Bcl-2 Phosphorylation Required for Anti-apoptosis Function*

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The protooncogene Bcl-2 functions as a suppressor of apoptosis in growth factor-dependent cells, but a post-receptor signaling mechanism is not known. We recently reported that interleukin 3 (IL-3) and erythropoietin, or the protein kinase C activator bryostatin-1 (Bryo), not only suppresses apoptosis but also stimulates the phosphorylation of Bcl-2 (May, W. S., Tyler, P. G., Ito, T., Armstrong, D. K., Qatsha, K. A., and Davidson, N. E. (1994) J. Biol. Chem. 269, 26865-26870). To test whether phosphorylation is required for Bcl-2 function, conservative serine → alanine mutations were produced at the seven putative protein kinase C phosphorylation sites in Bcl-2. Results indicate that the S70A mutant fails to be phosphorylated after IL-3 or Bryo stimulation and is unable to support prolonged cell survival either upon IL-3 deprivation or etoposide treatment when compared with wild-type Bcl-2. In contrast, a Ser → Glu mutant, S70E, which may mimic a potential phosphate charge, more potently suppressed the etoposide-induced apoptosis than wild type in the absence of IL-3. Since the loss of function S70A mutant can heterodimerize with its partner protein and death effector Bax, these findings demonstrate that Bcl-2:Bax heterodimerization is not sufficient and Bcl-2 phosphorylation is required for full Bcl-2 death suppressor signaling activity.

Hematopoietic growth factors such as the multipotential hematopoietic interleukin (IL) 3 promote net cell growth by stimulating proliferation and suppressing apoptosis (1, 2). A great deal is understood about the molecular components and biochemical mechanisms that regulate cell proliferation, including activation of non-receptor protein-tyrosine kinases like JAKs and coupling of the post-receptor molecular signals generated through the cytoplasmic signal transducers and activators of transcription and/or the Ras/Raf-1/mitogen-activated protein kinase cascade (3). However, little is known about the signal transduction pathway involved in suppressing apoptosis.

The protooncogene Bcl-2 functions as an apoptosis suppressor in many systems (4, 5), although the mechanism is not yet known. We recently discovered that IL-3 and erythropoietin or the potent activator of PKC, bryostatin-1 (Bryo), could support survival of factor-dependent myeloid cells in a PKC-dependent pathway (6). It was discovered that IL-3 and Bryo-induced survival under these conditions correlated tightly with serine phosphorylation of Bcl-2. Direct phosphorylation of Bcl-2 in vitro was shown to be Ca2+-dependent indicating a role for a classical PKC isoform. Thus, whether IL-3 induced Bcl-2 phosphorylation is required for the anti-death phenotype of Bcl-2 is a critical issue that was addressed. Here, we present results obtained with site-specific phosphorylation-negative Bcl-2 mutants and a putative phosphorylation-equivalent mutant to test the biological significance of Bcl-2 phosphorylation in a physiologically relevant IL-3-dependent myeloid cell line.

EXPERIMENTAL PROCEDURES

Cell Transfections—Murine Bcl-2 cDNA (6) was cloned in pUC19, and nucleotides corresponding to each serine residue were substituted to create a conservative alteration to alanine with a site-directed mutagenesis kit (Transformer, CLONTECH) using 25-base pair oligonucleotides. Each single mutant was verified by sequencing the cDNA and then cloned into the pMKTNeo expression vector used to transfact murine IL-3-dependent NSF/N1.H7 cells (7) as described previously (6). Ten to twenty clones from each mutation were selected for G418 resistance, and the expression level of exogenous Bcl-2 was compared by Western blot analysis. Clones expressing similar amounts of exogenous Bcl-2 were selected to study.

Immunoprecipitation and Immunoblotting Analysis—Cells were labeled with [32P]orthophosphoric acid, and Bcl-2 proteins were immunoprecipitated using rabbit anti-mouse Bcl-2 antibody as described (6). The sample was subjected to 10–20% gradient SDS-polyacrylamide gel electrophoresis gel, transferred to a nitrocellulose membrane, and exposed to Kodak X-Omat film for 16 h at 80 °C. The same blot was then used to perform immunoblotting analysis with the anti-Bcl-2 antibody and developed using the ECL kit (Amersham) by exposing a film for 5 s as described (6). The anti-murine Bax antibody was raised and characterized as described (8). For Bax:Bcl-2 heterodimerization analysis, immunoprecipitation and Western blotting were performed as described above except that 0.25% Nonidet P-40 was used to prepare the cell lysates.

Analysis of Cell Viability—Growing cells were deprived of IL-3, and the cell viability was measured by the trypan blue dye exclusion method at the indicated times.

RESULTS AND DISCUSSION

Analysis of the primary sequence identified seven potential PKC serine phosphorylation sites at Bcl-2 residues 24, 70, 102, 158, 164, 202, and 213 (9) (Fig. 1A). Following conservative Ser → Ala mutagenesis at each site, the resulting plasmids were used to transfect murine IL-3-dependent NSF/N1.H7 cells (7). These factor-dependent myeloid cells were used because they express very low but detectable levels of endogenous Bcl-2 and thus represent a physiologically relevant system within which to test the Bcl-2 mutant phenotype with respect to IL-3 signaling. Clones were selected for stable expression of quantitatively comparable levels of wt or mutant Bcl-2 (i.e. 6–8-fold increase above the endogenous level of Bcl-2 as determined by densitometry; Fig. 1C). Following metabolic labeling with [32P]orthophosphoric acid and stimulation with 10 nM Bryo, six Ser → Ala mutants (S24A, S102A, S158A, S164A, S202A, S213A) and wild-type Bcl-2 (wt) could be identified as phosphoproteins (Fig. 1B). By contrast, no incorporation of labeled phosphate could be detected in the S70A mutant after either Bryo or IL-3 stimulation (Figs. 1B, 2A, and 2B). In addition, the pp1 and -2A phosphatase inhibitor okadaic acid, which has been found to enhance Bcl-2 phosphorylation (10), was unable to effect phosphorylation of the S70A mutant Bcl-2 (data not available on line at http://www-jbc.stanford.edu/jbc/11671

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These data indicate the potential involvement of regulatory kinase and phosphatase activities in phosphorylation of Ser70 and Bcl-2 function. Therefore, although it is not clear whether PKC is the only kinase that can phosphorylate Bcl-2 in vivo, these results demonstrate that Ser 70 is the critical Bcl-2 phosphorylation site. Since the Ser 70 residue is evolutionarily conserved in Bcl-2 expressed by various species including human, rat, chicken, and mouse (11), these findings support the conclusion that Ser70 is the physiologically relevant Bcl-2 phosphorylation site.

Next, the death suppressor activity of the Bcl-2 mutants was assessed by depriving cells of IL-3 (Fig. 1D). After 48 h, less than 10% of the vector alone control cells survived. More than 80% survival was observed for cells expressing any of the six mutants (S24A, S102A, S158A, S164A, S202A, S213A) or wild-type Bcl-2. By contrast, only 10–30% of S70A-expressing cells survived (Figs. 1 and 2), suggesting that the majority of the apoptosis-suppressing activity of Bcl-2 is regulated by phosphorylation. Changing serine to glutamate (E) is thought to be able to mimic, at least in part, the phosphorylation charge conferred on the polypeptide. Similar to the S70A clones tested, no significant increase in phosphate incorporation was detected in metabolic labeling studies conducted with S70E Bcl-2 after either Bryo or IL-3 treatment (Fig. 2, A and B). Functionally, however, the S70E Bcl-2 mutant was able to support survival as well as cells expressing wt and S24A Bcl-2 (Fig. 2C). These results strongly suggest that the phosphorylation-defective S70A Bcl-2 represents a loss of function mutation which is unable to suppress apoptosis.

IL-3 deprivation may cause apoptosis of the IL-3-dependent cells by mechanisms other than the loss of Bcl-2 phosphorylation. To assess the significance of the phosphorylation induced by IL-3 on the anti-apoptotic activity of Bcl-2, wt and mutant Bcl-2-expressing cells were treated with etoposide (VP-16) either in the presence or absence of IL-3. In the presence of IL-3, more than 70% survival was observed for wt and S70E Bcl-2-expressing cells while less than 30% of the S70A Bcl-2-expressing cells survived (Fig. 3A). In the absence of IL-3, however, the survival advantage of wt Bcl-2-expressing cells was significantly reduced and resembled that of the S70A clone (Fig. 3B). By contrast, the S70E Bcl-2-expressing cells could support prolonged survival with etoposide even in the absence of IL-3 (Fig. 3C). These data support and help to explain the previously reported findings demonstrating that addition of IL-3 protects hematopoietic cell viability following irradiation when compared with controls deprived of IL-3 (12).

The Bcl-2 protein family shares conserved domains (4). Since mutations of Bcl-2 at the BH1 or BH2 domain (Fig. 1A) were found to disrupt heterodimerization with the proapoptotic protein Bax (13) and abrogate the death suppressor activity of Bcl-2, it has been held that heterodimerization of Bcl-2:Bax, in the appropriate ratio, may neutralize Bax and be essential for cell survival (14). Thus, whether Bcl-2 phosphorylation might...
alter the interaction between these crucial partner molecules was tested since a failure to associate would identify a functional effect for Ser70 phosphorylation. When Bax was quantitatively immunoprecipitated from lysates of cells disrupted by non-ionic detergent following treatment with Bryo, no significant difference in the amount of Bcl-2 co-immunoprecipitated could be detected (Fig. 4). Moreover, all three phosphorylation-deficient S70A clones tested were capable of associating with endogenous Bax in ratios similar to that observed for the phosphorylation-competent wt and S24A Bcl-2 (Fig. 4). These data indicate that Ser70 phosphorylation apparently does not alter the affinity of Bcl-2 for Bax. Thus, it can be concluded that heterodimerization is not sufficient and an intact Ser70 phosphorylation site appears to be required for the potent death suppressor activity of Bcl-2. Furthermore, results indicate that the phosphorylation-deficient S70A Bcl-2 has neither an altered half-life nor an abnormal subcellular localization pattern compared with the functional Bcl-2. Therefore, how phosphorylation may regulate Bcl-2 function is not yet clear. According to the recent structural analysis of the Bcl-2-related anti-apoptotic protein Bcl-X\textsubscript{L}, the corresponding Ser70 site in Bcl-2 would be predicted to be located within a flexible loop domain (15) with which activated protein kinase(s) can be envisioned to interact. Phosphorylation or any resulting charge alteration in this loop domain may initiate a structural change in the Bcl-2 molecule and/or may change the affinity for any partner protein. Since the S70E Bcl-2 mutant is fully functional, a negative charge associated with the Ser70 site may be required for the appropriate conformation of this death suppressor molecule.

Purified PKC phosphorylates the identical serine site on murine Bcl-2 in vitro as observed in vivo following agonist addition suggesting that PKC is a candidate Bcl-2 kinase (6). Alternatively, it has been reported that cells overexpressing exogenous Bcl-2 are more resistant to staurosporine, which can inhibit various protein kinases including PKC. We found that Bryo-induced Bcl-2 phosphorylation could not be fully inhibited by staurosporine even at concentrations (500–1000 nM) that Bryo-induced Bcl-2 phosphorylation could not be fully inhibited in the presence of IL-3. Cell viability was assayed by trypan blue dye exclusion as described in Fig. 1. B, representative clones were compared after 24 h of treatment with 25 \mu M etoposide either in the presence or absence of IL-3.

FIG. 3. Effect of etoposide on mutant Bcl-2 anti-apoptotic activity. A, the same clones used in Fig. 2C were treated with 25 \mu M etoposide for 48 h in the presence of IL-3. Cell viability was assayed by trypan blue dye exclusion as described in Fig. 1. B, representative clones were compared after 24 h of treatment with 25 \mu M etoposide either in the presence or absence of IL-3.

stimulation appears to be sufficient to allow some level of cell survival in cells overexpressing wt Bcl-2. However, the failure to indefinitely support survival under such conditions may be due, at least in part, to the non-maximal phosphorylation of Bcl-2. Interestingly, it has recently been reported that Bad, a Bcl-2-family member and death agonist, can be phosphorylated in response to IL-3 (17). Phosphorylation of Bad appears to inhibit binding to Bcl-X\textsubscript{L} where it may facilitate apoptosis but induces binding to the 14-3-3 protein, resulting in cytosolic sequestration of Bad to apparently allow membrane-associated Bcl-X\textsubscript{L} to remain functional (17). Furthermore, phosphorylation of Bad appears to be mediated by Raf-1 (18), which is required for cell growth and can be activated by PKC in response to IL-3 (19, 20). Taken together, these findings indicate that the signal(s) transduced by growth factors such as IL-3 may regulate apoptosis through phosphorylation of Bcl-2 and Bcl-2 family members.

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