Interleukin-3 Induces the Phosphorylation of a Distinct Fraction of Bcl-2

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Bcl-2-related proteins (i.e. Bcl-2 and Bax) regulate the effector stage of apoptosis and can modulate the entry of quiescent cells into the cell cycle. Phosphorylation of Bcl-2 is presumed to modify its apoptosis-inhibitory function. By utilizing an interleukin-3 (IL-3)-dependent hematopoietic cell line, we examined the structural requirements of Bcl-2 phosphorylation and the correlation of this post-translational modification with its function. In the presence of IL-3, constitutively expressed Bcl-2 was phosphorylated on serine residue(s), and phosphorylated Bcl-2 lost its capacity to heterodimerize with Bax. Whereas the majority of Bcl-2 resided in mitochondria, phosphorylation only affected a minor pool of total Bcl-2 that selectively partitioned into a soluble fraction. Cytosolic targeting of Bcl-2 greatly increased its ratio of phosphorylation. Bcl-2 phosphorylation was reduced during IL-3 deprivation, and its phosphorylation was also delayed after transient cytokine deprivation. This pattern of phosphorylation temporally correlated with the accelerated exit and delayed reentry of Bcl-2-expressing cells into the cell cycle upon transient IL-3 deprivation and subsequent cytokine restimulation. Thus, IL-3-induced phosphorylation of a distinct pool of Bcl-2 may contribute to the inactivation of its anti proliferative function.

The process of apoptosis is executed by a distinct genetic pathway that is apparently shared by all multicellular organisms. The family of Bcl-2-related proteins constitutes a class of apoptosis-regulatory gene products that act at the effector stage of apoptosis. In vertebrates, two functional classes of Bcl-2-related proteins exist that share highly conserved Bcl-2 homology (BH)1, BH2, BH3, and BH4 domains: (α) antiapoptotic members, including Bcl-2, that inhibit cell death; and (β) proapoptotic members, including Bax, that accelerate apoptosis (reviewed in Refs. 1–3). Several gene ablation studies confirm that in vertebrates, the balance between death-promoting and death-repressing members of these two major cell fates (20, 21). Here we show that in the presence of IL-3, a fraction of constitutively expressed Bcl-2 was phosphorylated on serine residue(s), and this phosphorylated pool of Bcl-2 lost its capacity to heterodimerize with Bax. Whereas the majority of Bcl-2 resided in mitochondria, phosphorylation involved a minor pool of total Bcl-2 that selectively partitioned into a soluble fraction. Cytosolic targeting of Bcl-2 by deletion of its membrane insertion domain greatly increased its ratio of phosphorylation. The reduced phosphorylation of Bcl-2 upon IL-3 deprivation and its delayed rate of phosphorylation upon cytokine restimulation temporally correlated with the accelerated exit and delayed reentry of Bcl-2-expressing cells into the cell cycle. Thus, above a threshold level of Bcl-2 expression, IL-3-induced phosphorylation...
artation of a distinct pool of Bcl-2 may represent a selective inactivating mechanism of its antiproliferative function.

**Experimental Procedures**

Cell Culture and Apoptosis Induction—The IL-3-dependent murine cell line FL5.12, a lymphoid progenitor clone, and all its derivatives were maintained in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal calf serum (Life Technologies, Inc.) and 10% WEHI-3B conditional medium as a source of IL-3 (19). To induce apoptosis, FL5.12 cells were washed three times in serum-free medium to remove the growth factor and cultured in the absence or presence of 25 IU/ml recombinant murine IL-3 (Genzyme). Antibodies—mAbs 6C8 (a human Bcl-2-specific hamster mAb; Ref. 19), 4D2 (a murine Bax-specific hamster mAb; Ref. 22), and 3F11 (a murine Bcl-2-specific hamster mAb; Ref. 23) were used. mAb 124 (a human Bcl-2-specific murine mAb) was purchased from DAKO and used for Western immunostaining at a dilution of 1:100.

**Metabolic Labelings and Immunoprecipitation**—Before metabolic labeling with [35S]methionine, cells were washed three times in prewarmed, serum-free, methionine-free Dulbecco’s medium (Life Technologies, Inc.). Cells were resuspended at 3–5 × 10^6 cells/ml in methionine-free Dulbecco’s medium supplemented with 10% phosphate-buffered saline (PBS)-dialized fetal calf serum with or without 50 IU/ml recombinant murine IL-3 (Genzyme). Metabolic labeling was performed with 40 μCi/ml [35S]methionine and [35S]cysteine (Translabel; ICN) for the indicated times before lysis. For phosphorylation studies, cells were carried out as described previously (22, 24), except that some gels containing 32P-labeled proteins were transferred on polyvinylidine difluoride membrane and immunostained (see below) before autoradiography.

**Subcellular Fractionation**—Cells were metabolically labeled as described above and lysed in hypotonic buffer (42.5 mM KCl, 5 mM MgCl_2, and 10 mM HEPES, pH 7.4) by passing them four times through a 30-gauge needle. Isotonicity was reestablished by adding an equal volume of hypertonic buffer (242.5 mM KCl, 5 mM MgCl_2, and 10 mM HEPES, pH 7.4). Nuclei and unlysed cells were pelleted twice at 200 × g for 10 min. The supernatant was centrifuged at 10,000 × g for 10 min to collect the heavy membrane pellet. That supernatant was centrifuged at 100,000 × g for 60 min, and the final supernatant was collected as the soluble fraction, and the pellet was collected as the light membrane fraction. The heavy membrane pellet was washed twice in H medium (0.25 mM mannitol, 0.075 M sucrose, 1 mM EGTA, 5 mM HEPES, pH 7.4, and 0.1% fatty acid-free bovine serum albumin). Both heavy and light membrane pellets were lysed in RIPA buffer and, together with the soluble fraction, were immunoprecipitated with mAb 6C8 as described above.

**Western Blotting and Immunostaining**—For immunoblots, proteins were electrotransferred at 4 °C on polyvinylidine difluoride membrane and immunostained (see below) before autoradiography.

**Results**

Phosphorylation of Bcl-2 Is Independent of Its Capacity to Heterodimerize with Bax—Phosphorylation of Bcl-2 was previously observed in selected cell lines (12–18). However, the physiologic role of this post-translational modification is controversial (15, 16, 18). Also, the structural features of Bcl-2 required for this post-translational modification were not examined in detail.

Our studies utilized the IL-3-dependent early lymphoid progenitor murine cell line FL5.12, whose viability and proliferation is maintained by a minimum of 25 IU/ml recombinant murine IL-3 (data not shown). In the absence of IL-3, FL5.12 dies by apoptosis, but overexpression of Bcl-2 significantly extends its survival without maintaining its proliferation (19). The Bcl-2 expression level in these FL5.12-Bcl-2 clones (26, 27) was approximately the same as that seen in a cell line established from a patient with t(14;18)-bearing follicular B-cell lymphoma or in pre-B cells (19), thus representing physiologically relevant protein levels. Of note, FL5.12 cells do express a significant amount of endogenous Bax as well as a low amount of endogenous Bcl-2 that is insufficient to provide protection against IL-3 deprivation-induced apoptosis (24).

To investigate whether phosphorylation of Bcl-2 is dependent on its capacity to heterodimerize with Bax, FL5.12 clones stably expressing either wild type human Bcl-2 (Bcl-2) or a BH1 substitution mutant of human Bcl-2 (mI-3/G14A) were examined. This mutant fails to counterapoptosis in FL5.12 cells (26) and heterodimerize with Bax in a yeast two-hybrid assay (22). Equal numbers of FL5.12-Bcl-2 and FL5.12-Bcl-2 mI-3 cells were metabolically labeled for 6 h with either [32P]orthophosphoric acid or [35S]methionine in the presence of recombinant murine IL-3 and subsequently immunoprecipitated with the human Bcl-2-specific 6C8 mAb (19). FL5.12-Bcl-2 and FL5.12-Bcl-2 mI-3 cells contained a comparable amount of [35S]methionine-labeled (Fig. 1A, lanes 2 and 3) and [32P]orthophosphoric acid-labeled (Fig. 1A, lanes 1 and 4) pro-
NeoR cells were immunoprecipitated with the 4D2 mAb for murine Bax cells and analyzed by SDS-polyacrylamide gel electrophoresis. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis.

The Native Conformation of Bcl-2 Is Altered by Its Phosphorylation—in FL5.12 cells, the antiapoptotic Bcl-2 molecule resides predominantly in the mitochondria, whereas the majority of proapoptotic Bax is located in the cytosol in monomeric form (28). Upon apoptosis induction, cytosolic Bax undergoes a change in its conformation and translocates to mitochondria (28–30). Here it can heterodimerize with Bcl-2 (28, 31), presumably involving interactions between their BH1, BH2, and BH3 domains (10, 11, 32). In the absence of proapoptotic signals, nonionic detergents, such as Nonidet P-40, are able to induce this conformational change of Bax and permit Bcl-2/Bax dimerization in cell lysates (33, 34).

The majority of [35S]methionine-labeled Bcl-2 localized to the heavy membrane fraction, with only minor amounts observed in the light membrane and S100 fractions, whereas Bcl-2 ΔTM localized to the S100 fraction (Fig. 3A, left panels), as described previously (27, 36, 37). However, phosphorylated Bcl-2 and Bcl-2 ΔTM were both found almost exclusively in the S100 fraction (Fig. 3A, right panels). We conclude that whereas the majority of Bcl-2 is localized to the heavy membrane fraction in FL5.12-Bcl-2 cells, the membrane integration of phosphorylated Bcl-2 is compromised.

Cytosolic Targeting Substantially Enhances Bcl-2 Phosphorylation—Because phosphorylated Bcl-2 localized to the S100 cytosolic fraction, we next asked whether targeting Bcl-2 to the cytosol by removal of its membrane insertion domain enhances its rate of phosphorylation. To this end, FL5.12-Bcl-2, FL5.12-Bcl-2 mI-3, and FL5.12-Bcl-2 ΔTM cells were labeled with [35S]methionine or [32P]orthophosphoric acid in the presence of IL-3 for 6 h, lysed in RIPA lysis buffer, and immunoprecipitated with the human Bcl-2-specific mAb 6C8. Immunoprecipitates of [35S]methionine-labeled cell lysates revealed a comparable amount of Bcl-2 expression in all three cell lines (Fig. 3B, top panel). However, Bcl-2 ΔTM proved ~50× more phosphorylated than the mostly membrane-bound Bcl-2 and Bcl-2 mI-3 proteins (Fig. 3B, bottom panel). Thus, targeting Bcl-2 into the cytosol by preventing its membrane association substantially...
enhances its level of phosphorylation.

Bcl-2 Delays IL-3-induced Cell Proliferation in FL5.12 Cells—Constitutively expressed Bcl-2 prevents the apoptosis of FL5.12 cells upon IL-3 withdrawal (19, 24). As only a minor S100 fraction of Bcl-2 is phosphorylated in the presence of IL-3, the physiologic role of this post-translational modification may not directly relate to the molecule’s apoptosis-inhibitory function in these cells. Besides inhibiting apoptosis, several studies demonstrated that Bcl-2 can also delay the entry of cells into the cell cycle (reviewed in Ref. 2). Thus, we wished to examine the potential relationship between Bcl-2's phosphorylation status and its antiproliferative effect in these cells.

First, the cell cycle status of FL5.12-NeoR and two FL5.12-Bcl-2 clones was tested after transient IL-3 deprivation and subsequent IL-3 restimulation, in a manner similar to that described previously (40). Before IL-3 deprivation, the apparent rate of cell proliferation and the ratio of cells in S phase + G2/M phase were essentially identical in all clones (Fig. 4A, 0hr), although the proportion of Bcl-2-expressing cells in S phase was somewhat lower than that in FL5.12-NeoR cells (Fig. 4B, 0hr). Upon transient IL-3 withdrawal, Bcl-2-expressing clones started to accumulate in the G1 phase of the cell cycle faster than NeoR cells, and the ratio of Bcl-2-expressing cells in G2/M phase also increased. Because control FL5.12-NeoR cells remain fully viable for only 12 h after cytokine deprivation (24), IL-3 was added back to all clones at this time. 12 h after the readdition of IL-3, a lower proportion of FL5.12-Bcl-2 cells was in the S phase + G2/M phase of the cell cycle compared with FL5.12-NeoR cells (Fig. 4A, 0hr). At this time, FL5.12-NeoR cells demonstrated a synchronous entry of cells into S phase, which was delayed in FL5.12-Bcl-2 cells (Fig. 4B, 0hr). By 24 h after the readdition of IL-3, this transient difference had disappeared (Fig. 4, A and B). These data demonstrate that similarly to that seen before (40), Bcl-2 is able to provoke a temporary refractoriness to IL-3-stimulated cell proliferation in FL5.12 cells.

Phosphorylation of Bcl-2 Is Dependent on the Presence of Interleukin-3—To determine the temporal correlation of Bcl-2’s phosphorylation status and its antiproliferative function, FL5.12-Bcl-2 and FL5.12-NeoR cells were metabolically labeled with either [%32P]orthophosphoric acid or [%35S]methionine in the presence or absence of recombinant murine IL-3 and solubilized with RIPA lysis buffer at distinct time points thereafter. When lysates of FL5.12-NeoR cells were immunoprecipitated with the endogenous murine Bcl-2-specific mAb 3F11, no phosphorylation of endogenous Bcl-2 was detected during the first 8 h of [%32P]orthophosphoric acid labeling (Fig. 5A, top panel) in either the presence (+) or absence (−) of IL-3. Similarly, lysates of FL5.12-NeoR (or FL5.12-Bcl-2) cells did not demonstrate any phosphorylation of Bax when precipitated with the anti-Bax mAb within the same time frame (Fig. 5A, middle panel). In contrast, efficient phosphorylation of constitutively expressed Bcl-2 was detected in the presence of IL-3 when it was immunoprecipitated with the human Bcl-2-specific 6C8 mAb (Fig. 5A, bottom panel, +). However, in the absence of IL-3 during metabolic labeling, a significant reduction in the amount of phosphorylated human Bcl-2 was observed (Fig. 5A, bottom panel, −).

To ascertain that the differences seen in the phosphorylation of overexpressed Bcl-2 in the presence or absence of IL-3 were not due to differences in the rate of its de novo protein synthesis, [%35S]methionine labeling was performed within the same
time course. Of note, the amount of endogenous murine Bcl-2 is about 10% compared with the amount of overexpressed human Bcl-2 (19), whereas endogenous Bax levels are comparable to that of overexpressed Bcl-2 (24). Both endogenous Bcl-2 and Bax incorporated a detectable amount of radiolabeled methionine at the same rate in the presence or absence of IL-3 (Fig. 5B, top and middle panels). Similarly, metabolic labeling demonstrated identical amount of newly synthesized constitutively expressed Bcl-2 in the presence or absence of the cytokine (Fig. 5B, bottom panel). We conclude that above a threshold level of protein expression, a distinct pool of Bcl-2 is phosphorylated in the presence of IL-3, but upon cytokine deprivation, Bcl-2 is either dephosphorylated or its phosphorylation is inefficient.

**IL-3-induced Bcl-2 Phosphorylation Correlates with Reentry of FL5.12 Cells into the Cell Cycle—**To further examine the temporal correlation of Bcl-2’s phosphorylation status and its cell cycle inhibitory function, we determined the phosphorylation status of Bcl-2 after transient IL-3 deprivation and subsequent IL-3 restimulation. To this end, FL5.12-Bcl-2 cells were first transiently deprived of IL-3 for 12 h. These cells were then metabolically labeled with [32P]orthophosphoric acid or [35S]methionine in the presence of recombinant IL-3 for 3, 6, and 12 h (Fig. 6, IL-3 Depr.). As controls, identical metabolic labeling was performed on FL5.12-Bcl-2 cells that were incubated in the continuous presence of IL-3 (Fig. 6, Control). Samples were lysed in RIPA lysis buffer and immunoprecipitated with the human Bcl-2-specific 6C8 mAb at the indicated time points.

Compared with control cells, the amount of phosphorylated Bcl-2 was reduced in transiently IL-3-deprived cells at all time points tested. This difference was most pronounced at 3 h after the initiation of metabolic labeling. However, at 6 h, but not at 12 h, the phosphorylation level of Bcl-2 was still weaker in the transiently IL-3-deprived cells than in control cells (Fig. 6, top panel). To ascertain that the differences seen in Bcl-2’s level of phosphorylation were not due to differences in the rate of its de novo protein synthesis, [35S]methionine labeling was performed within the same time course. As shown in Fig. 6, bottom panel, both transiently IL-3-deprived and control cells incorporated radiolabeled methionine at similar rates at all time points. Thus, after transient cytokine withdrawal, the delayed rate of IL-3-stimulated phosphorylation of Bcl-2 (Fig. 6) correlated to a certain degree (3 and 6 h) with the reduced sensitivity of FL5.12-Bcl-2 cells to IL-3-stimulated cell proliferation (Fig. 4).

**DISCUSSION**

In vertebrates, death-promoting and death-repressing members of the Bcl-2-related proteins are important regulators of the effector stage of apoptosis. The regulation of their biologic activity is poorly understood, but differential subcellular targeting of these molecules is clearly involved. For instance, Bcl-2 is an integral membrane protein that resides in mitochondria, endoplasmic reticulum, and nuclear membranes (19, 41, 42). In contrast, the majority of proapoptotic Bax is located in the cytosome in monomeric inactive form (28, 29). Upon apoptosis induction, cytosolic Bax translocates from the cytosol to the mitochondria, where it displays its apoptosis-inducing function (28–30), which perhaps involves its heptamerization (43). Alternatively, mitochondrial Bax can heterodimerize with Bcl-2 (28, 31), an association that is likely to involve interactions between their BH1, BH2, and BH3 domains. NMR and x-ray crystallography structure of Bcl-xL monomer (10) and of a Bcl-xL-Bak BH3 peptide complex (32) revealed both hydrophobic and electrostatic interactions between the BH3 amphipathic α-helix and the Bcl-xL hydrophobic pocket formed by its BH1, BH2, and BH3 domains (32). Selective BH1 mutations that abolish Bcl-2’s heterodimerization capacity with Bax in a yeast two-hybrid assay (22) can also reduce its antiapoptotic function in mammalian cells (26, 44), further underlining the functional significance of this interaction.

Another type of functional regulation of Bcl-2 family members involves their post-translational modification by phosphorylation. For instance, phosphorylation of proapoptotic Bad (45) on two serine residues in response to IL-3 stimulation promotes its cytosolic targeting and association with the 14-3-3 family of proteins (46). Similarly, a number of studies demonstrated the phosphorylation of Bcl-2 in a variety of cell lines, but the functional consequence of this post-translational modification remains unclear. In some studies, chemotherapeutic-induced apoptosis correlated with concomitant phosphorylation of Bcl-2 (14, 15, 17, 47, 48), whereas in others phosphorylation of Bcl-2 correlated with its antiapoptotic func-
Bcl-2 kinase

IL-3

Bcl-2 kinase

IL-3-stimulated Phosphorylation of Bcl-2

dooplasmic reticulum that may not be dependent on its C-terminal membrane insertion domain (35). In the second scenario, Bcl-2 that is already inserted in the membrane may show a differential sensitivity to kinase activity according to its site of integration. Thus, a small pool of mitochondria- or endoplasmic reticulum-localized Bcl-2 with subtle differences in its protein conformation may be selectively accessible to IL-3-stimulated Bcl-2 kinase, whereas the majority of its mitochondrial counterpart is shielded from it. Expression of Bcl-2 mutants targeted selectively to the endoplasmic reticulum or mitochondrial subregions in FL5.12 cells will be needed to clarify this issue.

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Among these, cytokine-induced survival of an IL-3-dependent myeloid cell line temporally correlated with serine phosphorylation of Bcl-2 (12), suggesting growth factor-initiated modulation of its function.

Besides regulating apoptosis, Bcl-2-related proteins can also modulate the entry of quiescent cells into the cell cycle (reviewed in Ref. 2), suggesting a cell autonomous coordination between proliferation and cell death. In light of the emerging dual role of Bcl-2 as both an apoptosis- and cell cycle-inhibitory protein, we considered whether phosphorylation of Bcl-2 may differentially alter just one function of the molecule in certain cell types. To examine the temporal correlation of Bcl-2’s phosphorylation status and its antiapoptotic function, we have utilized an IL-3-dependent lymphoid cell line in which Bcl-2 blocks apoptosis after growth factor withdrawal.

The data presented in this paper suggest that IL-3-induced phosphorylation of Bcl-2 may temporally correlate better with abrogation of its cell cycle-inhibitory effect than with regulation of its apoptosis-inhibitory function. First, transient IL-3 deprivation resulted in a reduced level of Bcl-2 phosphorylation and, in time, correlated with the accelerated exit of Bcl-2-expressing cells from the cell cycle (Figs. 4 and 5). Similarly, upon cytokine restimulation, Bcl-2-expressing clones exhibited a temporary refractoriness to IL-3-induced cell proliferation that correlated with the delayed rate of Bcl-2 phosphorylation (Figs. 4 and 6). Thus, in both cases, the phosphorylation status of Bcl-2 correlated with its antiapoptotic effect. Also, in FL5.12 cells, Bcl-2 provides an extended protection from IL-3 deprivation-induced apoptosis (19). Taken together, these data suggest that IL-3-induced phosphorylation of Bcl-2 may contribute to the inactivation of its antiproliferative function rather than altering Bcl-2’s antiapoptotic effect. To directly test this hypothesis, identification of IL-3-induced Bcl-2 phosphorylation sites and the creation and testing of phosphorylation-deficient Bcl-2 mutants will be needed.

Several considerations also suggest that in FL5.12 cells, IL-3-stimulated Bcl-2 kinase may not affect the majority of constitutively expressed Bcl-2. First, in FL5.12 cells in the presence of IL-3, Bcl-2 predominantly localized to the mitochondria-rich heavy membrane fraction, in accordance with that described previously (Refs. 19 and 28; Fig. 3A, left panel). However, almost all phosphorylated Bcl-2 resided in the soluble S100 fraction (Fig. 3A, right panel). This demonstrates that phosphorylation-induced conformational change of Bcl-2 results in the loss of its capacity for firm membrane integration. Second, Bcl-2 ATM, a Bcl-2 mutant that is predominantly cytosolic due to deletion of its membrane insertion domain, was ~50% more phosphorylated than membrane integrated Bcl-2 when both exhibited a similar overall protein expression level (Fig. 3B). This suggests that IL-3-stimulated Bcl-2 kinase is sufficiently active to phosphorylate perhaps all intracellular Bcl-2. Consequently, the majority of Bcl-2 is apparently shielded from this kinase activity and integrates to the mitochondria in the presence of IL-3.

But what shields Bcl-2 from this kinase effect? Two separate scenarios can be envisioned. First, IL-3-induced Bcl-2 kinase may require sufficient time to phosphorylate cytosolic Bcl-2. Thus, rapid mitochondrial integration of newly synthesized Bcl-2 may prevent such an effect. However, slower integration to alternative sites, such as endoplasmic reticulum membranes or selected mitochondrial subregions, may allow sufficient time for phosphorylation to take place that subsequently prevents Bcl-2’s membrane integration (Fig. 7). Of note, in vitro targeting experiments demonstrated that efficient insertion of Bcl-2 into the mitochondrial outer membrane is mechanistically different from its comparatively low-affinity association with endoplasmic reticulum that may not be dependent on its C-terminal membrane insertion domain (35). In the second scenario, Bcl-2 that is already inserted in the membrane may show a differential sensitivity to kinase activity according to its site of integration. Thus, a small pool of mitochondria- or endoplasmic reticulum-localized Bcl-2 with subtle differences in its protein conformation may be selectively accessible to IL-3-stimulated Bcl-2 kinase, whereas the majority of its mitochondrial counterpart is shielded from it. Expression of Bcl-2 mutants targeted selectively to the endoplasmic reticulum or mitochondrial subregions in FL5.12 cells will be needed to clarify this issue.

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