

Bcl-2 Prevents CD95 (Fas/APO-1)-induced Degradation of Lamin B and Poly(ADP-ribose) Polymerase and Restores the NF- κ B Signaling Pathway*

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In the study presented here, we investigated the possible interactions between CD95 (Fas/APO-1) and Bcl-2 by studying the effects of Bcl-2 on the modulation of cellular pathways activated by CD95 using HeLa cells as a model system. We report that stable expression of Bcl-2 in HeLa cells is associated with multiple phenotypic changes. Treatment of HeLa cells with anti-CD95 monoclonal antibody (mAb) resulted in preferential degradation of lamin B compared with lamins A and C. Significant lamin B degradation was detected as early as 1 h after anti-CD95 mAb treatment. In contrast, lamins A and C as well as actin remained unchanged until 4 h after treatment with anti-CD95 mAb, a time point that correlated with the period of DNA fragmentation. These results indicate that selective degradation of lamin B is an early cellular event in response to activation of the CD95 pathway and that it precedes DNA fragmentation. Overexpression of Bcl-2 resulted in prevention of lamin B degradation and DNA fragmentation into oligonucleosome fragments in response to the apoptotic signal by anti-CD95 mAb. In addition, in Bcl-2-overexpressing cells that were protected against apoptosis, anti-CD95 mAb-induced cleavage of poly(ADP-ribose) polymerase was completely blocked. Overexpression of Bcl-2 also resulted in restoration of the CD95-mediated signaling pathway involving activation of the transcription factor NF- κ B (p50/RelA). These findings suggest that Bcl-2 prevents apoptosis in part by preventing the degradation of major nuclear polypeptides such as lamin B and poly(ADP-ribose) polymerase. In addition, our results demonstrate that CD95-mediated signaling involves activation of NF- κ B (p50/RelA).

is dependent on both pre-existing proteins and *de novo* protein synthesis and is characterized in part by nuclear membrane blebbing, chromatin condensation, and internucleosomal DNA degradation into oligonucleosome fragments (1). In addition, we and others have shown that chromatin condensation during apoptosis is accompanied by degradation of the nuclear lamins, which are involved in maintaining the integrity of the nuclear envelope (4–8), and also by degradation of poly(ADP-ribose) polymerase (PARP)¹ (9, 10), which is thought to be involved in DNA repair.

The occurrence of apoptosis is a highly regulated process. The human CD95 receptor, a member of the TNF/nerve growth factor receptor superfamily, is a cell-surface antigen that triggers apoptosis when activated by agonist anti-CD95 mAb and by a specific CD95 ligand (CD95L) (11). Recent deletion studies have identified an 80-residue homology region (death domain) in the carboxyl-terminal part of CD95 and the TNF receptor (12, 13) that appears to play an essential role in transduction of the apoptotic signal. The TNF receptor still retains its apoptotic activity when this region is replaced with the death domain of CD95, raising the possibility of a similar mechanism(s) in induction of apoptosis by these two receptors. Despite this structural similarity, data from the literature suggest that CD95 and TNF receptors may utilize distinct signal transduction pathways, as TNF (but not CD95) induces activation of the transcription factor NF- κ B (14, 15). In spite of these differences, both anti-CD95 mAb and TNF trigger apoptosis. The common pathway of cell death appears to involve activation of interleukin-1 β -converting enzyme (ICE) or ICE/*ced-3* family proteases (16–18). The observations that overexpression of ICE in mammalian cells potentiates CD95-mediated apoptosis and that inhibition of ICE activity suppresses CD95-mediated apoptosis in human cells (18) illustrate the critical importance of this protease pathway in CD95-induced apoptosis.

Another important regulator of apoptosis is Bcl-2, 26-kDa protein that protects cells against apoptosis in a number of experimental systems (1). A number of Bcl-2 homologs have been identified, including Bcl-X_L and BAG-1, which suppress apoptosis, and Bcl-X_s, Bax, and Bad, which promote apoptosis. Current models suggest that the ratios of these anti- to pro-apoptotic proteins may play a regulatory role in apoptosis (19–21). Recent studies suggest that cells derived from a variety of human cancers may have a decreased ability to undergo apoptosis in response to various physiological stimuli (3, 4), and

In recent years, it has become accepted that programmed cell death or apoptosis is a normal physiological phenomenon that plays an important role in the regulation of tissue development, metamorphosis, organ involution, and homeostasis (1, 2). The deregulation of apoptosis has been shown to contribute to the pathogenesis of a number of human diseases including cancer and autoimmune disorders (3, 4). Activation of apoptosis

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¹ The abbreviations used are: PARP, poly(ADP-ribose) polymerase; TNF, tumor necrosis factor; mAb, monoclonal antibody; ICE, interleukin-1 β -converting enzyme; EMSA, electrophoretic mobility shift assay; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

thus, a defect in apoptosis may be involved in the aberrant survival and/or development of cancer.

The mechanism by which Bcl-2 exerts its anti-apoptotic effect is not as yet fully resolved. One view suggests that Bcl-2 may function as an antioxidant and blocks oxidative damage induced by reactive oxygen species generated during apoptosis (1). Accordingly, overexpression of Bcl-2 has been shown to inhibit activation of NF- κ B caused by oxidative stress (22). In contrast, recent studies have shown that apoptosis by a number of agents including anti-CD95 mAb (23) can be induced even in the absence of reactive oxygen, and Bcl-2 could effectively inhibit such apoptosis (24, 25). There is also evidence indicating that activation of NF- κ B is not exclusively restricted to oxidative damage, as TNF has been shown to induce NF- κ B in T lymphocytes without involving reactive oxygen (25). Furthermore, Bcl-2 has been shown to protect mouse L929 cells from TNF-induced oxidative damage and apoptosis without interfering with activation of NF- κ B (26). In brief, these observations suggest that Bcl-2 might protect cells without altering signaling pathways activated during apoptosis.

In this study, we investigated the regulatory interactions between the CD95 and Bcl-2 pathways. We report that the deregulation of Bcl-2 in HeLa cells results in several important phenotypic changes that accompany the inhibition of apoptosis. First, we examined the effect of Bcl-2 on lamin cleavage during CD95-mediated apoptosis. We observed that CD95-mediated DNA fragmentation is preceded by selective degradation of lamin B (compared with lamins A and C) and that Bcl-2 overexpression inhibits lamin degradation as well as DNA fragmentation. Second, we examined the kinetics of PARP cleavage in CD95-activated cells and demonstrated that Bcl-2 overexpression completely blocked anti-CD95 mAb-induced cleavage of PARP. Finally, we observed that treatment of Bcl-2-expressing cells with anti-CD95 mAb was accompanied by activation of NF- κ B (p50/RelA) and also *de novo* synthesis of NF- κ B-regulated proteins. These findings indicate that Bcl-2 deregulation is accompanied by multiple phenotypic changes, each of which might contribute to the inhibition of apoptosis.

MATERIALS AND METHODS

Cells and Expression of Bcl-2—HeLa cells (27, 28) were maintained in modified Eagle's medium supplemented with 10% fetal bovine serum. HeLa cells at a density of 10^6 cells/100-mm diameter plate were transfected with plasmid DNA containing the full-length human Bcl-2 cDNA and a selectable marker, the neomycin phosphotransferase gene (pSSFbcl-2) (29), by calcium phosphate precipitation procedures as described (30). Expression of Bcl-2 in individually isolated clones was determined by immunoblotting with a Bcl-2 mAb (Neomarkers, Inc.). Once a stable cell line from each clone had been established, the drug was removed from the culture medium. The clonal lines have been maintained in drug-free medium since then, and expression of Bcl-2 was periodically examined.

Induction of Apoptosis—Anti-human CD95 mAb (clone CH-11) (31) was obtained from Kamiya Biochemical. Recombinant human interferon- γ (specific activity = 4×10^6 IU/mg) was obtained from Genentech Inc. For induction of apoptosis, cells were treated with interferon- γ (500 IU/ml) for 12 h, and some cultures were treated with anti-CD95 mAb (100 ng/ml) from 1–6 h as described (31). After the desired treatment, both floating and attached cells were collected and analyzed.

Cell Extracts and Immunoblotting—All experiments were performed with cells in logarithmic phase by controlling the plating density. Viability of cells was assayed by trypan blue dye exclusion. Cells extracts were prepared as described (27). Cell lysates containing equal amounts of total protein (15–25 μ g) were resolved on a 10% SDS-polyacrylamide gel, followed by probing with the desired antibodies (27), and immune complexes were detected using an ECL method (Amersham Corp.). For immunoblotting of lamins, we used well characterized chicken antibodies to lamins A and C (recognized by the same antibody) and lamin B (32). For PARP immunoblotting, we used a mAb (C-2-10) against PARP (33). As an internal control, the same blot was reprobed with an unrelated actin antibody (Sigma). Low molecular mass colored markers

(Amersham Corp.) were used as molecular mass standards. Quantitation of specific protein bands was performed using a protein data base scanner (Molecular Dynamics, Inc.).

DNA Fragmentation Assay—For the DNA fragmentation assay, low molecular size DNA was isolated. Briefly, cells (3×10^6 /plate) were seeded in 100-mm plates and treated as desired. Both floating and attached cells were scraped and collected in medium, washed three times with phosphate-buffered saline, and resuspended in 1 ml of lysis buffer (20 mM Tris-HCl, pH 8, 10 mM EDTA, pH 8, and 0.5% Triton X-100). After a 30-min incubation on ice, the lysates were centrifuged at 12,000 rpm in an Eppendorf microcentrifuge for 10 min. Low molecular size DNA in the supernatant was extracted with an equal volume of phenol/chloroform for 1 h at 4 °C. Ammonium acetate (2 M) was added to the aqueous phase, and DNA was precipitated with 2 volumes of ethanol at -20 °C overnight. DNA was treated with RNase A (1 mg/ml) at 37 °C for 1 h, and total DNA was analyzed using 1.5% agarose gel and visualized by ethidium bromide staining of gel.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)—HeLa cells were washed with phosphate-buffered saline and then collected by centrifugation. Nuclear extracts were prepared as described previously (34). EMSA was performed by incubating the nuclear extracts (5 μ g) with a 32 P-radiolabeled high affinity palindromic κ B probe, κ B-pd (coding stand sequence, 5'-CAACGGCAGGGGAAT-TCCCCTCTCTCCTT-3'). The DNA-protein complexes were resolved on 5% native polyacrylamide gels (34). For antibody "supershift" assays, 1 μ l of anti-peptide antiserum specifically recognizing each of the NF- κ B subunits was added to the EMSA reaction 10 min prior to electrophoresis.

RESULTS

Effect of Anti-CD95 mAb on Integrity of Lamins—To explore the possible involvement of nuclear envelope proteins during CD95-mediated apoptosis, we examined the effect of CD95 activation on the integrity of lamins. Previous results have demonstrated that proteolytic cleavage of lamins occurs in several cell types undergoing apoptosis (see Introduction) and is mediated by an ICE family member (7). The results in Fig. 1A demonstrate the kinetics of anti-CD95 mAb-mediated degradation of DNA in HeLa cells. As expected, treatment of HeLa cells with anti-CD95 mAb stimulated internucleosomal DNA degradation into oligonucleosome fragments in a time-dependent manner starting at 4 h post-treatment (Fig. 1A, lane 6). The results of a quantitative enzyme-linked immunosorbent assay-based apoptotic assay (30) that measures cytoplasmic histone-bound DNA complexes generated during apoptotic DNA fragmentation demonstrated lack of DNA fragmentation up to 3 h after anti-CD95 mAb treatment (data not shown). To examine the relationship between the degradation of DNA and lamins, we analyzed the cellular content of the lamins by immunoblotting HeLa cells treated with or without anti-CD95 mAb. As shown in Fig. 1 (B and C), treatment of HeLa cells with anti-CD95 mAb resulted in preferential degradation of lamin B compared with lamins A and C. Significant lamin B degradation (62% degradation compared with levels in control cells) was detected as early as 1 h after anti-CD95 mAb treatment. In contrast, lamins A and C as well as actin remained unchanged until 4 h after treatment with anti-CD95 mAb, a time point that correlated with the period of DNA fragmentation. These results indicate that selective degradation of lamin B is an early cellular event in response to activation of the CD95 pathway.

Bcl-2 Prevents CD95-mediated Degradation of Lamin B—To further explore the possible involvement of lamin B in anti-CD95 mAb-mediated apoptosis, we next examined the effect of Bcl-2 on anti-CD95 mAb-mediated lamin B degradation and DNA fragmentation. For these studies, HeLa cells were transfected with a Bcl-2 expression vector (29), and a number of clonal cell lines stably overexpressing Bcl-2 were generated. Fig. 2A shows the levels of Bcl-2 protein in three out of seven isolated clones, HeLa/Bcl-2.1 (lane 2), HeLa/Bcl-2.7 (lane 3), and HeLa/Bcl-2.5 (lane 4). Clones 1 and 7 express the highest

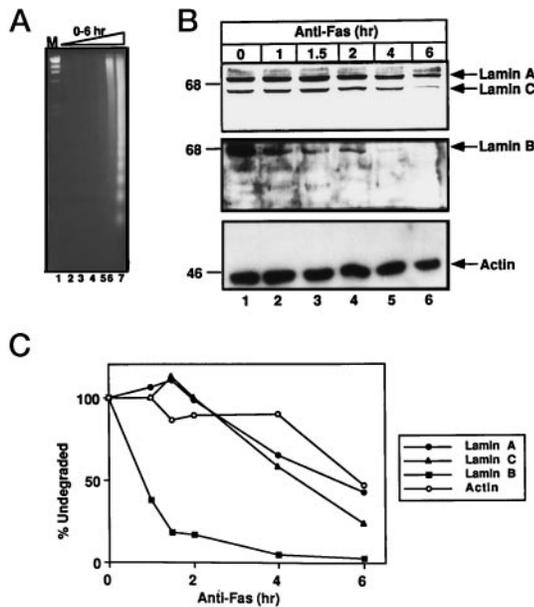


FIG. 1. CD95-induced fragmentation of oligonucleosomal DNA follows degradation of lamin B. *A*, DNA fragmentation. HeLa cells were treated with anti-CD95 mAb (100 ng/ml) for 0–6 h. Ethidium bromide staining of low molecular size DNA from 3×10^6 cells is shown. Lane 1, [λ] DNA (*Hind*III digest) molecular size marker (*M*); lane 2, 0 time; lane 3, 1 h; lane 4, 1.5 h; lane 5, 2 h; lane 6, 4 h; lane 7, 6 h. *B*, effect of anti-CD95 mAb on lamin levels. Samples containing equal amounts of protein (20 μ g) were immunoblotted using well characterized chicken antibodies to lamins A and C (recognized by the same antibody) and lamin B. As an internal control, the lamin B blot was reprobbed with an unrelated actin antibody. *C*, quantitation of degradation of lamins. The relative intensities of the specific lamin bands shown in *B* were quantified using a protein data base scanner. Results are presented as percent lamin contained in the specific lamin band at the various times after anti-CD95 mAb treatment. Results shown in *A* and *B* are from the same experiment and are representative of three independent experiments.

(4–6-fold) levels of Bcl-2 compared with the levels detected in the parental HeLa cells (Fig. 1A, lane 1) or in the control HeLa/PKR.w1 neo clone (lane 5) expressing an unrelated gene. To compare the fate of the lamins in the control and Bcl-2-expressing HeLa cells, cells were treated with anti-CD95 mAb. As shown in Fig. 2, Bcl-2 overexpression prevented the degradation of lamin B (panel C) and DNA fragmentation into oligonucleosome fragments (panel B) in response to the apoptotic signal by anti-CD95 mAb (see also Fig. 4, lanes 8 and 9). The observed protection of cells from anti-CD95 mAb-mediated apoptosis resulted from overexpression of Bcl-2 since it was detected in both clones 1 and 7, but not in control HeLa cells or in HeLa cells overexpressing an unrelated PKR/neo gene (HeLa/PKR.w1 cells) (data not shown). Taken together, these results suggest that (i) CD95-mediated degradation of DNA is preceded by selective degradation of lamin B, and (ii) degradation of lamin B, like DNA fragmentation, can be effectively blocked by Bcl-2 expression.

Bcl-2 Prevents CD95-mediated Degradation of PARP—Previous studies have shown that PARP is also cleaved by the ICE family proteases as an early event during apoptosis in a number of cell types (5, 9, 10). As illustrated in Fig. 3A, incubation of HeLa cells with anti-CD95 mAb resulted in a time-dependent cleavage of PARP and the appearance of an immunoreactive 85-kDa cleavage product. Quantitation of uncleaved PARP indicated that anti-CD95 mAb cleaved ~22 and 53% of PARP by 1 and 1.5 h post-treatment, respectively, suggesting that the cleavage of PARP is also an early cellular marker during CD95-induced apoptosis. More important, in Bcl-2-overexpressing

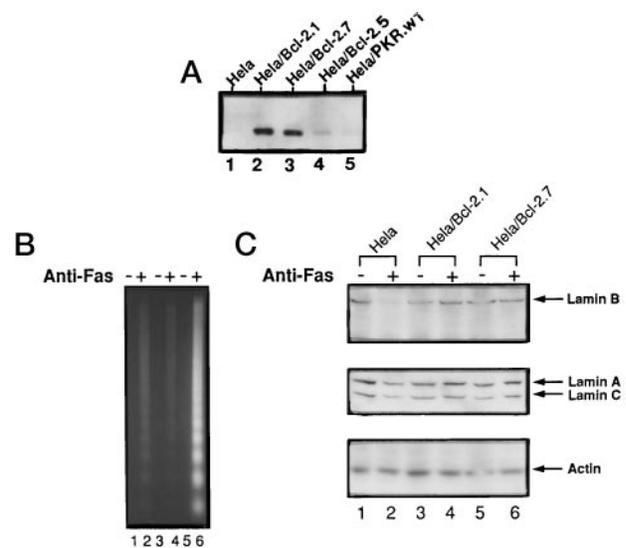


FIG. 2. Bcl-2 suppresses CD95-mediated DNA fragmentation as well as degradation of lamin B. *A*, shown is overexpression of Bcl-2. Lane 1, control HeLa cells; lane 2, clone 1; lane 3, clone 7; lane 4, clone 5; lane 5, control neo clone expressing an unrelated PKR.w1 gene. *B*, HeLa/Bcl-2.1 (lanes 1 and 2), HeLa/Bcl-2.7 (lanes 3 and 4), and HeLa (lanes 5 and 6) cells were treated with (+) or without (–) anti-CD95 mAb (100 ng/ml) for 6 h. Ethidium bromide staining of low molecular size DNA from 3×10^6 cells is shown. *C*, cells were treated with anti-CD95 mAb (100 ng/ml) for 6 h. Whole cell extracts were made, and equal amounts of protein (20 μ g) were immunoblotted with antibodies specific for different types of lamins. As an internal control, the lamin B blot was reprobbed with an unrelated actin antibody. Results shown are representative of five independent experiments.

HeLa cells that were protected against apoptosis, anti-CD95 mAb-induced cleavage of PARP was completely blocked (Fig. 3B; see also Fig. 4, lanes 8 and 9). Taken together, the results from Figs. 1–3 suggest that the enhanced survival of Bcl-2-overexpressing HeLa cells in response to the apoptotic CD95 signal is correlated with the suppression of degradation of lamin B and PARP.

Effect of Protease Inhibitors on Anti-CD95 mAb-induced Degradation of PARP and Lamin B—To understand the biochemical nature of proteases that may be inactivated and/or suppressed by Bcl-2 expression in HeLa cells, we examined the effect of different protease inhibitors on CD95-induced cleavage of PARP and lamin B degradation. The results in Fig. 4 show the effects of the intracellular Ca^{2+} -chelating agent BAPTA, the ICE family protease inhibitor YVAD-cmk, and the serine protease inhibitor TPCK on CD95-induced cleavage of PARP (panel A) and lamin B (panel B). Inclusion of these inhibitors at the concentration used in Fig. 4 had no effect on the viability of cells (data not shown). In these experiments, Bcl-2-overexpressing HeLa cells were used as a negative control (Fig. 4, lanes 8 and 9). In general, BAPTA and YVAD-cmk were more effective in inhibiting anti-CD95 mAb-triggered cleavage of PARP (42% uncleaved in BAPTA and 94% uncleaved in 1 mM YVAD-cmk) compared with lamin B (55% uncleaved in BAPTA and 63% uncleaved in 1 mM YVAD-cmk). Analysis of DNA fragmentation indicated that treatment with both BAPTA and YVAD-cmk (but not TPCK) inhibited the CD95-induced DNA fragmentation as expected from the previous studies (data not shown). In brief, these results not only demonstrate the possible involvement of Ca^{2+} -sensitive cellular events and ICE family proteases in CD95-induced cleavage of PARP and lamin B degradation, but also indicate that these pathways may not be active in Bcl-2-expressing cells (Fig. 4, compare lanes 9 and 8).

Bcl-2 Restores Anti-CD95 mAb-induced NF- κ B Activation—Recent studies have suggested that CD95 and TNF receptors,

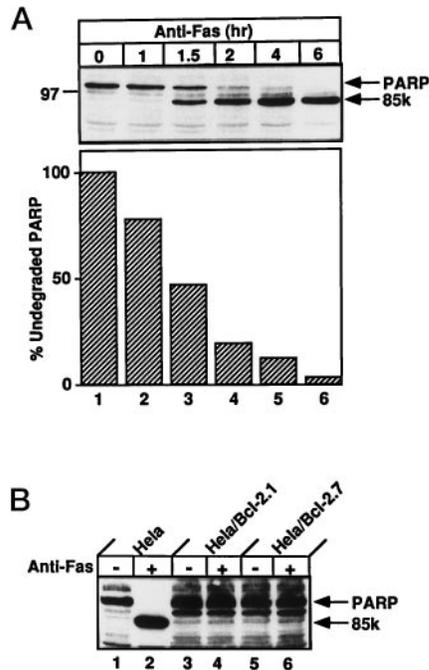


FIG. 3. Bcl-2 prevents CD95-mediated cleavage of PARP. *A*, samples of HeLa cells treated with anti-CD95 mAb for different times were immunoblotted with a mAb (C-2-10) against PARP. The positions of PARP (113 kDa) and the 85-kDa proteolytic cleavage product of PARP (85 kDa) are shown (*upper panel*). The relative intensities of the upper 113-kDa PARP bands were quantified using a protein data base scanner and are presented as percent PARP contained (uncleaved) in the upper band at the various times after anti-CD95 mAb treatment (*lower panel*). *B*, HeLa cells and their Bcl-2-overexpressing clones were treated with anti-CD95 mAb (100 ng/ml) for 6 h. Cell extracts were made and subjected to immunoblot analyses with anti-PARP mAb. An overexposed film demonstrating the lack of the 85-kDa band in Bcl-2-overexpressing clones is shown here. The positions of PARP and its 85-kDa cleaved fragment are shown on the right. Results shown are representative of four independent experiments.

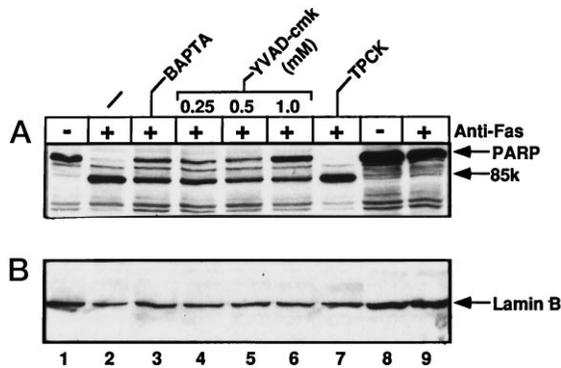


FIG. 4. Effect of protease inhibitors on anti-CD95-induced degradation of PARP and lamin B. Cells were pretreated for 6 h with 400 μ M BAPTA (*lane 3*); 0.25, 0.5, or 1 mM YVAD-cmk (*lanes 4–6*, respectively); 100 μ M TPCK (*lane 7*); or mock 0.1% (v/v) methanol solvent (*lane 2*). Then the cells were incubated with (+) or without (-) anti-CD95 mAb (100 ng/ml) for 2 h. All agents were dissolved in methanol. Shown is the immunoblotting of cell extracts with anti-PARP mAb (*A*) and anti-lamin B antibody (*B*). *Lanes 1–7*, HeLa cells; *lanes 8 and 9*, HeLa/Bcl-2.1 cells. Results shown are representative of three independent experiments.

despite significant homology, may utilize different signal transduction pathways. In particular, TNF (but not CD95) induces activation of NF- κ B (15). Since CD95-induced apoptosis is known to be inhibited by Bcl-2 expression, we hypothesized that the deregulation of Bcl-2 may modulate the signal transduction pathway associated with activation of CD95. We there-

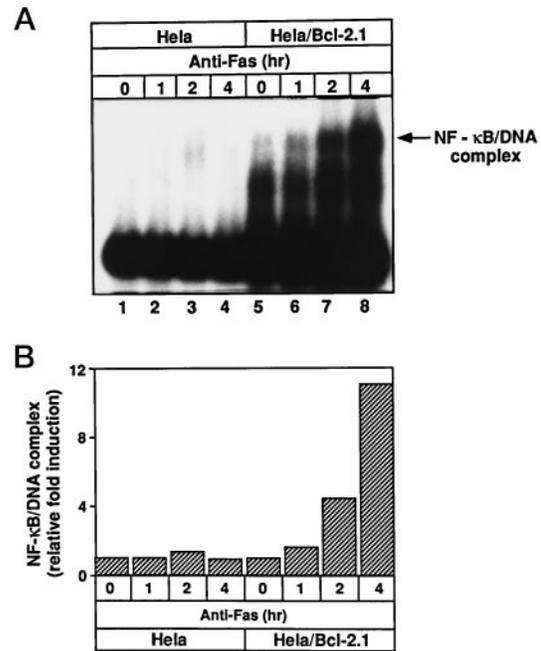


FIG. 5. Anti-CD95 mAb induces NF- κ B activation. *A*, the parental HeLa (*lanes 1–4*) or Bcl-2-overexpressing HeLa/Bcl-2.1 (*lanes 5–8*) cells were treated with anti-CD95 mAb (100 ng/ml) for the indicated time periods. Nuclear extracts were prepared. Equal amounts of protein (5 μ g) were incubated with a 32 P-radiolabeled κ B probe, and the DNA-protein complexes were fractionated by 5% native polyacrylamide gel electrophoresis. The arrow indicates the position of the NF- κ B-DNA complex. *B*, the relative intensities of the NF- κ B-DNA complex band were quantified and are presented as -fold activation over the levels in the control untreated cells. Results shown are representative of four separate experiments.

fore examined the effect of anti-CD95 mAb on activation of NF- κ B in HeLa cells and the Bcl-2-overexpressing HeLa clone using electrophoresis mobility shift assay with the nuclear extracts (Fig. 5). Consistent with previous studies (15), no NF- κ B-DNA-bound activity was detected in HeLa cells stimulated with anti-CD95 mAb (Fig. 5, *lanes 1–4*). Interestingly, treatment of Bcl-2-expressing HeLa/Bcl-2.1 cells with anti-CD95 mAb resulted in potent activation of the NF- κ B-DNA complex within 1 h (1.7-fold), which continued to increase up to 11-fold by 4 h post-treatment (Fig. 5, *lanes 5–8*). The observed κ B-binding complex resulted from the specific DNA-protein interaction since it was competed by 100-fold molar excess of unlabeled κ B probe (data not shown).

We next examined the nature of NF- κ B subunits in the major NF- κ B-DNA complex activated by anti-CD95 mAb in Bcl-2-expressing HeLa cells (Fig. 6, *A* and *B*). The results of antibody supershift experiments demonstrated that incubation of DNA-binding mixtures with either a p50-specific (Fig. 6C, *lane 2*) or a RelA-specific (*lane 4*) antiserum resulted in the supershift of the κ B-binding complex (Fig. 6C, indicated by *arrowheads*). In contrast, the anti-CD95 mAb-inducible NF- κ B-DNA complex only slightly immunoreacted with anti-p52 serum (Fig. 6C, *lane 3*) and had no immunoreactivity with anti-c-Rel serum (*lane 5*). Similarly, a preimmune serum did not affect the formation of the NF- κ B-DNA complex. Parallel immunoblotting analysis of the subcellular extracts revealed that activation of the NF- κ B-DNA binding activity by anti-CD95 mAb resulted from the induction of RelA nuclear translocation (Fig. 7A). Immunoblotting of cell extracts from anti-CD95 mAb-treated HeLa cells indicated that RelA was proteolytically degraded in these cells (data not shown). To determine whether anti-CD95 mAb-mediated NF- κ B was functional, we examined the expression of NF- κ B target gene products such as I κ B α (35). As observed in

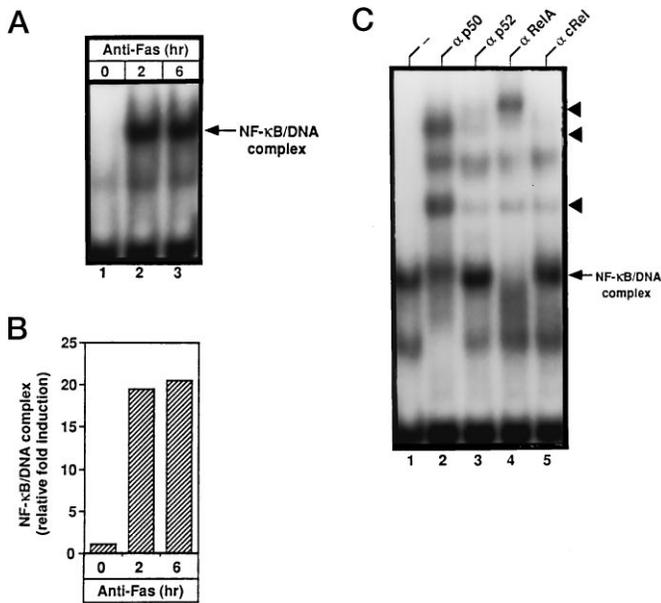


FIG. 6. Activation of p50/RelA NF- κ B heterodimer by anti-CD95 in HeLa/Bcl-2.1 cells. A, cells were treated with anti-CD95 mAb (100 ng/ml) for the indicated time periods. Nuclear extracts were analyzed by EMSA as described in the legend to Fig. 6. The position of the NF- κ B-DNA complex is indicated by the arrow. B, shown is the quantitation of the NF- κ B-DNA activation from A. C, shown is the antibody supershift analysis of anti-CD95 mAb-induced NF- κ B-DNA complex formation. To determine the NF- κ B species in the DNA-protein complex, nuclear extracts (5 μ g) from anti-CD95 mAb-treated (2 h) HeLa cells were incubated for 10 min with or without specific antisera recognizing different NF- κ B subunits and were analyzed by EMSA. The major supershifted bands are indicated by arrowheads. Results shown are representative of three separate experiments.

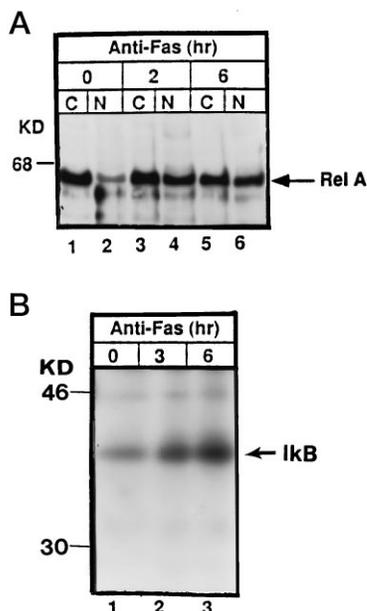


FIG. 7. Effect of anti-CD95 mAb on nuclear translocation of RelA and expression of target gene product I κ B α . A, cytosolic (C) and nuclear (N) extracts were made, and equal amounts of protein (20 μ g) were immunoblotted with RelA antibody. B, cells were stimulated with anti-CD95 mAb (100 ng/ml) for 6 or 3 h and metabolically labeled with [35 S]methionine during the last 1.5 h before harvesting the cells. Lysates containing equal amounts of trichloroacetic acid-precipitable cpm were immunoprecipitated with the I κ B α -specific antiserum.

TNF- α -induced cells (35), anti-CD95 mAb potently induced the *de novo* synthesis of I κ B α in a time-dependent manner. Similarly, anti-CD95 mAb also induced the *de novo* synthesis of

p105, another target gene product of NF- κ B (36). In brief, these results suggest that in Bcl-2-expressing cells, anti-CD95 mAb-mediated signaling involves functional activation of the p50/RelA NF- κ B heterodimer, a prototypic form of NF- κ B induced by various agents including TNF- α (36).

Since CD95-induced apoptosis involves activation of ICE family proteases (15, 18) and inclusion of the specific ICE inhibitor YVAD-cmk is known to prevent anti-CD95 mAb-induced cleavage of PARP (Fig. 4), we also determined whether inhibition of ICE/ICE-like proteases by YVAD-cmk could restore activation of NF- κ B by anti-CD95 mAb in HeLa cells. Our results indicated that pretreatment (6 h) of HeLa cells with YVAD-cmk (0.5 mM) could effectively block anti-CD95 mAb-mediated cleavage of PARP and DNA fragmentation, but had no effect on activation of NF- κ B (data not shown), suggesting that, perhaps, activation of NF- κ B may not be an essential early event in anti-CD95 mAb-mediated apoptosis in HeLa cells. This view is also supported by Ponton *et al.* (37) (who reported their results while this study was in progress), demonstrating that activation of NF- κ B by anti-CD95 mAb is not related to the apoptotic property of CD95.

DISCUSSION

It is now well accepted that apoptosis is a normal physiological phenomenon that plays an important role in the maintenance of tissue homeostasis. Apoptosis is regulated by specific cellular pathways including Bcl-2 and CD95, and the deregulation of apoptosis contributes to the pathogenesis of a number of human diseases including cancer. Although recent studies have shown the inhibitory effect of Bcl-2 against anti-CD95 mAb-induced apoptosis both *in vivo* and *in vitro* (38, 39), the possible biochemical pathways involved remain poorly understood. This investigation was undertaken to examine the regulatory interactions between CD95 and Bcl-2 pathways by studying the effects of Bcl-2 on the possible modulation of cellular pathways activated by CD95 using HeLa cells as a model system. We have now demonstrated that the deregulation of Bcl-2 in HeLa cells is closely linked to several important phenotypic changes including prevention of degradation of lamin B and PARP in response to activation of cells by anti-CD95 mAb.

To explore the biochemical basis of the protective effects of Bcl-2 against an apoptotic signal such as CD95, we compared the effect of anti-CD95 mAb treatment on the cleavage of lamins and PARP in control and Bcl-2-transfected cells. In control cells, selective degradation of lamin B and PARP was observed to precede CD95-mediated DNA fragmentation (Figs. 1 and 3A). Interestingly, the early degradation of lamin B was in contrast to the fate of lamins A and C, which were cleaved at the time of internucleosomal DNA fragmentation. These results suggest that apoptotic signals may first degrade the nuclear components involved in protection of chromosomes, and this in turn might promote DNA fragmentation. To our knowledge, this is the first demonstration that the A-type and B-type lamins are handled differently during CD95-mediated apoptosis. The basis for the differences in timing of lamin cleavage are currently unknown. Previous studies using a cell-free system that recapitulates the nuclear changes observed in apoptotic nuclei have revealed that PARP is cleaved by one ICE family protease (10), possibly CPP32 (40, 41), whereas lamins are cleaved by another ICE family protease (7). Interestingly, the lamin protease was more sensitive to the ICE family inhibitor YVAD-cmk than the PARP protease in these previous cell-free studies. The cleavage of nuclear polypeptides appears to be more complicated in anti-CD95-treated HeLa cells. First, the observation that lamin B is cleaved before lamins A and C in HeLa cells raises the possibility that B-type lamins are cleaved

by one protease and A-type lamins are cleaved by a second protease. Second, based on the differences in sensitivity to YVAD-cmk (Fig. 4), it appears that the lamin B protease and PARP protease are also distinct. This raises the possibility that as many as three distinct ICE family proteases might be active in nuclei of anti-CD95 mAb-treated HeLa cells during the course of apoptosis. Finally, although proteolysis of PARP and lamins in the cell-free system is insensitive to chelation of divalent cations (7), our results show that proteolysis of these polypeptides in anti-CD95-treated HeLa cells can be partially inhibited by the calcium chelator BAPTA. These observations raise the possibility that Ca^{2+} -dependent processes might play a role in protease activation in anti-CD95 mAb-treated cells, although the participation of Ca^{2+} -dependent proteases in the degradation process *in situ* cannot be ruled out.

Recent results from several laboratories (18, 42) have indicated that Bcl-2 expression prevents activation of ICE family proteases. Consistent with these results, we observed that Bcl-2 expression abolished CD95-mediated cleavage of the lamins as well as PARP (Figs. 2 and 3). This work is the first to show that Bcl-2 expression is associated with failure to cleave major polypeptides that are cleaved in most cell types undergoing apoptosis. These results are consistent with the hypothesis that Bcl-2 acts on steps that occur upstream from the action of ICE family proteases in these cells (18). The finding that the degradation of lamin B and PARP was completely prevented by Bcl-2 expression (as opposed to partial inhibition by YVAD-cmk; Fig. 4) suggests that the deregulation of Bcl-2 may inactivate not only YVAD-cmk-sensitive ICE family proteases, but also other unidentified proteases that still may be active in YVAD-cmk-treated HeLa cells stimulated with anti-CD95 mAb.

Another notable finding in this study was the activation of NF- κ B by anti-CD95 mAb in Bcl-2-overexpressing cells. Previous studies have demonstrated that CD95-mediated signaling does not involve activation of NF- κ B (14). However, the existence of significant homology between the death domains of CD95 and TNF receptors raises the possibility of a similar mechanism(s) of signaling by these two receptors. It is possible that during CD95-induced apoptosis, cellular proteolytic changes might preclude detection of the NF- κ B activation, and in the presence of Bcl-2, which prevents proteolysis, activation of NF- κ B may be restored. We therefore examined the hypothesis that Bcl-2 deregulation may modulate signal transduction pathway(s) associated with activation of the CD95 receptor. In agreement with the previous study (14), there was no activation of NF- κ B by anti-CD95 mAb in HeLa cells. Interestingly, anti-CD95 mAb treatment of Bcl-2-overexpressing HeLa cells resulted in the potent early activation of NF- κ B, suggesting that the regulation of Bcl-2 could also restore the NF- κ B signaling by CD95. Our results also show that CD95-induced activation of the NF- κ B-DNA complex consisted of the p50/RelA NF- κ B heterodimer, a prototypic form of NF- κ B induced by various agents including TNF- α (34). Since overexpression of Bcl-2 resulted in restoration of NF- κ B activation by CD95, these findings suggest that the observed activation of NF- κ B by CD95 may be not sufficient to result in apoptosis in HeLa cells treated with anti-CD95 mAb. This notion is in agreement with the earlier observations demonstrating the effectiveness of Bcl-2 in preventing apoptosis in the absence of reactive oxygen (24). Furthermore, our conclusion that activation of NF- κ B may not be an essential component in anti-CD95 mAb-mediated apoptosis in HeLa cells is also supported by the finding that YVAD-cmk, an ICE inhibitor, effectively blocked anti-CD95

mAb-induced DNA fragmentation and PARP cleavage, but did not allow detection of CD95-mediated activation of NF- κ B in HeLa cells. However, it is also possible that some ICE family proteases escape inhibition by YVAD-cmk and degrade NF- κ B in the cells even in the presence of YVAD-cmk. Studies are in progress to address these possibilities further.

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