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Fas Stimulation Induces RB Dephosphorylation and Proteolysis That Is Blocked by Inhibitors of the ICE Protease Family

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Abstract Fas antigen is a member of the tumor necrosis factor/nerve growth factor receptor family. Stimulation of Fas by Fas ligand or agonistic antibodies results in the activation of interleukin-1β converting enzyme-like (ICE-like) proteases, and proteolytic cleavage of poly(ADP-ribose) polymerase (PARP). Ultimately, Fas activation leads to apoptotic cell death. The importance of PARP cleavage to the death process remains unclear. We have hypothesized that the cleavage of other cellular substrates may be important for Fas-mediated apoptosis. Here we show that stimulation of Fas results in significant alterations of retinoblastoma protein (RB). Treatment of Jurkat cells, a human leukemic T cell line, with anti-Fas induces dephosphorylation of RB, followed by proteolytic cleavage. These events precede internucleosomal DNA fragmentation. Dephosphorylation and cleavage of RB are inhibited by a specific tetrapeptide inhibitor of ICE-like proteases or by expression of cowpox virus CrmA protein or the Bcl-2 oncoprotein. Inhibition of these RB changes correlates with inhibition of apoptosis. We propose that cleavage of RB may represent an important step in the pathway of Fas-mediated apoptotic cell death. J. Cell. Biochem. 64:586–594.

Key words: Fas; apoptosis; RB; ICE; protease

Apoptosis is an active form of cell death that is regulated by cellular gene products. The elimination of cells via apoptosis plays an important role in numerous biological processes, including the development, maintenance, and function of the human immune system [Ellis et al., 1991; Thomspon, 1995]. Thus, the identification of specific gene products that are involved in regulating apoptotic processes is vital to deciphering the complexity of immune system function.

In a variety of different models, treatment of cells with apoptotic stimuli has been shown to activate members of the interleukin-1β converting enzyme (ICE) family of cysteine proteases [Thornberry et al., 1992; Cerretti et al., 1992; Yuan et al., 1993]. To date, several members of this unique family of proteases have been identified and cloned, including ICE, CED-3, ICE rel II/ICH-2, ICE rel III, CPP32, Mch2, Mch3, Mch4, Nedd-2/ICH-1, and MACH/FLICE [reviewed in Martin and Green, 1995; Fraser and Evan, 1996]. The activation of intracellular ICE-like proteases leads to apoptotic cell death, possibly due to the proteolytic cleavage of important intracellular substrates [Martin and Green, 1995].

In the mammalian immune system, Fas mediates apoptosis in activated T cells and in target cells recognized by cytotoxic T lymphocytes expressing Fas ligand [Trauth et al., 1989; Itoh et al., 1991; Nagata and Golstein, 1995]. Activation of CPP32, an ICE-like protease, has been shown to be critically important in Fas-mediated apoptosis [Schlegel et al., 1996; Hasegawa et al., 1996; Enari et al., 1996]. One of the known substrates of CPP32 is PARP protein [Lazebnik et al., 1994; Tewari et al., 1995;

Abbreviations used: Ac-YVAD-CMK, acetyl-YVAD-chloromethyl ketone; ICE, interleukin-1β converting enzyme; kDa, kilodaltons; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; RB, retinoblastoma protein.

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Nicholson et al., 1995]. Expression of the cowpox virus protein CrmA [Ray et al., 1992], a direct inhibitor of ICE-like proteases (Komiyama et al., 1994), can block Fas-mediated cleavage of PARP [Tewari et al., 1995] and also inhibits Fas-mediated apoptosis [Tewari et al., 1995; Tewari and Dixit, 1995; Enari et al., 1995; Los et al., 1995]. These findings suggest that the cleavage of specific intracellular proteins such as PARP may be important for Fas-mediated apoptotic execution. Presently, however, the full breadth of proteins that are cleaved by ICE-like proteases is unknown. Furthermore, it remains to be determined which substrate proteins, including PARP, represent key proteins that must be cleaved in order to trigger complete execution of Fas-mediated apoptosis.

Recent work indicates that RB protein may play an important role in regulating apoptosis. Overexpression of RB protects SAOS-2 human osteosarcoma cells from radiation-induced apoptosis [Haas-Kogan et al., 1995]. By contrast, functional inactivation of RB promotes apoptosis in the central nervous system and fetal liver of $Rb^{-/-}$ mouse embryos [Lee et al., 1992; Jacks et al., 1992; Clarke et al., 1992], in developing mouse lens [Morgenbesser et al., 1994], in differentiating P19 embryonal carcinoma cells [Slack et al., 1995], and in drug-treated mouse embryo fibroblasts [Almasan et al., 1995]. In addition, we have shown that drug treatment of HL-60 or U937 cells results in cleavage of hypophosphorylated RB into at least two peptide fragments with molecular weights of 48 and 68 kDa [Dou et al., 1995; An and Dou, 1996]. Thus, drug treatment may facilitate functional inactivation of RB.

In the present study we demonstrate that anti-Fas treatment of a human leukemic T cell line induces RB dephosphorylation and cleavage. We also show that Fas-mediated RB dephosphorylation and cleavage can be inhibited by acetyl-YVAD-chloromethyl ketone (Ac-YVAD-CMK) tetrapeptide, CrmA protein, or Bcl-2 protein, implicating the involvement of ICE-like protease(s) in both of these processes. We propose that inactivation of RB by proteolytic cleavage is an important step in Fas-mediated apoptosis.

MATERIALS AND METHODS

Cell Lines and Reagents

Jurkat, a human leukemic T cell line, was kindly provided by Dr. N. Vujanovic (University of Pittsburgh Cancer Institute). Jurkat cells were grown in an atmosphere of 5% CO_2 and 37°C in RPMI 1640 media containing 10% heat-inactivated fetal calf serum, penicillin, streptomycin sulfate, and L-glutamine. Media and media supplements were obtained from Gibco/BRL (Gaithersburg, MD).

Agonistic antihuman Fas monoclonal antibody (clone CH-11) and antihuman Fas blocking monoclonal antibody (clone ZB4) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-RB monoclonal antibody (G3-245) was purchased from PharMingen (San Diego, CA). The tetrapeptide inhibitor, acetyl-YVAD-chloromethyl ketone (Ac-YVAD-CMK) was purchased from Bachem Bioscience Inc. (King of Prussia, PA) and dissolved in water.

Treatment of Cells With Anti-Fas Antibody

Cells were counted, pelleted, and resuspended in culture media at a density of 5×10^6 cells per milliliter. To the media was added 1/10 volume of 2,000 ng/ml anti-Fas in PBS, to give a final concentration of 200 ng/ml anti-Fas. For control cultures, 1/10 volume of PBS was added. Cells were then placed at 37°C for varying lengths of time. At the indicated time points, cells were centrifuged, resuspended in PBS, and pelleted again. Cell pellets were then processed for analysis of protein or genomic DNA.

Western Blot Analysis of RB Protein

Cell pellets were lysed and analyzed by Western blotting using anti-RB monoclonal antibody G3-245 as previously described [An and Dou, 1996]. Proteins were electrophoresed on 6.5% SDS-PAGE gels, and filters were incubated with 5 μg/ml anti-RB, followed by development using an enhanced chemiluminescence kit from Amersham (Arlington heights, IL).

Analysis of Genomic DNA

Aliquots of treated cells were used to prepare genomic DNA as previously described [An and Dou, 1996]. DNA samples (3 μg/lane) were electrophoresed on 2% agarose gels and stained with ethidium bromide.

Construction of Expression Vectors Encoding the CrmA-KT3 or Bcl-2-KT3 Epitope-Tagged Proteins

Polymerase chain reaction technology (PCR) [Saiki et al., 1988] was used to fuse sequences encoding the KT3 epitope tag [MacArthur and

Walter, 1984] to sequences encoding the carboxyl terminus of full-length CrmA or murine Bcl-2 protein. KT3 is an 11 amino acid epitope tag sequence derived from SV40 virus T antigen [MacArthur and Walter, 1984]. PCR reactions were performed using full-length *CrmA* cDNA (kindly provided by Dr. D. Pickup, Duke University Medical Center, Durham, NC) or full-length murine *Bcl-2* cDNA as templates. For amplification of *CrmA-KT3*, the 5′ amplimer was 5′-GCCCGGGCAAAATCGATTGCCATGGA-3′, and the 3′ amplimer was 5′-TACCCGGGTCAAGTTTCCGGTTCCGGCGGCGGAGTCGGCGGTTTATTAGTTGTTGGAGA-3′. For amplification of *Bcl-2-KT3*, the 5′ amplimer was 5′-CTGACCCGGGGAATTCGTACCTGCAGCTTCTTTTCGGGGAACCATGGCGCAAGCCGGGAGAACA-3′, and the 3′ amplimer was 5′-CTGTAAGCTTCCCGGGTCTAGATCAAGTTTCCGGTTCCGGCGGCGGAGTCGGCGGTTTCTTGTGGCCCAGGTATGCA-3′. PCR reaction products were subcloned into Bluescript (Stratagene, La Jolla, CA) and verified by DNA sequencing [Sanger et al., 1977]. The *CrmA-KT3* and *Bcl-2-KT3* fusion cDNAs were then excised from Bluescript and subcloned individually into the CMV/neo expression vector, downstream from a cytomegalovirus promoter. The CMV/neo vector contains a cytomegalovirus promoter for expression of the insert cDNA, a polyadenylation signal from the human β-globin gene, and a gene encoding resistance to G418 (neo) under the control of a thymidine kinase promoter.

Generation of Jurkat Cell Lines Expressing the CrmA-KT3 or Bcl-2-KT3 Fusion Protein

To generate stable cell lines expressing epitope-tagged proteins, the CMV/neo/CrmA-KT3 and CMV/neo/Bcl-2-KT3 expression constructs were introduced individually into Jurkat cells by electroporation (250 V, 960 μF). As a control, the CMV/neo vector alone was electroporated into Jurkat cells. Following electroporation, transfected cells were selected in media containing 0.5 mg/ml G418. After 2 weeks of selection, independent clones were isolated by limiting dilution. Expression of the CrmA-KT3 or Bcl-2-KT3 proteins in independent clonal cell lines was assessed by Western blotting of whole cell lysates using anti-KT3 monoclonal antibody.

Determination of Cell Viabilities

Cells treated with anti-Fas antibody were scored for viability based on trypan blue exclusion. Strict criteria were used for scoring. Briefly, in addition to bright blue cells, cells which exhibited only a faint blue-green hue but showed morphological changes characteristic of apoptosis (nuclear condensation and membrane blebbing) were also scored as dead. Using these strict criteria, rapid kinetics of cell death were observed.

RESULTS

Anti-Fas Treatment of Jurkat Cells Induces RB Dephosphorylation and Cleavage

In exponentially growing Jurkat cells we found that most of the cellular RB protein was present in a hyperphosphorylated form with an approximate molecular weight of 120 kDA (p120/hyper) (Fig. 1A, lane 1). Treatment of these cells for 1 h with 200 ng/ml of agonistic anti-Fas monoclonal antibody, however, resulted in the conversion of about 50% of p120/hyper RB to a 115 kDa form (p115/hypo) (Fig. 1A, lanes 2, 3). By 2 h, virtually all of the detectable RB had been converted to the 115 kDa form (Fig. 1A, lane 4). In previous studies we have shown that treatment of HL-60 human promyelocytic leukemia cells with chemotherapeutic drugs results in a similar conversion of RB protein and that the 115 kDa form represents a hypophosphorylated form of RB [Dou et al., 1995; An and Dou, 1996]. The drug-induced dephosphorylation of RB requires the action of a calyculin A–inhibitable, serine/threonine phosphatase [Dou et al., 1985].

By 4 h of anti-Fas treatment, the amount of p115/hypo/RB in Jurkat cells began to decline, concomitant with the appearance of a 48 kDa RB proteolytic fragment, p48 (Fig. 1A, lanes 5–7; longer exposure of the gel shown in Fig. 1A revealed that some p48 could be detected by 2 h). The monoclonal antibody used in these studies specifically recognizes the p48 proteolytic fragment of RB but not p68, another RB proteolytic fragment detected in drug-treated cells using a different monoclonal antibody [An and Dou, 1996]. Treatment of Jurkat cells for 8 h with antibody diluent (PBS) alone or treatment simultaneously with agonistic anti-Fas and blocking anti-Fas antibody did not result in RB dephosphorylation or proteolysis (Fig. 1A, lanes 8, 9). Thus, the RB changes seen following treatment with agonistic anti-Fas were specifically mediated by Fas antigen on the surface of Jurkat cells.

Analysis of genomic DNA from the samples shown in Figure 1A revealed that internucleosomal DNA fragmentation was first detected

between 2 and 4 h after addition of anti-Fas (data not shown; see Fig. 3B). The extent of DNA fragmentation increased with longer anti-Fas treatment, reaching a maximum after about 8 h. Comparisons of kinetic data indicate that Fas-mediated RB cleavage occurs immediately prior to degradation of genomic DNA to oligonucleosomal-length fragments.

Acetyl-YVAD-Chloromethyl Ketone Blocks Fas-Mediated RB Dephosphorylation and Proteolysis

Previous studies have shown that ICE-like proteases are important in Fas-mediated apoptosis [Schlegel et al., 1996; Hasegawa et al., 1996; Enari et al., 1995, 1996; Tewari et al., 1995; Tewari and Dixit, 1995; Los et al., 1995; Kuida et al., 1995]. To determine whether ICE-like proteases are involved in Fas-mediated changes in RB protein, we studied the effects of Ac-YVAD-CMK, a well-characterized, membrane-permeable inhibitor of ICE-like proteases [Thornberry et al., 1992; Lazebnik et al., 1994, 1995; Enari et al., 1995]. In these studies we also sought to focus on signaling events that are more distal to Fas antigen signaling. To accomplish this, Jurkat cells were transiently exposed to anti-Fas and then washed three times in phosphate-buffered saline (PBS) and incubated for an additional 6 h in antibody-free media with or without Ac-YVAD-CMK. Using this procedure we found that transient exposure to anti-Fas for 25 min, followed by 6 h in antibody-free media, was sufficient to generate roughly equimolar amounts of p120/hyper and p115/hypo, as well as readily detectable p48 fragment (Fig. 1B, lanes 1, 2). Addition of Ac-YVAD-CMK to 1 or 5 μM at the start of the 6 h incubation completely blocked production of the p48 fragment but did not significantly affect RB dephosphorylation (Fig. 1B, lanes 3, 4). By contrast, addition of Ac-YVAD-CMK to 20 μM almost completely blocked the production of the p115/hypo form of RB (Fig. 1B, lane 5).

Taken together, our studies with Ac-YVAD-CMK inhibitor indicate that the Fas-mediated independent processes of RB dephosphorylation and RB proteolysis both involve the action of an ICE-like protease. Moreover, since RB proteolysis is inhibited by Ac-YVAD-CMK concentrations of less than or equal to 1 μM, while inhibition of dephosphorylation requires 20 μM

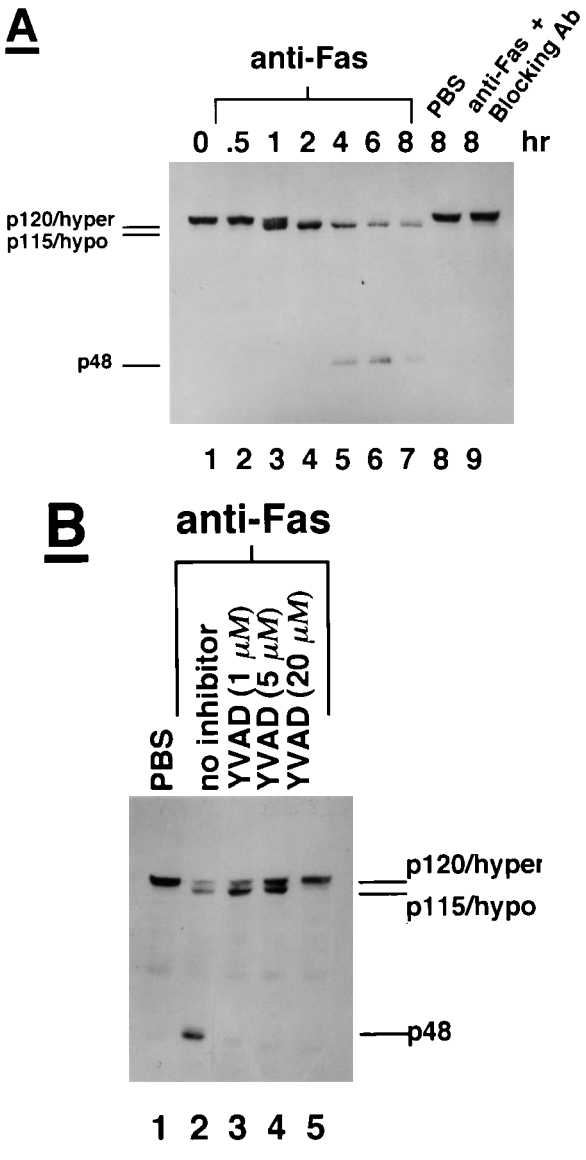

Fig. 1. **A:** Fas stimulation induces RB dephosphorylation and cleavage in Jurkat cells. Jurkat cells were plated in medium at 5×10^6 cells/ml. Agonistic anti-Fas antibody (anti-Fas), diluted in PBS, was added to a final concentration of 200 ng/ml (*lanes 2–7, 9*). PBS alone was added to cells in *lane 8*. In lane 9, anti-Fas blocking antibody (Blocking Ab) was added to a final concentration of 500 ng/ml simultaneously with agonistic anti-Fas. Following the addition of antibodies or PBS, cells were incubated at 37°C. At the indicated times, whole cell extracts were prepared and analyzed by Western blotting using anti-RB monoclonal antibody G3-245. The locations of p120/hyper/RB, p115/hypo/RB, and the p48 RB proteolytic fragment are indicated. **B:** Inhibition of Fas-mediated RB dephosphorylation and cleavage by Ac-YVAD-CMK. Jurkat cells, plated at 5×10^6 cells/ml in complete media, were pretreated for 25 min at 37°C with 200 ng/ml anti-Fas (*lanes 2–5*) or PBS alone (*lane 1*). Following incubation, cells (5×10^6 per sample) were washed three times with 1.0 ml of PBS and then resuspended in fresh media. Ac-YVAD-CMK, diluted in water, was added to a final concentration of 1 (lane 3), 5 (lane 4), or 20 μM (lane 5). Water alone was added to cells depicted in lanes 1 and 2. Cells were then incubated for an additional 6 h at 37°C before whole cell lysates were prepared and analyzed by Western blotting.

of Ac-YVAD-CMK, it appears likely that different ICE-like proteases are involved in each process. In the case of proteolysis, the RB cleavage enzyme may itself be an ICE-like protease. However, we cannot rule out the possibility that activation of an upstream ICE-like protease leads to the activation of an RB protease that is not a member of the ICE family.

Expression of Epitope-Tagged CrmA or Bcl-2 in Jurkat Cells

To characterize Fas-mediated RB dephosphorylation and proteolysis in greater detail, we studied the effects of cowpox virus CrmA protein or the Bcl-2 oncoprotein on these two processes. CrmA has been shown to be a potent inhibitor of Fas-mediated apoptosis [Tewari et al., 1995; Tewari and Dixit, 1995; Enari et al., 1995; Los et al., 1995] and a direct inhibitor of ICE-like proteases [Ray et al., 1992; Komiyama et al., 1994]. Bcl-2, on the other hand, provides only partial protection against Fas-mediated apoptosis [Itoh et al., 1993; Takayama et al., 1995]. The mechanism whereby Bcl-2 inhibits certain ICE-like protease–mediated events remains unknown [Miura et al., 1993; Shimizu et al., 1996].

CrmA and Bcl-2 were expressed in Jurkat cells as epitope-tagged proteins under the control of a cytomegalovirus promoter (see Materials and Methods). In each case an 11 amino acid epitope tag, KT3 [MacArthur and Walter, 1984], derived from SV40 virus T antigen was fused to the carboxyl terminus of the full-length protein. Following electroporation, multiple independent clonal cell lines were isolated and analyzed (Figs. 2–5). In Figure 2, Western blotting with anti-KT3 monoclonal antibody demonstrated expression of CrmA-KT3 protein (40 kDa) in three clones of CrmA/Jurkat cells and Bcl-2-KT3 protein (29 kDa) in three clones of Bcl-2/Jurkat cells. No expression of the KT3 epitope was detected in three clones of vector-transfected control cells (Vector).

CrmA and Bcl-2 Inhibit Fas-Mediated RB Dephosphorylation, RB Cleavage, and Apoptosis in Jurkat Cells

Treatment of vector-transfected Jurkat cells (clone B) with anti-Fas resulted in changes in RB protein with kinetics similar to those seen in untransfected cells. Significant conversion of p120/hyper/RB to p115/hypo/RB was detected by 1 h, and complete dephosphorylation was

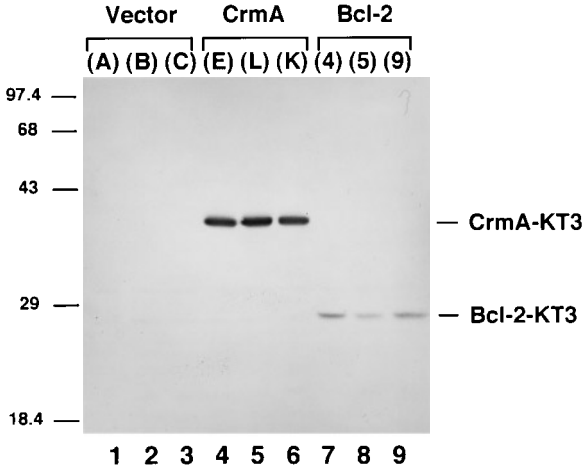

Fig. 2. Expression of epitope-tagged CrmA or Bcl-2 proteins in transfected Jurkat cells. Whole cell lysates were prepared from independent clones of Jurkat cell lines transfected with CMV/neo vector alone (Vector) (*lanes 1–3*), vector containing a cDNA encoding the CrmA-KT3 fusion protein (CrmA) (*lanes 4–6*), or vector containing a cDNA encoding the Bcl-2-KT3 fusion protein (Bcl-2) (*lanes 7–9*). Protein samples (100 μg/lane) were resolved on a 12.5% SDS-PAGE gel and then analyzed by Western blotting using an anti-KT3 monoclonal antibody. The locations of CrmA-KT3, Bcl-2-KT3, and molecular weight markers are indicated. The clone names are indicated in parentheses.

observed by 2 h (Figs. 3A, 5A, lanes 1–3). The p48proteolytic fragment of RB was first detected after 2 h treatment, with even greater levels seen after 4 h (Figs. 3A, 5A, lanes 3–5).

By contrast, in CrmA cells (clone L), Fas-mediated RB dephosphorylation was substantially inhibited (Fig 3A, lanes 7–12). After 8 h of anti-Fas treatment, only a minority of RB protein in CrmA cells was present in the p115/hypo form. In addition, the p48 fragment was only barely detectable after 8 h. A similar pattern of inhibition by CrmA was found in three independent clonal cell lines (Fig. 3C).

The inhibition by CrmA of RB dephosphorylation and cleavage also correlated with inhibition by CrmA of other markers of Fas-mediated apoptosis. With Vector cells (clone B), internucleosomal fragmentation of DNA was clearly evident after 4 h of treatment, whereas no fragmentation was seen with CrmA cells (clone L) even after 8 h (Fig. 3B). Also, similar to what others have observed [Tewari et al., 1995; Tewari and Dixit, 1995; Enari et al., 1995; Los et al., 1995], when cells were scored for morphological evidence of apoptosis, greater than 90% of Vector cells were apoptotic after 8 h, while only 11% of CrmA cells were apoptotic (Fig. 4).

Cells expressing Bcl-2 (clone 4), like CrmA cells, exhibited inhibition (albeit reduced) of

Fas-mediated RB dephosphorylation and cleavage (Fig. 5A). After 8 h anti-Fas treatment of Bcl-2 cells, p120/hyper and p115/hypo forms of Rb were present in roughly equimolar amounts. The p48 RB fragment was readily detectable after 4 h. A similar pattern of inhibition by Bcl-2 was seen in three independent clonal cell lines (Fig. 5B).

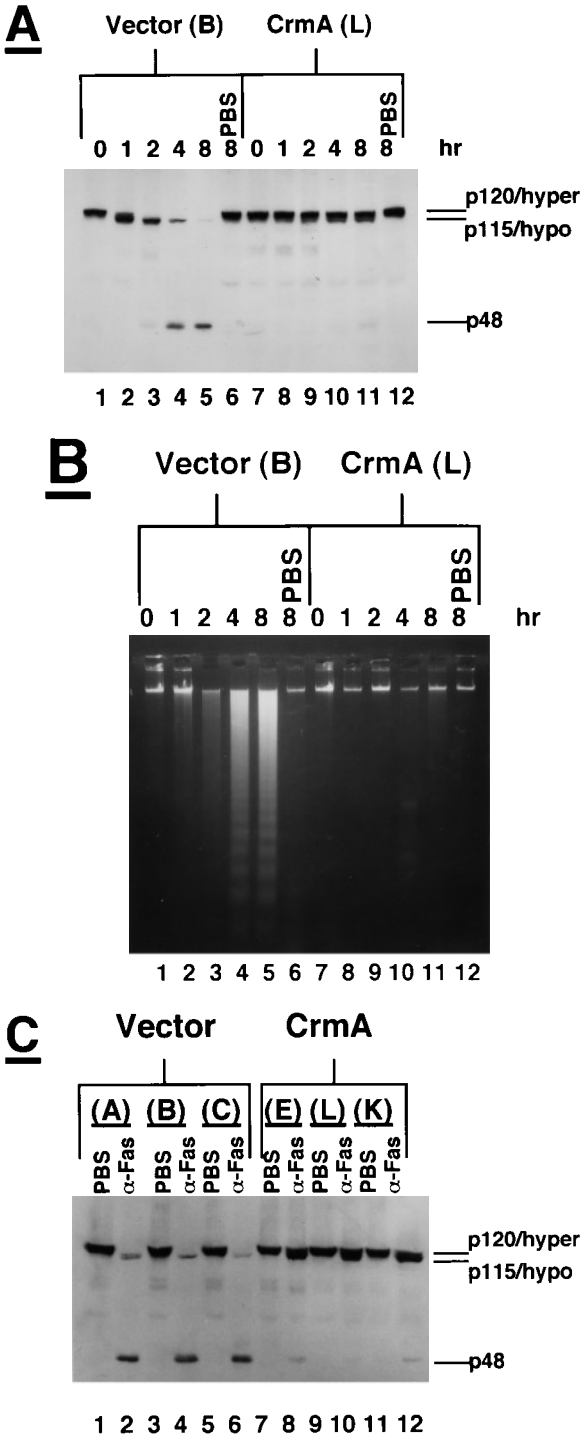

Fig. 3. **A:** CrmA inhibition of Fas-mediated RB dephosphorylation and cleavage. Vector (clone B) or CrmA (clone L) cells were plated at 5×10^6 cells/ml in media containing 200 ng/ml anti-Fas (*lanes 2–5, 8–11*) or PBS control (*lanes 6, 12*). Cells were then incubated at 37°C for the indicated times before preparation of whole cell extracts and Western blot analysis, as described in Fig. 1. **B:** CrmA inhibition of RB changes correlates with inhibition of DNA fragmentation. Aliquots of cell lysates from panel A were used to prepare genomic DNA. Genomic DNA samples were electrophoresed on an agarose gel and stained with ethidium bromide **C:** CrmA inhibition of Fas-mediated RB changes in multiple independent clonal cell lines. Vector clones A, B, C (*lanes 1–6*) or CrmA clones E, L, K (*lanes 7–12*) were treated with 200 ng/ml anti-Fas (even-numbered lanes) or PBS control (odd-numbered lanes) for 6 h at 37°C before preparation of whole cell extracts and Western blotting.

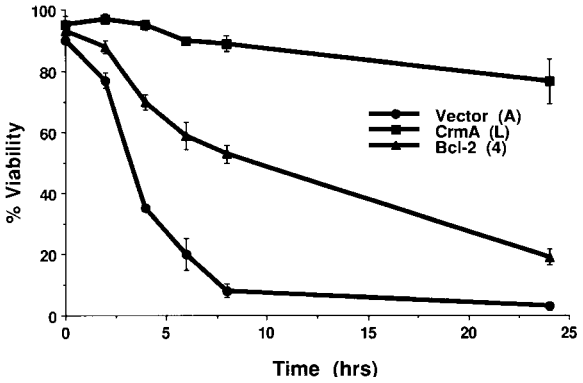

Fig. 4. Viabilities of transfected Jurkat cell lines in the presence of anti-Fas. Vector (clone A), CrmA (clone L), or Bcl-2 (clone 4) cells (5×10^6) were incubated at 37°C in 1.0 ml of complete media containing 200 ng/ml anti-Fas antibody. At the indicated times, cell viabilities were determined by trypan blue exclusion as described in Material and Methods. Each data point represents the mean of triplicate samples, with a minimum of 100 total cells counted per sample. Error bars represent standard deviations.

The reduced ability of Bcl-2, relative to CrmA, to inhibit Fas-mediated RB changes also correlated with a reduced ability of Bcl-2 to inhibit other markers of Fas-mediated apoptosis. Minor levels of internucleosomal DNA fragmentation were seen in Bcl-2 cells after only 4 h, and 47% of Bcl-2 cells were scored as apoptotic after 8 h (Fig. 4). The finding that Bcl-2 is only partially effective at blocking Fas-mediated apoptosis is consistent with the results of others [Itoh et al., 1993; Takayama et al., 1995].

DISCUSSION

The Fas antigen plays important roles in the function of immune system. Fas mediates apoptosis in activated T cells and in target cells

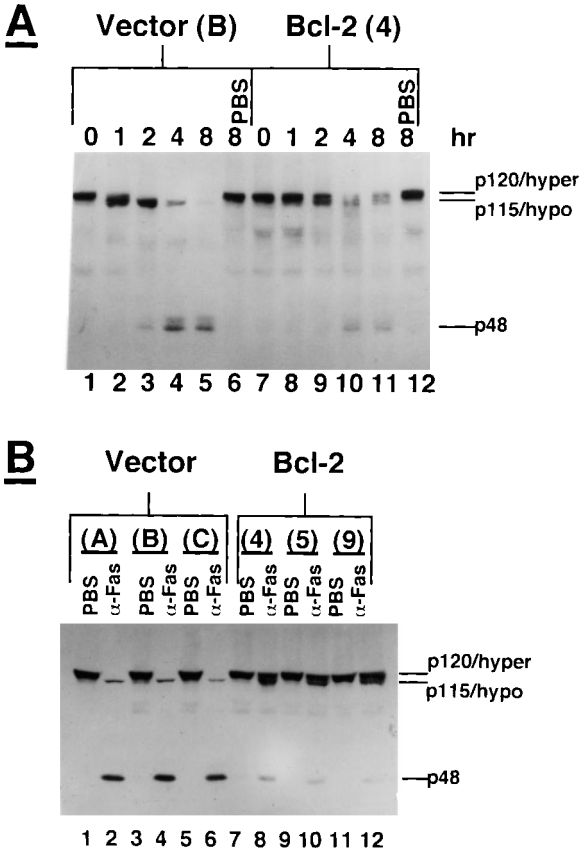

Fig. 5. **A:** Bcl-2 inhibition of Fas-mediated RB dephosphorylation and cleavage. Vector (clone B) (*lanes 1–6*) or Bcl-2 (clone 4) (*lanes 7–12*) cells were treated with anti-Fas or PBS for varying lengths of time and then analyzed as described in Fig. 3A. **B:** Bcl-2 inhibition of Fas-mediated RB changes in multiple independent clonal cell lines. Vector clones A, B, C (*lanes 1–6*) or Bcl-2 clones 4, 5, 9 (*lanes 7–12*) were treated with anti-Fas or PBS and analyzed as described in Fig. 3C.

attacked by Fas ligand–expressing cytotoxic T cells. The stimulation of Fas by Fas ligand or agonistic antibodies results in the activation of intracellular proteases that are members of the ICE protease family. Recent evidence demonstrates that certain members of the ICE protease family such as FLICE/MACH1 directly associate with the activated Fas signaling complex [Boldin et al., 1996; Muzio et al., 1996]. A cascade of activation of different ICE-like proteases then proceeds [Schlegel et al., 1996; Enari et al., 1996; Shimizu et al., 1996].

While the activation of ICE-like proteases has been shown to be essential for Fas-mediated apoptosis, it remains undetermined, from a molecular standpoint, how the activation of these enzymes results in cell death. It will be important to identify the substrates of proteases that are activated during Fas-dependent apoptosis to answer this question. Studies done to date have identified the PARP protein as a substrate of CPP32, an ICE-like protease that is activated relatively late in the Fas-dependent protease cascade [Lazebnik et al., 1994; Tewari et al., 1995; Nicholson et al., 1995; Schlegel et al., 1996; Hasegawa et al., 1996; Enari et al., 1996; Shimizu et al., 1996]. The significance of PARP cleavage, however, remains unclear.

We now report that stimulation of Fas antigen results in significant changes in cellular RB protein. Treatment of Jurkat cells with anti-Fas resulted in dephosphorylation of p120/hyper RB to a p115/hypo form. The dephosphorylation of RB was followed by proteolytic cleavage of the p115/hypo form to smaller peptide fragments, including a readily observable 48 kDa fragment. Kinetic analyses demonstrate that dephosphorylation and cleavage of RB are relatively distal events in Fas-mediated signaling yet still precede internucleosomal fragmentation of genomic DNA. This raises the possibility that degradation of RB may be important for the ultimate genetic demise of the cell.

Both dephosphorylation and cleavage of RB were inhibited by Ac-YVAD-CMK tetrapeptide, CrmA, or Bcl-2. This suggests the involvement of ICE-like proteases in both of these processes. Furthermore, it appears likely that different ICE-like proteases are involved. While 1 μM Ac-YVAD-CMK was sufficient to completely block RB cleavage, 20 μM Ac-YVAD-CMK was required to inhibit dephosphorylation. In the case of RB dephosphorylation, an upstream ICE-like protease could be involved in phosphatase activation, while in the case of RB cleavage it is possible that the RB protease itself may be an ICE-like protease. Verification that the RB protease is a member of the ICE family, however, will require mapping of the cleavage site and purification and cloning of the enzyme.

Although the consequences of RB cleavage have not yet been tested, it is reasonable to propose that cleavage of RB may result in functional inactivation of the protein. In this regard it is important to note that deletion of functional RB protein via gene inactivation has been shown to promote cellular apoptosis in a variety of different systems [Lee et al., 1992; Jacks et al., 1992; Clarke et al., 1992; Morgenbesser et al., 1994; Slack et al., 1995; Almasan et al., 1995]. Thus, functional RB protein is an inhibitor of apoptosis. The destruction of RB via Fas-mediated signaling pathways may there-

fore be an important step in the Fas-dependent death process.

Perhaps the destruction of RB significantly alters the transcriptional potential of the cell. RB is known to bind to and negatively regulate the transcriptional activity of E2F-1. Several recent studies have shown that E2F-1, in direct contrast to RB, can act to promote apoptosis [Qin et al., 1994; Shan and Lee, 1994; Wu and Levine, 1994; Kowalik et al., 1995; Field et al., 1996; Yamasaki et al., 1996]. The physical destruction of RB during Fas-mediated cell death may free up E2F-1 protein, thereby allowing E2F-1 to function as an apoptosis-promoting factor. Further studies will be necessary to test these hypotheses.

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