly and strongly inhibited. One way to determine such a target is to analyze the kinetics of CrmA inhibition of the candidate caspases Mch2, Yama, LAP3, and FLICE that have been implicated in the Fas-mediated pathway to cell death.

EXPERIMENTAL PROCEDURES

Expression and Purification of Proteins—Full-length cDNAs encoding Yama, Mch2, and LAP3 and a cDNA encoding the catalytic chains of FLICE (starting with Ser-217 and ligated downstream of the vector sequence MGSSHHHHHHSSGLVPRGSH) were cloned into PET23b or pET15b (Novagen) and expressed in Escherichia coli strain BL21(DE3)pLysS (15). Codon 217 of the FLICE precursor was chosen since the fragment 1–216 is found associated with the presumptive pro-FLICE activator complex in vivo. Yama, Mch2, and LAP3 contained C-terminal His 6 tags, and FLICE contained an N-terminal His 6 tag. N-terminal sequencing of each recombinant protease confirmed purity and demonstrated that the following cleavage sites resulted from removal of the N-terminal peptides and generation of the large and small subunits: Mch2 at Asp-23 and -193; Yama at Asp-28 and -175 (minor cleavage at Asp-179); LAP3 at Asp-23 and -206 (minor cleavage at Asp-198); and FLICE at Asp-385 (no removal of the N-terminal vector sequence). His 6 tagged CrmA was expressed using E. coli strain TG1 as described previously (16). All tagged proteins were purified by affinity chromatography on Ni 2⁺-nitrilotriacetate-agarose (Qiagen) or Pharma Biotech Inc. chelating Sepharose charged with NiSO 4 according to the manufacturer’s instructions.

Substrates for Enzyme Assays—The fluorogenic substrate benzoylxylo-carbonyl-Tyr-Val-Ala-Asp-7-amino-4-trifluoromethyl-coumarin (Z-YVAD-FMK) for ICE and benzoylxylo-carbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl-coumarin (Z-DEVD-FMK) for the other four caspases were purchased from Enzyme Systems Products and dissolved in dimethyl formamide as a 10 mM stock solution.

Enzyme Assays—Caspase activity was determined by measuring the release of AFC from the synthetic substrates using continuous record-

This paper is available on line at http://www-jbc.stanford.edu/jbc/
ing instruments. Enzyme activities at high concentrations were analyzed using a SpectraMax 340 plate reader (Molecular Devices) at 380 nm in the kinetic mode at 37 °C. Assay buffer was 50 mM Hepes, pH 7.4; 100 mM NaCl, 0.1% Chaps, 10 mM dithiothreitol, and 10% sucrose. Enzyme activities at lower concentrations were assayed by using a LSS50B fluorometric plate reader (Perkin-Elmer) in the kinetic mode at 37 °C. The excitation and emission wavelengths were 400 nm and 505 nm, respectively. Assay buffer is the same as described above. Values of $K_i$ for the cleavage of Z-DEVD-AFC by FLICE, Mch2, Yama, and LAP3 were calculated in the substrate range of 10–1000 μM, and data were analyzed by Eadie-Hofstee plots.

Kinetic Analysis of Enzyme Assay Data—Inhibition rates and equilibria were determined by the method of progress curves, where a reaction was initiated by the addition of enzyme to a mixture of substrate and inhibitor. Inhibitor concentration was kept at a large excess of enzyme to render the reaction pseudo-first order for convenience of analysis. Calculations of kinetic parameters are based on a single-step reaction scheme (see “Results and Discussion” for more details). The observed inhibition constant in the presence of substrate, $K_{\text{app}}$, is given by,

$$v_0/b_i = 1 + b_i K_{\text{app}}$$  \hspace{1cm} (Eq. 1)

where $v_0$ is the steady state rate of substrate hydrolysis in the presence of inhibitor concentration $I$, and $b_i$ is the uninhibited rate. The overall equilibrium constant is obtained by taking into account the substrate concentration $S$ according to the following equation.

$$K_i = K_{\text{app}}/(1 + S/K_m)$$ \hspace{1cm} (Eq. 2)

The first order rate constant ($k_{\text{obs}}$) was derived from,

$$\ln\left(\frac{[P]_{t,\infty} - [P]}{[P]_t}\right) = C - k_{\text{obs}}t$$ \hspace{1cm} (Eq. 3)

where $[P]_{t,\infty}$ is the product concentration at time $t + \Delta t$ of the association reaction and $[P]_t$ is the product concentration at time $t$ (17). The second order rate constant ($k_{a0}$) was derived according to the following relationship.

$$k_a = \left(\frac{k_{\text{obs}}}{I} + S/K_m\right)$$ \hspace{1cm} (Eq. 4)

Polyacrylamide Gel Electrophoresis (PAGE)—Protein samples were analyzed by non-denaturing PAGE or reduced SDS-PAGE in gels of 5–15% linear acrylamide gradients (18). Protein bands were visualized by Coomassie Blue R250 staining.

RESULTS AND DISCUSSION

Relative Rates and Affinities of Inhibition—The caspases were obtained predominantly as the processed two chain forms with yields of 0.5–1.0 mg/liter of induced culture and judged to be more than 95% pure by SDS-PAGE and N-terminal sequence analysis. Caspase concentrations were determined from their observed absorbance at 280 nm and molar extinction coefficients were calculated according to Ref. 19. The mechanism of processing that results in the conversion of the precursors to active two-chain proteases in E. coli is unknown. The CrmA recombinants were obtained in purified form with a yield of about 3.7 mg/liter of induced culture.

When CrmA was incubated with ICE the cleavage of substrate was almost completely abrogated (Fig. 1), consistent with previous analysis of the ICE-CrmA interaction (2). Significantly, inhibition of FLICE by CrmA was also very potent. Inhibition of Mch2 and Yama was much weaker and slower, while no inhibition of LAP3 was observed for up to 6.7 μM CrmA. Representative progress curves for the inhibition of FLICE and Mch2 are shown in Fig. 2. After variable relaxation times, corresponding to the association of protease with inhibitor, steady states of product formation were observed from which $k_a$ values were calculated according to Equations 1 and 2, and tabulated in Table 1.

The first order rate constants ($k_{\text{obs}}$) for the interaction of CrmA with FLICE, Mch2, Yama, and LaP3 were determined by analysis of the association phase (Fig. 2) as described in Equation 3 and plotted against inhibitor concentration to derive $k_a$ values, according to Equation 4. Fig. 3 demonstrates the analysis of association rates of CrmA with FLICE, Mch2, and Yama, and the respective $k_a$ values determined from these plots are listed in Table 1. The inhibition rates correlated with $K_i$ values to demonstrate the same order of efficiency of inhibition. The equilibrium and rate constants derived from the data are global constants, the exact meanings of which depend upon the reaction mechanisms. As is the case with most protein inhibitors of proteases, the mechanism is probably two-step. Indeed, this is indicated by deviation from linearity in the $k_{\text{obs}}$ plot at high inhibitor concentration, most obvious in the case of Mch2 (Fig. 3). Irrespective of the number of steps in the inhibitory pathway of the caspases, their relative specificity of inhibition by CrmA would not be dependent on the mechanisms involved. In each case we were able to observe final steady states of substrate hydrolysis in large inhibitor excess, indicating that the inhibition of FLICE, Mch2, and Yama by CrmA is reversible. This was not detectable with ICE, as previously reported (2), because of the extreme strength of that interaction.

Complex Formation and Analysis by PAGE—FLICE or Mch2 were incubated with an excess of CrmA and samples analyzed by SDS- and non-denaturing PAGE. Sample gels for FLICE and Mch2 are shown in Fig. 4, which demonstrates that a unique protein band consistent with a complex between each of the caspases and CrmA can be identified in non-denaturing PAGE. The complex is dissociated by boiling in SDS. Turnover of CrmA is visible on SDS-PAGE as denaturation of the complexes results in the appearance of a cleavage product of CrmA, which probably resides in the reactive site loop as observed with most serpin-protease-serpin complexes (20, 21). Similar results were obtained with Yama, and as expected, no complex was observed with LAP3 (data not shown). Because complexes of CrmA with the caspases are kinetically reversible and not stable to SDS, they are unlike most serpin-protease-serpin complexes. The detailed mechanism of inhibition of caspases by CrmA is under investigation in our laboratory, but our observations confirm that the cross-class inhibitory activity of serpins first demonstrated with ICE (2) is a general phenomenon for a number of caspases.

In Vivo Implications—Since CrmA is an extremely good inhibitor of ICE, it was originally thought that this would be the
inhibitor's target. This is probably correct in the context of interleukin-1β processing but not likely during apoptosis. Though CrmA does not protect against apoptosis triggered by all stimuli, the extent to which it protects against Fas-mediated death, for example, is remarkable. Cells that express CrmA survive and replicate in the presence of lethal levels of Fas antibody, and so proteolysis mediated by a CrmA-inhibitable protease is essential in the Fas death pathway (8, 22). We demonstrate here that three of four caspases implicated in apoptosis are inhibited by CrmA. This does not mean that all three are likely targets for CrmA in vivo. Earlier studies suggested that Yama might be a target of CrmA (22). Doubts were raised because the inhibition of Yama by CrmA, although appreciable under experimental conditions, would be kinetically unfavorable (23). The inhibition of Mch2 is faster and tighter than Yama, but it too is unlikely to be a target based on the same criteria.

Though the target of a protease inhibitor cannot be elucidated solely on kinetic evidence, it is more straightforward to rule out targets. Thus, if neither Mch2, Yama, nor LAP3 could be considered targets, they cannot be at a key location in the apoptotic pathway triggered by Fas ligation. Because CrmA

**FIG. 2.** A family of progress curves for CrmA inhibition on FLICE and Mch2. FLICE or Mch2 was added to a final concentration of 10⁻⁹ or 10⁻¹⁰ M to prewarmed mixtures of CrmA at the indicated final concentrations and 100–200 μM Z-DEVD-AFC to monitor the reaction. Product formation was recorded over the time course of the reactions, which demonstrate a slow binding process for FLICE (A) and Mch2 (B). The inhibition constant $K_i$ was calculated for FLICE (C) and Mch2 (D) by plotting $v_0/v_i - 1$ against $I$, where $v_0$ is the velocity of AFC formation in the absence of CrmA and $v_i$ is the steady state of product formation in the presence of CrmA.

|        | ICE* | FLICE | Mch2 | Yama | LAP3 |
|--------|------|-------|------|------|------|
| $K_i$ (M) | <1 x 10⁻¹ⁱ | 9.5 x 10⁻¹⁰ | 1.1 x 10⁻⁷ | 5.0 x 10⁻⁷ | >4 x 10⁻⁵ |
| $k_a$ (M⁻¹ s⁻¹) | 1.7 x 10⁷ | 9.1 x 10⁴ | 4.4 x 10² | 1.6 x 10² | <10 |

* Data for ICE are from Ref. 2.

**FIG. 3.** Relationships between $k_{obs}$ and $[I]$. The first order rate constant $k_{obs}$ increased with increasing concentrations of CrmA for all the three caspases shown. A linear relationship was assumed (see "Results and Discussion"), and the overall second order rate constant, $k_o$, was determined by equations described under "Experimental Procedures."
prolongs the activation of these three caspases in vivo (24, 25), the CrmA target must be upstream. FLICE is inhibited more than 2 orders of magnitude faster and tighter than Mch2 and on this basis is a much likelier candidate for the CrmA target. Thus, of the four caspases implicated in Fas-mediated apoptosis, FLICE is the clearest CrmA target.

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