Fas-induced Activation of the Cell Death-related Protease CPP32 Is Inhibited by Bcl-2 and by ICE Family Protease Inhibitors

Robert C. Armstrong, Teresa Ajait, Jialing Xiang, Smita Gaur, Joseph F. Kreb, Kim Hoang, Xu Biat, Stanley J. Korsmeyer, Donald S. Karanewsky, Lawrence C. Fritz, and Kevin J. Tomaselli

From IDUN Pharmaceuticals, Inc., La Jolla, California 92037 and the Howard Hughes Medical Institute, Molecular Oncology, Washington University School of Medicine, St. Louis, Missouri 63110

The human proto-oncogene bcl-2 and its Caenorhabditis elegans homologue ced-9 inhibit programmed cell death. In contrast, members of the human interleukin-1β converting enzyme (ICE) family of cysteine proteases and their C. elegans homologue CED-3 promote the death program. Genetic experiments in C. elegans have shown that ced-9 is formally a negative regulator of ced-3 function, but neither those studies nor others have determined whether CED-9 or Bcl-2 proteins act biochemically upstream or downstream of CED-3/ICE proteases. CPP32, like all known members of the CED-3/ICE family, is synthesized as a proenzyme that is subsequently processed into an active protease with specificity for cleavage at Asp-X peptide bonds. In this report, we demonstrate that the CPP32 proenzyme is proteolytically processed and activated in Jurkat cells induced to die by Fas ligation. CPP32 activation is blocked by cell-permeable inhibitors of aspartate-directed, cysteine proteases, suggesting that pro-CPP32 is cleaved by active CPP32 or by other ICE family members. Heterologous expression of Bcl-2 in Jurkat cells prevents Fas-induced cell death as well as proteolytic processing and activation of CPP32. Thus, Bcl-2 acts at or upstream of the CPP32 activation step to inhibit apoptosis induced by Fas stimulation.

Virtually all eukaryotic cells are capable of activating an intrinsic cell death program (1). Genetic studies in both vertebrate and invertebrate systems have identified several genes that regulate programmed cell death (2). One set of cell death regulating genes, the ced-3/ICE family, encodes structurally related cysteine proteases which have the unusual substrate specificity for cleavage at Asp-X peptide bonds (3-11). Mutations in the Caenorhabditis elegans ced-3 gene block all developmentally programmed cell deaths in the C. elegans hermaphrodite, demonstrating the role of this protease as a cell death effector in this species (4). The role of CED-3/ICE family proteases in vertebrate cell death is evidenced by the ability of proteolytic processing to produce active, heterodimeric enzymes (3-11, 27). For two family members, pro-ICE and pro-CPP32, proteolytic processing has been shown to occur at Asp-X bonds (3, 27, 28), indicating that proenzyme cleavage involves proteases with substrate specificities similar to those of the CED-3/ICE proteases themselves. Recent studies have suggested that proteolytic cleavage and activation of the CPP32 protease may be functionally important in the induction of apoptosis (13, 27). For example, the cytotoxic T lymphocyte (CTL)13 serine protease, granzyme B, may cleave and activate CPP32 during the induction of apoptosis in CTL target cells (29).

In contrast to the death effector functions of CED-3/ICE family members, the bcl-2 gene family encodes several proteins that inhibit programmed cell death. In mammalian cells, Bcl-2 and Bcl-XL inhibit cell death induced by diverse stimuli in many cell types (30–32). In C. elegans, the bcl-2 homologue, ced-9, inhibits ced-3-dependent cell death and is thus genetically a negative regulator of ced-3 (33, 34). However, these genetic studies do not distinguish whether CED-9/Bcl-2 proteins act upstream of CED-3/ICE proteases to inhibit their activation or downstream to prevent the function of CED-3/ICE protease substrates (35). In the present report, we demonstrate that CPP32 is proteolytically activated in response to the cell death stimulus, Fas ligation. Activation of CPP32 following Fas stimulation requires endogenous aspartate-directed, cysteine protease activity, most likely of the CED-3/ICE family. Further, we show that in cells expressing Bcl-2, Fas-induced CPP32 activation is inhibited. These results demonstrate that Bcl-2 functions at or upstream of the CPP32 activation step in a cell death pathway.

MATERIALS AND METHODS

Cell Culture—Jurkat cells (clone E6-1, ATCC, Rockville, MD) were cultured in medium RPMI 1640 with 10% fetal bovine serum and penicillin/streptomycin (100 units/ml). Jurkat cells were stably transfected with the plasmid SFFV-human bcl-2 or the control plasmid SFFV-neo (31). Selection of the SFFV-Bcl-2 transfecants in G418 yielded a Bcl-2-transfected pool that, on limiting dilution, yielded several clonal Bcl-2 cell lines (representative clone 18 was used for further analysis). FACS and Western analysis of the Bcl-2 transfecants with observed following induction of apoptosis in vertebrate cells (24–26).

All of the known members of the CED-3/ICE protease family are synthesized as single-chain proenzymes that require proteolytic processing to produce active, heterodimeric enzymes (3-11, 27). For two family members, pro-ICE and pro-CPP32, proteolytic processing has been shown to occur at Asp-X bonds (3, 27, 28), indicating that proenzyme cleavage involves proteases with substrate specificities similar to those of the CED-3/ICE proteases themselves. Recent studies have suggested that proteolytic cleavage and activation of the CPP32 protease may be functionally important in the induction of apoptosis (13, 27). For example, the cytotoxic T lymphocyte (CTL)13 serine protease, granzyme B, may cleave and activate CPP32 during the induction of apoptosis in CTL target cells (29).

In contrast to the death effector functions of CED-3/ICE family members, the bcl-2 gene family encodes several proteins that inhibit programmed cell death. In mammalian cells, Bcl-2 and Bcl-XL inhibit cell death induced by diverse stimuli in many cell types (30–32). In C. elegans, the bcl-2 homologue, ced-9, inhibits ced-3-dependent cell death and is thus genetically a negative regulator of ced-3 (33, 34). However, these genetic studies do not distinguish whether CED-9/Bcl-2 proteins act upstream of CED-3/ICE proteases to inhibit their activation or downstream to prevent the function of CED-3/ICE protease substrates (35). In the present report, we demonstrate that CPP32 is proteolytically activated in response to the cell death stimulus, Fas ligation. Activation of CPP32 following Fas stimulation requires endogenous aspartate-directed, cysteine protease activity, most likely of the CED-3/ICE family. Further, we show that in cells expressing Bcl-2, Fas-induced CPP32 activation is inhibited. These results demonstrate that Bcl-2 functions at or upstream of the CPP32 activation step in a cell death pathway.

1 The abbreviations used are: CTL, cytotoxic T lymphocytes; PARP, poly(ADP)-ribose polymerase; ZVAD-FMK, benzylolcarbonyl-Val-Ala-Asp-fluoromethylketone; Z-FA-FMK, benzyloxycarbonyl-Phe-Ala-fluoromethylketone; FACS, fluorescence-activated cell sorting; AMC, amionomethylcoumarin; MES, 4-morpholineethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dr. Lawrence C. Fritz, IDUN Pharmaceuticals, Inc., 11085 N. Torrey Pines Rd., Suite 300, La Jolla, CA 92037. Tel.: 619-623-1330; Fax: 619-623-2765; E-mail: lfritz@idun.com.
The Bcl-2 monocalonal antibody, 6C8, demonstrated Bcl-2 levels 10–20-fold enhanced over the levels of Bcl-2 present in untransfected or SFFV-neo-transfected Jurkat cells. For cell death assays, 100,000 cells were placed in a 0.28-cm² tissue culture well and exposed to 100 ng/ml CH11 Fas monoclonal antibody (PanVera Labs, Madison, WI) in the absence or presence of benzo[aloxycarbonyl-Val-Ala-Asp-fluoromethylketone (ZFA-FMK). At the indicated times, cells were treated with 1 mg/ml MTT for 90 min and the absorbance of the dissolved reaction product measured at 650 nm. Control experiments demonstrated that the decline in MTT metabolism correlated closely with the appearance of apoptotic cells in the culture as assessed by the nuclear condensation and fragmentation observed using either phase-contrast microscopy or immunofluorescence of nuclei labeled with Hoechst dye no. 33342 (data not shown). FACS analysis demonstrated that both the ZVAD-FMK-treated and the Bcl-2-transfected Jurkat cells expressed normal levels of cell surface Fas (data not shown).

Clavage of in Vitro Translated Poly(ADP)-ribose Polymerase (PARP)—Cystolic extracts were prepared essentially as described (36). 
35S-Labeled, in vitro translated PARP was generated using the TNT-linked transcription/translation kit (Promega, Madison, WI) according to manufacturer’s instructions. Briefly, human PARP cDNA (a gift from A. Bürkle) in pBluescript SK (Stratagene, San Diego, CA) was linearized with Smal prior to use. Linearized template was incubated with Taq polymerase, 40 μCi of [35S]methionine, and TNT kit components to generate the translated PARP. In vitro translated (2%) or total TNT reaction) was incubated with 5 μg of cystolic extracts for 1 h. At 30°C in 25 mM HEPES, pH 7.5, 0.1% CHAPS, 1 mM dithiothreitol (Buffer A). Reactions were boiled in Laemmli buffer, separated on 4–20% gradient SDS-polyacrylamide gels, and the protein bands visualized by autoradiography.

Protease inhibitors and Substrates—ZVAD-FMK was synthesized as described (37). ZFA-FMK was prepared from Enzyme Systems Products (Dublin, CA). Ac-Tyr-Val-Ala-Asp-aminomethylcoumarin was purchased from Bachem (King of Prussia, PA). Ac-Asp-Glu-Val-Asp-aminomethylcoumarin was prepared by coupling Ac-Asp(OBu)-OH to H-Glu(OBu)-Val-OH (OBut)-semicarbazone (38) followed by catalytic hydrogenation over palladium on carbon. Ac-Asp-Glu-Val-Asp-H was prepared by coupling Ac-Asp(OBu)-OH to H-Glu(OBu)-Val-OH (OBut)-semicarbazone followed by sequential deprotection with 50% trifluoroacetic acid in CH3Cl, and 37% aq HCHO/ArOAc/MEOH (1:1:3). The requisite amine was prepared by coupling Z-Glu(OBu)-Val-OH to H-Glu(OBu)-Val-OH by catalytic hydrogenation over palladium on carbon. All couplings were performed using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-1-hydroxybenztriazol in either CH3Cl or CH2Cl2-dimethylformamide.

CPP32 and ICE Expression and Enzyme Assay—Recombinant human CPP32 and mouse ICE proteases were expressed in bacteria as described (11, 12) in the presence of the plasmids pET 21b (CPP32) or pET3a (ICE) (Novagen, Madison, WI) containing a BamHI/XhoI or a NdeI/BamHI fragment of the human CPP32 (5) or mouse ICE cDNAs, respectively. After induction with isopropyl-1-thio-galactopyranoside, bacteria were harvested and lysates prepared by sonication in ice-cold 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 50 mM KCl. Insoluble material was removed by centrifugation at 30,000 × g. CPP32 was purified from bacterial lysate by Ni2+-chelate chromatography (Hi-Trap Pharmacia Biotech) and elution with a 60 ml to 1 M gradient of imidazole following manufacturer’s instructions (Novagen, Madison, WI). Enzyme reactions contained 20 μM acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (DEVD-AMC) or acetyl-Tyr-Val-Ala-Asp-aminomethylcoumarin (YVAD-AMC). In some cases, serine protease inhibitors A and either 5 μg of Jurkat cell cystolic extracts or 0.3 μM recombinant CPP32 (rcCPP32) with or without the irreversible inhibitor acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-aldehyde). Fluorescent AMC product formation was measured at excitation 360 nm, emission 460 nm using a Cytofluor II fluorescent plate reader (Millipore, Bedford, MA).

In vitro translation—The inhibitory effects of ZVAD-FMK inhibitor on several cysteine proteases were examined: YVAD-AMC hydrolysis by mICE lysate, DEVD-AMC hydrolysis by CPP32, benzo[aloxycarbonyl-Arg-Arg-AMC (Sigma) hydrolysis by cathepsin B (Sigma), and succinyl-Leu-Tyr-AMC (Sigma) by calpain I (Calbiochem). All reactions were performed in buffer A except cathepsin B reactions which were performed in 50 mM MES, 1 mM EDTA, 2 mM dithiothreitol, pH 6.0. Calpain I reactions contained 10 mM CaCl2.

The rate of inactivation of the enzymes by the ZVAD-FMK inhibitor was determined from the time-dependent inhibition of hydrolysis observed after the addition of excess inhibitor to enzyme reactions (39). 50 μl of 0.1–250 μM inhibitor was added to 200-μl reactions containing 12.5 μM substrate and 2 units of enzyme (1 unit of enzyme will liberate 1 μM AMC product in 60 min). The observed rate constant for the inactivation, kobs, was calculated from the progress curves of the hydrolysis reaction during the first 10% of the reaction using Equation 1, where kobs is the initial hydrolysis rate (40).

\[
[\text{AMC}] = V_0 (1 - \exp(-k_{\text{obs}}/k_{\text{inact}}))
\]  

(1) The second order rate constant for inactivation, k_{\text{inact}}, is related to k_{\text{obs}} by Equation 2 (39).

\[
k_{\text{inact}} = k_{\text{obs}} [I] (1 + [S]/K_m)
\]  

(2)

Western Blotting—For anti-CPP32 immunoblot, cystolic extracts from Fas antibody-stimulated cells (15 μg/ml) or purified recombinant CPP32 were separated on 14% SDS-polyacrylamide gels under reducing conditions, transferred to nitrocellulose, and probed with mouse monoclonal anti-CPP32 (Signal Transduction Labs, Lexington, KY) followed by goat anti-mouse coupled to alkaline phosphatase, or by affinity-purified anti-CPP32 p12 N-terminal peptide antibodies followed by goat anti-rabbit coupled to alkaline phosphatase. Rabbis were immunized with the peptide CSGVDDMACHPKVE corresponding to the N-terminal 15 amino acids of CPP32 p12 coupled via cysteine residues to keyhole limpet hemocyanin. Serum antibodies were affinity-purified on peptide coupled to Sepharose 4B. Affinity-purified p12 antibodies were tested by Western blot on bacterial extracts containing human CPP32 and were found to be specific for the p12 subunit of CPP32 (data not shown). Further control experiments demonstrated that preabsorption of the p12 antibodies with the peptide used for immunization eliminated the Western blot immunoreactivity (data not shown). For PARP immunoblots, whole cell lysates from Jurkat cell lysate (25 μg of protein/lane) were separated on 8% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membrane, and probed with monoclonal mouse anti-human PARP (41) followed by goat anti-mouse horseradish peroxidase. Products were visualized using the ECL system (Amersham Corp.).

RESULTS

Treatment of Jurkat T cells with 100 ng/ml Fas monoclonal antibody CH11 induced death in >85% of the cells within 8 h, as measured by a metabolic assay (Fig. 1a). In order to determine if Bcl-2 inhibits Fas-induced Jurkat cell death, a similar experiment was performed using Bcl-2-transfected cells. Heterologous stable expression of Bcl-2 in a transfected pool as well as in clonal transfectants strongly inhibited Fas-induced Jurkat cell death (Fig. 1a).

The previous studies have indicated that Fas-induced cell death involves members of the CED-3/ICE family of proteases (13, 17, 22, 23). To further characterize the role of this protease family in Fas-induced killing of Jurkat cells, we utilized the protease inhibitor benzo[aloxycarbonyl-Val-Ala-Asp-fluoromethylketone (ZVAD-FMK). Peptidyl-fluoromethylketones are irreversible inhibitors of cysteine proteases (42, 43), and ZVAD-FMK contains an Asp in the P1 position, which has been shown to be critical for ICE inhibition (3). ZVAD-FMK has recently been shown to specifically inhibit apoptosis in a tissue culture model, whereas a peptidyl-fluoromethylketone inhibitor of cathepsin B that lacks the P1 Asp was inert (44). To further characterize the specificity of ZVAD-FMK, we measured the second order rate constants (k_{\text{inact}}) for inhibition of four cysteine proteases including two members of the ICE family (mouse ICE, human CPP32) as well as two non-members (cathepsin B and calpain 1). ZVAD-FMK inhibited both ICE-related proteases, with ICE showing the faster rate of inactivation (Table I). Compared to ICE, the rates of inactivation of cathepsin B and calpain 1 were 3–4 orders of magnitude slower. Compared to CPP32, which was inhibited more slowly than ICE, the rates for calpain 1 and cathepsin B were 440-fold and 75-fold slower, respectively. The selectivity of ZVAD-FMK for ICE-related proteases is further indicated by its lack of detectable inhibition of granzyme B, a mammalian serine pro-
Bcl-2 Inhibits Fas-induced Activation of the CPP32 Protease

Fig. 1. a, Fas antibody-induced death of Jurkat cells is inhibited by ZVAD-FMK or by Bcl-2 overexpression. Untransfected (open circle), neo-transfected control (open square), Bcl-2-transfected pool (closed triangle), Bcl-2 clone 18 (closed diamond), and ZVAD-FMK-treated (50 μM) neo-transfected (closed square) Jurkat cells were treated with Fas antibody for the indicated times and cell viability was measured. All values are mean ± S.E. of triplicate determinations made in parallel (error bars are smaller than the symbols used) and are expressed as percentage of signal obtained from cells in the absence of anti-Fas. Similar results were obtained with ZVAD-FMK as a free acid or as a methyl ester. b, Western analysis of endogenous Jurkat PARP cleavage in untransfected (left panel), ZVAD-FMK-treated (middle panel), and Bcl-2-transfected (right panel) Jurkat cells stimulated with Fas antibody for the indicated times. Undeaved PARP is indicated by open arrowhead; the 85-kDa PARP cleavage fragment is indicated by the open arrowhead.

Table I

| Protease | k_{inact} | Value ± range for duplicate determinations |
|----------|-----------|--------------------------------------------|
| Mouse ICE | | 150,000 ± 20,000 |
| Human CPP32 | | 6,600 ± 800 |
| Cathepsin B | | 87 ± 4 |
| Calpain 1 | | 15 ± 1 |

ZVAD-FMK showed a dose-dependent inhibition of Fas-induced Jurkat death, with maximal protection observed at concentrations above 10 μM (data not shown). Quantitatively similar results were obtained with either the free acid or methyl ester of ZVAD-FMK. The anti-apoptotic effects of 50 μM ZVAD-FMK on the time course of cell death is shown in Fig. 1a. As a control, we tested the ability of an irreversible cathepsin B inhibitor, benzoyloxy carbonyl-Phe-Ala-fluoromethylketone (ZFA-FMK) to block Fas-induced Jurkat cell death. ZFA-FMK inhibits cathepsin B with a second order rate constant 2 orders of magnitude higher than ZVAD-FMK (Ref. 45 and Table I). ZFA-FMK showed no detectable inhibition of cell death at any of the concentrations tested (0.1–200 μM; data not shown). Thus the effect of ZVAD-FMK on cell death due neither to a nonspecific action of the fluoromethylketone moiety, nor likely to inhibition of cathepsin B.

To assess whether the CPP32 protease is activated following Fas ligation, we first examined the proteolytic cleavage of the endogenous nuclear protein PARP. Recent evidence suggests that PARP is cleaved by CPP32 or a related enzyme in response to a number of cell death stimuli (9, 13, 27). Western blot analysis of whole cell extracts of Jurkat cells demonstrated cleavage of the 115-kDa PARP protein into the characteristic 85-kDa fragment at 1 h after Fas stimulation and complete cleavage by 4 h (Fig. 1b). Coincubation of Fas-stimulated Jurkat cells with ZVAD-FMK completely prevented PARP cleavage (Fig. 1b), confirming that PARP cleavage does require aspartate-directed cysteine protease activity. Bcl-2 overexpression also strongly suppressed PARP cleavage (Fig. 1b), placing Bcl-2 action upstream of this nuclear event.

To further investigate Fas-induced CPP32 activation, CPP32-like protease activity was measured in cytosolic extracts isolated from Jurkat cells by assaying their ability to cleave two exogenous CPP32 substrates: in vitro-translated, 35S-labeled human PARP (in vitro translated PARP), and the fluorescent tetrapeptide substrate, acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (DEVD-AMC) (13, 27). DEVD corresponds to the sequence in PARP that is cleaved in an in vitro model of apoptosis (24). Extracts prepared from cells exposed to anti-Fas and immediately processed (time 0) contained neither PARP cleavage activity nor DEVD-AMC cleavage activity (Fig. 2, a and b, respectively). In contrast, extracts prepared from cells exposed for 1, 4, or 8 h to anti-Fas were able to cleave these substrates. Protease activity was consistently observed at 1 h, although the magnitude of activation varied (Fig. 2b). Cleavage of in vitro translated PARP by either the 4- or 8-h extracts produced products of ~85 and ~25 kDa that comigrated in SDS gels with cleavage products generated by incubation of in vitro translated PARP with recombinant human CPP32 (Fig. 2a). The DEVD-AMC cleaving activity present in Fas-induced extracts was completely inhibited by a reversible inhibitor of CPP32, DEVD-aldehyde (27), with an IC_{50} (3 nM) nearly identical to that for inhibition of recombinant human CPP32 (Fig. 2c). Thus, a CPP32-like protease activity is induced in the cytosol of Jurkat cells as early as 1 h after Fas stimulation.

Jurkat extracts were also tested for ICE-like protease activity at 1, 4, and 8 h after Fas stimulation. There was no detectable cleavage by the extracts of either of two ICE substrates, YVAD-AMC or 35S-labeled, in vitro translated pro-interleukin-1β, while both substrates were readily cleaved by recombinant ICE (data not shown). Thus, in contrast to CPP32, ICE protease activity was not detectable at these times following Fas stimulation in Jurkat cells.

Proteolytic processing of the pro-CPP32 polypeptide in response to Fas stimulation was studied in the cytosolic extracts by Western blotting using a monoclonal antibody to the 32-kDa (p32) proenzyme and a polyclonal antibody to the N terminus of the mature, processed 12-kDa (p12) subunit. The p32 protein was detected in cells prior to anti-Fas treatment with either the monoclonal or polyclonal antibody (Fig. 3, a and b, respectively), but at the 4- and 8-h time points following anti-Fas exposure, p32 levels declined significantly (Fig. 3, a and b). In contrast, the mature p12 subunit of CPP32 was not observable in cells prior to anti-Fas treatment, but reached detectable levels by 4 h of anti-Fas exposure (Fig. 3b). The protein in the...
Bcl-2 Inhibits Fas-induced Activation of the CPP32 Protease

Fig. 2. A CPP32-like protease activity is induced in Fas-stimulated Jurkat cells. a, cleavage of in vitro translated, 35S-labeled PARP by cytosolic extracts of transfected control (left panel), ZVAD-FMK-treated (50 μM; middle panel), or Bcl-2-transfected (right panel) Jurkat cells treated with Fas antibody for the indicated times. For comparison, in vitro translated PARP (closed arrowhead) was incubated with recombinant CPP32 protease (rCPP32). PARP cleavage fragments produced by incubation with cytosolic extracts or with rCPP32 are indicated by open arrowheads (upper, ~85-kDa fragment; lower, ~25-kDa fragment); molecular weight markers are indicated (M). b, cleavage of DEVD-AMC by cytosolic fractions isolated from untransfected control (open circle), ZVAD-FMK-treated (50 μM; closed triangle), or Bcl-2-transfected (closed circle) Jurkat cells treated with Fas antibody for the indicated times. Each point represents the mean ± S.D. of three separate experiments and is expressed as the percentage of the maximum increase in DEVD-AMC cleaving activity observed in each experimental time course. The fold-stimulation in control cell extracts at 4 h compared to 0 h in four separate experiments was 342-, 200-, 28-, and 6-fold. c, inhibition by DEVD-aldehyde of DEVD-AMC cleaving activity present in cytosolic extracts from untransfected control Jurkat cells stimulated with anti-Fas for 8 h (closed circles) or in preparations of human, recombinant CPP32 (open squares). Equal amounts of Jurkat and CPP32 DEVD-AMC cleaving activities were used.

Jurkat extracts recognized by the p12 N-terminal antibody comigrated with immunoreactive p12 from active, purified CPP32 (Fig. 3b). These data indicate that the 32-kDa pro-CPP32 is processed to an active p12-containing form following Fas activation.

Active CPP32 protease is generated in the THP-1 monocytic cell line by cleavage of p32 at Asp-28 and Asp-175 (27), indicating that pro-CPP32 is cleaved by an aspartate-directed cysteine protease, possibly active CPP32 itself or another member of the CED-3/ICE family. To test this hypothesis, CPP32 activation was evaluated in cells coincubated with anti-Fas and ZVAD-FMK. ZVAD-FMK treatment blocked both the induction of CPP32-like protease activity (Fig. 2, a and b) and the processing of p32 into the p12 subunit (Fig. 3). Thus, inhibition of Asp-directed, cysteine proteases with ZVAD-FMK prevented the activation of CPP32 normally observed after Fas stimulation.

The effect of Bcl-2 overexpression on Fas-induced CPP32 activation was tested in Jurkat cells stably transfected with Bcl-2. Bcl-2 expression prevented the induction of PARP cleavage activity and DEVD-AMC cleavage activity (Fig. 2, a and b) as well as the conversion of pro-CPP32 to the p12 subunit (Fig. 3). Both the Bcl-2-transfected pool and a clonal line gave identical results. Thus, overexpression of Bcl-2, like treatment with ZVAD-FMK, prevented the activation of CPP32 induced by anti-Fas.

DISCUSSION

We have shown that the CPP32 protease is activated in Jurkat cells following stimulation with Fas antibody. Western blot analysis with antibodies specific for the p12 subunit of active CPP32 and analysis of CPP32-like enzymatic activities in Jurkat cell extracts indicate that activation begins at 1-2 h following Fas stimulation. This time course is consistent with a role for CPP32 in the cleavage of PARP observed in this and other Fas-induced apoptosis models (13). Fas-induced activation of CPP32 appears to require endogenous aspartate-directed, cysteine protease activity, suggesting that pro-CPP32 is processed either by active CPP32 or by other members of the CED-3/ICE family. Finally, our results provide strong biochemical evidence that Bcl-2 functions at or upstream of the CPP32 activation step in this cell death pathway.

Activation of CED-3/ICE family proteases appears to be a critical step in the control of apoptosis in mammalian cells. For example, recent studies have suggested a role for the activation of CPP32 in several apoptotic model systems (13, 27, 29). All of the known CED-3/ICE family members are synthesized as single-chain, inactive proenzymes that require proteolytic cleavage for activation (3–11). For two family members, ICE and CPP32, cleavage is known to occur at Asp-X bonds (3, 27, 28), suggesting that the proenzyme forms of these proteases are themselves substrates of activated family members. Indeed, evidence for both self-processing and heterologous processing of ICE family members has been presented (3, 13, 28). We have shown that CPP32 activation is blocked by the protease inhibitor ZVAD-FMK. Our kinetic data demonstrate that this compound effectively inhibits two CED-3/ICE family proteases but is a poor inhibitor of other cysteine proteases. Thus, the action of the ZVAD-FMK in our system suggests that cleavage of the CPP32 proenzyme is effected by a member or members of this protease family.

Our results clearly demonstrate that Bcl-2 expression regulates the activation of CPP32 normally observed following the Fas death stimulus. A variety of molecular mechanisms can be proposed to explain how Bcl-2 inhibits CPP32 activation. For example, Bcl-2 could be an inhibitor of a protease that cleaves pro-CPP32. Alternatively, Bcl-2 could interact directly or indirectly with pro-CPP32, rendering it a poorer substrate for the protease that activates it, or Bcl-2 could act even further upstream to inhibit signal transduction events that lead to CPP32 activation. However, the ability of Bcl-2 to effectively block programmed cell death induced by a wide variety of stimuli (46), indicates that it functions downstream of Fas-specific
signaling events.

Two recent reports (47, 48) demonstrate that extracts made from cells treated with Fas for 30 min to 1 h can induce apoptosis in exogenous test nuclei. Furthermore, these reports indicate that recombinant Bcl-2 protein can block this form of cell-free apoptosis. These observations suggest that Bcl-2 may function at a late step in the apoptotic pathway since in one of the studies, Bcl-2 acted after an ICE-dependent step (48) and in the other (47), Bcl-2 was functional after 1 h of Fas treatment, a time when our results suggest that significant CPP32 activation has already occurred. Further work will be necessary to reconcile the cell-free data with our results demonstrating the regulation of CPP32 activation by Bcl-2. For example, it will be critical to evaluate directly the degree of activation of CPP32 in the cell-free extracts.

The ability of the protease inhibitor ZVAD-FMK to inhibit Fas-induced apoptosis in Jurkat cells raises the question of which ICE-3/ICE protease or proteases are involved in cell death. Since ZVAD-FMK potently inhibits several ICE family proteases, including recombinant CPP32, we cannot say which family members are critical for cell death in this system. Our observation that CPP32 activation is prevented by Bcl-2 is consistent with, but does not demonstrate, a role for CPP32 in Fas-induced Jurkat cell death. We have found that high concentrations (200 μM) of the potent CPP32 inhibitor, DEVD-aldehyde block Fas-induced Jurkat cell death by about 50%. However, in addition to CPP32, DEVD-aldehyde is a potent inhibitor of several CED-3/ICE family members, including ICE and a novel ICE family member, MCH3, which is highly related to CPP32 (9). As all of these protease genes are expressed in Jurkat cells, it will require selective cell-permeable inhibitors to unambiguously define the specific roles of any of the protease family members in the cell death process.

Acknowledgments—We thank Dr. Bill Horne and Gary Wilson for expression and purification of CPP32 and helpful discussions, Dr. Larry Boise for advice and FACS analysis, Dr. Nathan Berger for the PARP monoclonal antibody, Dr. Alexander Bürkle for human PARP cDNA, Dr. John Reed and Dr. Bob Horvitz for helpful comments on the manuscript, and Linda Thompson and Heather Kondal for typing the manuscript.

Note Added in Proof—While this manuscript was under review, data were published demonstrating that Bcl-2 and ZVAD-FMK can block cleavage of the CPP32 protein in other apoptotic systems (49–51).

REFERENCES

1. Raff, M. C. (1992) Nature 356, 397–400
2. Steller, H. (1995) Science 267, 1445–1449
3. T. Aja and K. J. Tomaselli, unpublished observations.
4. Thornberry, N. A., Bull, H. D., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M., Miller, D. K., Molinaux, S. M., Weidner, J. R., Aunins, J. K., Ellison, K. O., Ayala, J. M., Casado, F. J., Chinn, J., Ding, G. J., Figer, E. L., Gaffen, E. P., Linjus, G., Palyha, O. C., Raju, S. M., Rolando, A. M., Sailey, J. P., Yamin, T.-T., Lee, T. D., Shively, J. E., MacCroskey, M., Mumford, R. A., Schmidt, J. A., and Tocci, M. J. (1992) Nature 359, 768–774
5. Y. J., Shaham, S., Ledoux, S., Ellis, H., and Horvitz, H. R. (1993) Cell 75, 641–652
6. Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1995) Cancer Res. 55, 2737–2742
7. Tewari, M., Telford, W. G., Miller, R. A., and Dixit, V. M. (1995) J. Biol. Chem. 270, 1885–1888
8. Enari, M., Hug, H., and Nagata, S. (1995) Nature 377, 1414–1422
9. Bump, N. J., Hackett, M., Hugunin, M., Seshagiri, S., Brady, K., Chen, P., Ferszt, C., Franklin, S., Gaytou, T., Li, P., Licari, P., Markovich, J., Shi, L., Greenberg, A. H., Miller, L. K., and Wong, W. W. (1995) Cell 81, 801–809
10. Tewari, M., Teford, W. G., Miller, R. A., and Dixit, V. M. (1995) J. Biol. Chem. 270, 22705–22708
11. Clerm, R. J., Fechheimer, M., and Miller, L. K. (1993) Science 258, 1388–1390
12. Beidler, D. R., Tewari, M., Friesen, P. D., and Bredesen, D. E. (1993) J. Neurochem. 61, 2318–2321
13. Bredesen, D. E., Salloum, M. E., Yamin, T. T., Yu, V. L., and Nicholson, D. W. (1995) J. Biol. Chem. 270, 15870–15876
14. Tewari, M., Quan, L. G., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S., and Dixit, V. M. (1995) Cell 81, 801–809
15. Tewari, M., Tocci, M., Horvitz, H. R., and Dixit, V. M. (1995) J. Biol. Chem. 270, 15626–15628
16. Ramage, P., Cheneval, D., Chvei, M., Graff, P., Hemmig, R., Heng, R., Kocher, H. P., Mackenzie, A., Memmert, K., Revesz, L., and Wishart, W. (1995) J. Biol. Chem. 270, 9378–9383
17. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Yu, V. L., and Nicholson, D. W. (1995) Nature 377, 891–893
18. Milligan, C. E., Prevette, D., Yagnimana, H., Homma, S., Cardwell, C., Fritz, L., Tomaselli, K. J., Oppenheim, R. W., and Schwartz, L. M. (1995) Nature 377, 248–251
19. Bump, N. J., Hackett, M., Hugunin, M., Seshagiri, S., Brady, K., Chen, P., Ferszt, C., Franklin, S., Gaytou, T., Li, P., Licari, P., Markovich, J., Shi, L., Greenberg, A. H., Miller, L. K., and Wong, W. W. (1995) Science 269, 1885–1888
20. Boudreau, N., Sympson, C. J., Web, Z., and Bissell, M. J. (1995) Science 267, 891–893
21. Miller, A. J., Fernandes-Alnemri, T., Takahashi, A., Armstrong, R., Krebs, J., Fritz, L., Tewari, M., Telford, W. G., Miller, R. A., and Dixit, V. M. (1995) J. Biol. Chem. 270, 15626–15628
22. Enari, M., Hug, H., and Nagata, S. (1995) Nature 375, 73–77
30. Vaux, D. L., Cory, S., and Adams, J. M. (1988) Nature 335, 440–442.
31. Hockenbery, D. M., Nunez, G., Milliman, C., Sreiber, R. D., and Korsmeyer, S. J. (1990) Nature 345, 334–336.
32. Böse, L. H., Gonzalez-García, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L., Mao, X., Nunez, G., and Thompson, C. B. (1993) Cell 74, 597–608.
33. Hengartner, M. O., Ellis, R. E., and Horvitz, H. R. (1992) Nature 356, 494–499.
34. Hengartner, M. O., and Horvitz, H. R. (1994) Cell 76, 665–676.
35. Horvitz, H. R., Shaham, S., and Hengartner, M. O. (1994) Cold Spring Harbor Symp. Quant. Biol. 111, 377–385.
36. Yang, T., Knappe, K., and Craig, R. (1995) J. Cell Biol. 128, 1173–1184.
37. Revesz, L., Briswalter, C., Heng, R., Leutwiler, A., Mueller, R., and Waethrich, H. (1994) Tetrahedron Lett. 35, 9693–9696.
38. Graybill, T. L., Dolle, R. E., Helasek, C. T., Miller, R. E., and Ator, M. E. (1994) Int. J. Peptide Protein Res. 44, 173–182.
39. Thornberry, N. A., Peterson, E. P., Zhao, J. J., Howard, A. D., Griffin, P. R., and Chapman, K. T. (1994) Biochemistry 33, 3934–3940.
40. Morrison, J. F., and Walsh, C. T. (1988) Enzymol. Relat. Areas Mol. Biol. 61, 201–301.
41. Ranjit, G. B., Cheng, M.-F., Mackay, W., Whitacre, C. M., Berger, J. S., and Berger, N. A. (1995) Clin. Cancer Res. 1, 223–234.
42. Demuth, H. U. (1990) J. Enzyme Inhibition 3, 249–278.
43. Esser, R. E., Angelo, R. A., Murphy, M. D., Watts, L. M., Thornburg, L. P., Palmer, J. T., Talhouk, J. W., and Smith, R. E. (1994) Arthritis Rheum. 37, 236–247.
44. Pronk, G. J., Ramer, K., Amiri, P., and Williams, L. T. (1996) Science 271, 808–810.
45. Rasnick, D. (1985) Anal. Biochem. 149, 461–465.
46. Vaux, D. L., Haecker, G., and Strasser, A. (1994) Cell 76, 777–779.
47. Martin, S. J., Newmeyer, D. D., Mathias, S., Farshchian, D. M., Wang, H. G., Reed, J. C., Kolesnik, R. N., and Green, D. R. (1995) EMBO J. 14, 5191–5200.
48. Enari, M., Hose, A., and Nagata, S. (1996) EMBO J. 14, 15021–15028.
49. Boulakia, C. A., Chen, G., Ng, F. W. H., Teodoro, J. G., Branton, P. E., Nicholson, D. W., Poirier, G. G., and Shore, G. C. (1996) Oncogene 12, 529–533.
50. Chinnaian, A. M., Orth, K., O’Rourke, K., Duan, H., Poirier, G. G., and Dixit, V. M. (1996) J. Biol. Chem. 271, 4573–4576.
51. Slee, E. A., Zhu, H., Chow, S., MacFarlane, M., Nicholson, D. W., and Cohen, G. M. (1996) Biochem. J. 315, 21–24.