Overexpression of the Bcl-2 Protein Increases the Half-life of p21Bax*

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Bcl-2 and Bax are homologous proteins which can heterodimerize with each other. These proteins have opposing effects on cell survival when overexpressed in cells, with Bcl-2 blocking and Bax promoting apoptosis. Here we demonstrate that gene transfer–induced elevations in Bcl-2 protein levels result in a marked increase in the steady-state levels of endogenous p21Bax protein as determined by immunoblotting in the Jurkat T-cell and 697 pre-B-cell leukemia cell lines, but not in several other cell lines including CEM T-cell leukemia, 32D.3 myeloid progenitor, PC12 pheochromocytoma, and NIH-3T3 fibroblasts. Steady-state levels of p21Bax protein were also elevated in the lymph nodes of Bcl-2 transgenic mice in which a Bcl-2 transgene is expressed at high levels in B-cells. Northern blot analysis of Bcl-2-transfected and control-transfected Jurkat and 697 leukemia cells revealed no Bcl-2-induced alterations in the steady-state levels of BAX mRNAs. In contrast, l-[35S]methionine pulse-chase analysis indicated a marked increase in the half-life (t1⁄2) of the p21Bax protein in Bcl-2–transfected 697 cells compared to control-transfected cells (t1⁄2 > 24 h versus ~4 h), whereas the rate of Bax degradation was unaltered in Bcl-2-transfected CEM cells. The results demonstrate that levels of the proapoptotic p21Bax protein can be post-translationally regulated by Bcl-2, probably in a tissue-specific fashion, and suggest the existence of a feedback mechanism that may help to maintain the ratio of Bcl-2 to Bax protein in physiologically appropriate ranges.

Among the more prominent regulators of Programmed cell death and apoptosis are Bcl-2 and its homologs. Gene transfer studies have shown that elevated levels of Bcl-2 protein can block or delay apoptotic cell death induced by a wide variety of stimuli and insults, suggesting that Bcl-2 controls a distal step in what may represent a evolutionarily conserved, final common pathway for cell death (reviewed in Ref. 1).

The Bcl-2 protein physically interacts with several other proteins, including several homologous proteins that constitute the Bcl-2 protein family (2–4). Some of the members of this protein family are blockers of cell death akin to Bcl-2, whereas others promote apoptosis and antagonize the function of Bcl-2 (reviewed in Ref. 1). The Bax protein shares ~20% amino acid identity with Bcl-2 and accelerates apoptosis when overexpressed by gene transfer methods (3). Bax forms heterodimers (or oligomers) with Bcl-2, and mutagenesis studies have demonstrated that the ability of Bcl-2 to heterodimerize with Bax is critical for the function of Bcl-2 as a suppressor of cell death (5).

Immunohistochemical studies have demonstrated that the BAX gene is widely expressed in vivo, but is subject to tissue-specific and differentiation stage-dependent regulation, which sometimes but not always correlates with the apoptotic tendencies of cells in vivo (6). Expression of BAX is induced in some types of tumor cells by radiation and chemotherapeutic drugs, in a p53-dependent manner, suggesting an important role for the BAX protein in apoptosis induced by genotoxic stress (7–9). Furthermore, reductions in BAX protein levels have been observed in ~35% of adenocarcinomas of the breast and correlate with poor responses to chemotherapy in some subgroups of patients (10). In addition, rapid and striking elevations in p21Bax protein levels have been described in ischemia-damaged neurons, implying a role for Bax in at least some types of neuronal cell death that occur in the setting of stroke (11).

Taken together, these observations suggest that BAX plays an important role in the control of cell death in several clinically relevant contexts and suggest a need for a greater understanding of the mechanisms that regulate the expression of this gene. At present, however, relatively little is known about the mechanisms that control the levels of the Bax protein. Clearly, transcriptional mechanisms can play an important role, given that the BAX gene promoter contains several p53-binding sites and can be strongly trans-activated by p53, at least in some types of cells (9). Here, we demonstrate that the levels of Bax protein can also be controlled by post-translational mechanisms, with the Bcl-2 protein reducing the rate of p21Bax protein degradation in some but not all types of cells.

MATERIALS AND METHODS

Cells and Metabolic Labeling—The preparation, characterization, and maintenance in culture of Bcl-2-transfected and Neo-control cell lines have been described previously (12–16). For l-[35S]methionine labeling, cells in log-phase growth were washed twice in methionine-free RPMI medium (Mediatech, Inc.), supplemented with 1 mM l-glutamine, and cultured for 1 h at 37 °C with 5% CO2 in methionine-free RPMI containing 10% dialyzed fetal calf serum (FCS) at 3 × 106 cells per ml. This medium was then removed and the cells were cultured for 2 h at 3 × 106 cells per ml in methionine-free RPMI, 10% dialyzed FCS containing 0.2 mM/ml Trans4S-label (ICN Radiochemicals, Inc.). For chasing out the radiolabeled l-[35S]methionine, cells were recovered by centrifugation, resuspended at 106 cells per ml in regular RPMI with 10% non-dialyzed FCS, and returned to culture for 6 to 24 h.

Immunoprecipitations and Immunoblot Assays—The preparation and characterization of rabbit anti-peptide antisera specific for amino acid sequence homology with other proteins whose mechanism of action is known.

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The abbreviations used are: FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); kbp, kilobase pair(s).
acids 43 to 61 of the mouse Bax and 43 to 61 of the human Bax proteins have been described (6, 10). For immunoprecipitations, 3 × 10⁶ cells that had been labeled with [³⁵S]methionine as above were resuspended on ice in 0.1 ml of 1% Triton X-100 lysis solution (10 ml Tris, pH 7.0–7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% phenylmethylsulfonyl fluoride, 0.23 unit/ml aprotinin, 10 µM leupeptin, 1 mM benzamidine) for 15 min, centrifuged at 16,000 × g for 1 min, and the supernatant was transferred to a fresh tube containing 40 µl of 1% Triton X-100 solution:protein A-Sepharose (1:1, v/v) (Sigma) that had been preadsorbed with 2 µl of preimmune serum. After rotating overnight at 4 °C, the supernatant was transferred to a fresh tube, and 2 µl of rabbit anti-human Bax antisera was added. After rotating end-over-end at 4 °C for 2–4 h, 40 µl of 1:1 Triton X-100 solution:protein A-Sepharose was added and the samples were rotated for an additional 2 h at 4 °C. Finally, immune complexes on protein A-Sepharose were recovered by centrifugation at 16,000 × g for 1 min, the supernatant was removed and discarded, and the pellet was washed 4 times with 1 ml each of 1% Triton X-100 lysis buffer before resuspending the protein A-Sepharose beads in 50 µl of 2 × Laemmli sample buffer containing 10% 2-mercaptoethanol, boiling for 5 min, and subjecting the samples to SDS-PAGE (12% gels). Gels were fixed in 25:65:10 (isopropyl alcohol:water:acetic acid for 30 min, then incubated in fluorographic reagent (Amplitz, Amersham, Inc.) for 15–30 min, and dried under vacuum before exposure to x-ray film.

Immunoblotting was performed as described in detail previously (6, 17). Briefly, cells were lysed in 1% Triton X-100 solution as above, normalized for total protein content, and subjected to SDS-PAGE/immunoblot assay using either 0.1% (v/v) or 0.05% (v/v) rabbit anti-mouse Bax or anti-human Bax antisera, respectively. Antibodies were detected using either biotinylated goat anti-rabbit IgG and an avidin-biotin-complex (ABC) reagent (Vector Laboratories, Inc.) containing horseradish peroxidase, followed by Vector SG-substrate or 3,3-diaminobenzidine for colorimetric detection, or using horseradish peroxidase-conjugated goat anti-rabbit IgG and an ECL kit from Amersham, Inc. for autoradiographic detection.

RNA Blot Assays—Total cellular RNA was isolated using the TriZol reagent (Life Technologies, Inc.), and 20 µg per lane was subjected to electrophoresis using 1% agarose, 6% formaldehyde gels and transferred to nylon membranes (GeneScreen; DuPont NEN) as described (4). Prehybridizations, hybridizations, and washes were performed as described previously (4), using a 579-bp human BAX cDNA clone which was obtained by polymerase chain reaction amplification from human hippocampal cDNA (Clontech, Inc.; Quickclone cDNA) using 1 primer (5'-GGAATTCGCGGTGATGGACGGGTCCGG-3') and reverse (5'-GGAATTCTCAGCCCATCTTCTTCCAGA-3') primers, 200 µM deoxynucleotide triphosphates, 1.5 mM MgCl₂, 75 mM KCl, 10 mM Tris, pH 9.2. The resulting polymerase chain reaction product was digested with EcoRI and cloned into the EcoRI site of pcDNA3 (Invitrogen, Inc.). DNA sequence analysis of this cloned human cDNA confirmed that it contained the complete open reading frame for the human p21Bax protein.

RESULTS

We noticed that stable transfectants of the Jurkat human T-cell leukemia cell line which overexpress Bcl-2 contained markedly higher steady-state levels of endogenous p21Bax protein compared to control transfectants ("Neo"). Fig. 1 shows a representative example of immunoblot results obtained for Jurkat-Bcl-2 and Jurkat-Neo cells. For this experiment, detergent lysates were prepared from Bcl-2 (B) and Neo (N) transfectants of Jurkat and CEM cells, normalized for total protein content (50 µg/lane), and subjected to SDS-PAGE/immunoblot assay using anti-human Bax antisera followed by biotinylated secondary antibody, ABC-horseradish peroxidase reagent, and 3,3-diaminobenzidine for color detection (brown), which revealed a 21-kDa band corresponding to the human Bax protein (closed arrowhead). The same blot was then incubated with anti-human Bcl-2 antisera (24), and colorimetric detection was achieved similarly, except that Vector SG-substrate was employed (black), revealing the presence of the expected 26-kDa band corresponding to human Bcl-2 (open arrowhead). (The original brown (Bax) and black (Bcl-2) colors are not retained in the black and white photograph shown.) Staining of the top of the blot (~50 kDa) with Ponceau S confirmed loading of equivalent amounts of protein samples for the pairs of Bcl-2 and Neo transfectants (not shown). In addition, a faint spurious band of ~40–45 kDa can be seen at equal intensity in Jurkat-Bcl-2 and Jurkat-Neo cells, thus providing further confirmation that equivalent amounts of protein samples were loaded.

This analysis of steady-state p21Bax protein levels was then extended to additional pairs of Bcl-2- and Neo-transfected cell lines, using immunoblot assays and antibodies specific for either the human or mouse Bax proteins. As shown in Fig. 2, BCL-2 transfaction resulted in increases in the steady-state levels of p21Bax only in the Jurkat T-cell and 697 pre-B-cell leukemia cell lines. In contrast, the relative steady-state levels of p21Bax in BCL-2-transfected PC12, NIH-3T3, 32D.3, and CEM cells were not detectably different from their Neo control transfected counterparts. Incubation of these blasts with control antibodies such as anti-Fc/γR ATPase or anti-actin confirmed the loading of equivalent amounts of total protein for the pairs of samples (not shown). These results therefore suggest that the influence of Bcl-2 on p21Bax protein levels is cell type-specific.

To explore the in vivo relevance of the observation that Bcl-2 can produce elevations in the steady-state levels of p21Bax in some cell lines, we compared the relative levels of Bax protein in lymph nodes derived from Bcl-2/Ig transgenic and nontransgenic littermate control mice (18). Fig. 2 shows a representative experiment demonstrating markedly elevated levels of p21Bax protein in Bcl-2/g transgenic lymph nodes compared to the nontransgenic littermate control. Similar results were obtained in 3 of 3 transgenic/nontransgenic pairs of age-matched animals (not shown).

Northern blot analysis was performed to explore the effects of BCL-2 transfaction on the steady-state levels of BAX mRNA in Jurkat and 697 cells, where gene transfer-mediated elevations in Bcl-2 resulted in substantial increases in p21Bax. As shown in Fig. 3 (left panel), the relative levels of the major ~1.0-kbp as well as the minor ~1.5-kbp BAX mRNAs were not higher in the BCL-2 transfecteds compared with the Neo control transfecteds. Reprobing the same RNA blot using a [³²P]glycer-
In the absence of alterations in the relative levels of BAX mRNA, it seemed likely that Bcl-2 causes elevations in p21Bax protein levels by either increasing the rate of p21Bax synthesis or by decreasing the rate of p21Bax degradation. To explore this possibility, 697-Bcl-2 and 697-Neo cells were metabolically labeled with \[^{35}S\]methionine for 2 h, and p21Bax protein was immunoprecipitated immediately (0 h) or at various times after chasing with unlabeled L-methionine. As shown in Fig. 4A, the relative rates of \[^{35}S\]methionine incorporation into p21Bax were not reduced in 697-Neo cells relative to 697-Bcl-2 cells, based on analysis of the Bax protein at 0 h before beginning the chase with unlabeled L-methionine. Thus, the rate of synthesis of p21Bax protein was not appreciably different for 697-Neo and 697-Bcl-2 cells. In contrast to 0 h, at 6 h after chasing out \[^{35}S\]methionine, \[^{35}S\]Bax protein levels were substantially reduced in 697-Neo cells but not in 697-Bcl-2 cells (Fig. 4A). At 12 h, \[^{35}S\]Bax had almost disappeared from 697-Neo cells and by 24 h no \[^{35}S\]Bax was detected, whereas \[^{35}S\]Bax protein levels were nearly constant for the first 24 h after chasing out \[^{35}S\]methionine in the 697-Bcl-2 cells (t½ \(~= 4\) h for Neo versus \(~> 24\) h for Bcl-2). In parallel with the disappearance of the 21-kDa Bax band in 697-Neo cells upon chasing with unlabeled methionine, a new species of \(~17\) to \(18\) kDa appeared, suggesting a possible precursor-product relation. Immunoprecipitations performed using normal rabbit serum (N) instead of anti-Bax antiserum or using anti-Bax antiserum that had been preadsorbed with Bax peptide (P) confirmed the specificity of these results, in that no 21-kDa band corresponding to Bax was detected. An additional 26-kDa band was also co-immunoprecipitated with p21Bax in 697-Bcl-2 but not 697-Neo lysates (Fig. 4A). This 26-kDa band represents Bcl-2 in physical association with p21Bax, based on experiments where the Bax immunoprecipitates were analyzed by immunoblotting using Bcl-2-specific antibodies (data not presented). Note that a slightly faster migrating protein at the \(~25\)-kDa band is also seen in 697-Neo cells which appears to be nonspecific, since it was also present when normal rabbit serum was employed instead of anti-Bax antiserum. This nonspecific protein is also seen to a lesser extent in 697-Bcl-2 cells just below the 26-kDa band corresponding to Bcl-2.) Similar results were obtained for J urkat-Neo and J urkat-Bcl-2 cells, except that the rate of degradation of Bax in J urkat-Neo cells was faster than in 697 cells, precluding an accurate estimate of the half-life of Bax protein under the experimental conditions employed here (not shown).

In addition, the effects of Bcl-2 transfection on p21Bax protein stability were compared in 697 cells with CEM cells. Since Bcl-2 did not cause elevations in p21Bax in CEM cells, we would not expect the half-life of p21Bax to be prolonged in these leukemic cells, unlike in 697 cells where Bcl-2 did increase the relative levels of p21Bax. Again, a \[^{35}S\]methionine pulse-chase analysis was performed, comparing the relative amounts of \[^{35}S\]Bax at 0 h before chasing with \[^{35}S\]methionine and at 4 h after chase, which was chosen for these comparisons because it represents the approximate t½ of Bax in 697-Neo cells. As shown in Fig. 4B, the relative levels of \[^{35}S\]Bax remained essentially
unchanged in CEM-Neo and CEM-Bcl-2 cells at 4 h after chasing with unlabeled l-methionine, suggesting that the $t_{1/2}$ of Bax is comparatively long in these cells regardless of their levels of Bcl-2 protein. In contrast, relative levels of 35S-Bax were reduced by approximately one-half at 4 h after chasing with unlabeled [35S]methionine in 697-Neo cells but not in 697-Bcl-2 cells.

DISCUSSION

The data presented here provide the first evidence that levels of a Bcl-2 family protein, in this case p21Bax, can be regulated through post-translational mechanisms. Gene transfer-mediated elevations in Bcl-2 protein resulted in increases in p21Bax and decreasing the rate of Bax protein degradation in some types of cell lines. A reasonable speculation therefore is that the interaction of Bcl-2 with p21Bax, leading to formation of heterodimers or complexes with other stoichiometry, somehow stabilizes the Bax protein in some types of cells, thus leading to increases in the steady-state levels p21Bax protein. We are currently attempting to express a variety of Bcl-2 mutants in Jurkat T-cells that either do or do not retain the ability to bind to Bax as an approach to testing this hypothesis directly. Consistent with this notion, a 26-kDa protein representing Bcl-2 co-immunoprecipitated with Bax in 697-Bcl-2 cells where p21Bax had a long half-life ($t_{1/2} > 24$ h) but not in 697-Neo cells where p21Bax turnover was considerably more rapid ($t_{1/2} = 4$ h).

It remains to be determined what the explanation is for the cell type specificity observed, where Bcl-2-mediated up-regulation of p21Bax protein levels in some cell lines but not others. Interestingly, even closely related cells such as the human T-cell acute lymphocytic leukemia lines Jurkat and CEM exhibited markedly different responses to Bcl-2 in terms of effects on p21Bax protein levels. Despite the fact that Jurkat and CEM are both T-cell acute lymphocytic leukemias, however, they possess immunophenotypes consistent with different stages of thymic development, i.e., CD4+CD8+ (mature thymocytes; Jurkat) and CD4+CD8− (immature thymocytes; CEM). Presumably, whatever factors that contribute to rapid degradation of Bax in cell lines such as 697 and Jurkat are not operative in CEM and other cell lines where Bcl-2 did not induce increases in Bax protein levels. These factors could include differences in the levels or activity of (i) proteases, (ii) kinases or phosphatases that theoretically could control phosphorylation of p21Bax, or (iii) non-Bcl-2 family proteins that might bind to p21Bax. Of particular note with regard to mechanisms of protein degradation is the observation that several recently described Bcl-2 binding proteins contain either PEST sequences (Nip-1, Nip-2, Nip-3) or ubiquitin-like domains (BAG-1) which conceivably might target protein complexes that contain these Bcl-2 binding proteins for proteolytic degradation (19, 20). Moreover, the Bcl-2 homologous protein Mcl-1 contains PEST sequences and has been shown to bind to p21Bax (4, 21). Alternatively, it could be that in addition to Bcl-2, other antiapoptotic members of the Bcl-2 protein family such as Bcl-X or Mcl-1, which can also heterodimerize with Bax (2, 4), are constitutively present at higher levels in cell lines such as CEM and these proteins perhaps may similarly stabilize p21Bax in a manner similar to Bcl-2. In this case, the supposition would be that these other Bcl-2-like proteins are already present at optimal levels for suppressing p21Bax degradation and that gene transfer-mediated increases in Bcl-2 protein therefore provide no further benefit. Immuno blot experiments not shown here indicate that all of the cell lines tested here express Bcl-X, Mcl-1, or both, although no correlations with Bax levels were apparent. Nevertheless, the more rapid degradation of Bax may be to some extent analogous to the rapid degradation of unpaired subunits of multipolypeptide complexes (22, 23).

Gene transfer studies in mammalian cells have shown that elevations in Bcl-2 protein increase resistance of cells to various apoptotic stimuli, whereas increases in Bax can accelerate cell death in the setting of growth factor deprivation. Presumably, therefore, the ratio of Bcl-2 to Bax controls the relative sensitivity or resistance of cells to apoptosis. One prediction of the finding that Bcl-2 can induce elevations in p21Bax, therefore, is that it may represent a feedback mechanism for maintaining Bcl-2/Bax ratios within a physiologically appropriate range. Since experimental manipulations that increase Bcl-2 relative to Bax have been shown to prolong cell survival and block apoptosis induced by many insults, the positive effects of Bcl-2 on p21Bax half-life may help to limit cell longevity and thus reduce the risk of tumorigenesis. Alternatively, it has not been excluded that Bcl-2/Bax heterodimers represent a molecular entity that promotes cell survival, not unlike the α- and β-chains of some cell surface receptors such as integrins and T-cell antigen receptors. In this case, Bcl-2-mediated up-regulation of p21Bax would constitute a positive feedback loop. Thus, until the functional properties of Bax/Bax homodimers, Bax/Bcl-2 heterodimers, and Bcl-2/Bcl-2 homodimers are better understood in terms of their biochemical influence on cell death pathways, it is difficult to predict the physiological consequences of the Bcl-2-mediated stabilization of the p21Bax protein described here. Nevertheless, these findings indicate that it may be important to determine the effects of Bcl-2 on Bax protein levels before interpreting the biological significance of experiments where gene transfer-mediated elevations in Bcl-2 are employed to assess the relative Bcl-2 dependence or independence of various apoptotic stimuli and pathways.

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