Selective regulation of *Bcl-X_L* by a Jak kinase-dependent pathway is bypassed in murine hematopoietic malignancies

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Bcl-2 family proteins are key regulators of apoptosis and function as cell death antagonists (e.g., Bcl-2, Bcl-X_L, and Mcl-1) or agonists (e.g., Bax, Bad, and Bak). Here we report that among the Bcl-2 family of proteins tested (Bcl-2, Bcl-X_L, Mcl-1, Bax, Bad, and Bak), Bcl-X_L was unique in that its protein levels were tightly regulated by hemopoietins in both immortal and primary myeloid progenitors. Investigating signaling pathways utilized by cytokine receptors established that the regulation of Bcl-X_L protein levels is mediated by the Jak kinase pathway and is independent of other signaling effectors including STATs, PI-3′ kinase, and Ras. Moreover, we provide the first direct evidence that *Bcl-X* is altered in cancer, because *bcl-X* expression was activated selectively by retroviral insertions in murine myeloid and T-cell hemopoietic malignancies. Tumors harboring *bcl-X* insertions had altered *bcl-X* RNAs, expressed elevated levels of Bcl-X_L protein, and lacked the requirements for cytokines normally essential for cell survival. Finally, overexpression of Bcl-X_L effectively protected IL-3-dependent myeloid cells from apoptosis following removal of trophic factors. Therefore, Bcl-X_L functions as a key cytokine regulated anti-apoptotic protein in myelopoiesis and contributes to leukemia cell survival.

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Committed hematopoietic progenitors continuously require hemopoietins for both growth and survival. Depletion of these trophic factors leads to apoptosis, or programmed cell death (PCD), and this endogenous cell suicide mechanism ensures strict control of hematopoietic cell numbers (Williams et al. 1990). In leukemia, cytokine-dependent signals required for survival are circumvented by oncoproteins that provide signals that suppress apoptosis. Thus, a major emphasis of leukemia research has been focused on identifying cytokine-dependent signaling pathways that govern cell survival and defining the effectors targeted by these pathways that regulate apoptosis.

There are many initiators of apoptosis, but the ultimate downstream effectors of the suicide program are a family of conserved cysteine-dependent, aspartate-specific proteases, termed caspases, that degrade key targets required for cell and nuclear integrity, including lamin A, hnRNPs, poly(ADP-ribose) polymerase, and DNA-dependent protein kinase (for review, see Villa et al. 1997). Genetic and biochemical evidence has demonstrated that the activation of caspases is influenced by the Bcl-2 family of proteins, which either suppress or induce apoptosis (for review, see Yang and Korsmeyer 1996). For example, the family members Bcl-2, Bcl-X_L, Mcl-1, and A-1 function as cell death antagonists against a variety of apoptotic stimuli, whereas the pro-apoptotic members Bax, Bak, and Bad augment the apoptotic program. Thus, some Bcl-2 family members would be predicted to be key targets for activation, whereas others would be targets for inactivation in cancer. Indeed, *BCL-2* expression is activated by the t(14:18) reciprocal translocation of *BCL-2* into the immunoglobulin heavy-chain locus in human follicular lymphoma (Bakshi et al. 1985; Tsujimoto et al. 1985; Cleary and Sklar 1985), and high levels of *BCL-2* have been reported in other cancers without obvious chromosomal alterations (Pezella et al. 1990). Elevated levels of BCL-2 in these scenarios are thought to suppress apoptosis by sequestering Bax, which dimerizes with many Bcl-2 family members, yet is a potent

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inducer of apoptosis as a homodimer (Oltvai et al. 1993; Sedlak et
al. 1995). In support of this concept, Bax functions as a tumor suppressor in murine model systems (Yin et al. 1997) and is down-regulated or inactivated by mutations in some human malignancies including leukemia and colon and breast carcinomas (Krajewski et al. 1995; Rampino et al. 1997; Brimmell et al. 1998). However, to date, other Bcl-2 family members have not been shown to be mutated directly in cancer.

Because Bcl-2 family members are key gatekeepers of the cell-death pathway, a reasonable expectation would be that their activity and/or expression is regulated by signaling pathways that suppress or induce apoptosis, and there is mounting evidence that this is the case. First, in some cell contexts Bcl-2 family members are regulated transcriptionally by mitogens and by other signals that regulate apoptosis. In these cases, the mRNA levels of the anti-apoptotic members Bcl-2, Bcl-X, A-1, and Mcl-1 are generally mitogen dependent (Reed et al. 1987; Boise et al. 1993; Lin et al. 1993; Broome et al. 1995; Kozopas et al. 1993; Yang and Korsmeyer 1996; Lomo et al. 1997), whereas the expression of the pro-apoptotic family members Bax and Bak is induced under some scenarios that lead to apoptosis (Zhan et al. 1994; Miyashita and Reed 1995; Ossina et al. 1997). Second, the activity of certain family members is regulated by post-translational modifications. For example, phosphorylation of Bcl-2 following treatment of some cells with paclitaxel has been associated with apoptosis (Haldar et al. 1994). More compelling is the recent demonstration that phosphorylation of the pro-apoptotic family member, Bad, ablates its activity as an apoptotic agonist in response to survival factors by inhibiting its association with Bcl-X_L (Zha et al. 1996). Moreover, phosphorylation of Bad occurs via the Akt serine/threonine kinase (Datta et al. 1997; Delpeso et al. 1997), a key signaling effector of the PI-3′ kinase pathway (Franke et al. 1995) essential for the survival of at least some cell types (Yao and Cooper 1995; Dudek et al. 1997). Finally, it has been also reported that Bcl-2 and Bcl-X_L are direct substrates for caspases, and that they are cleaved into pro-apoptotic forms during apoptosis induced by specific stimuli, suggesting a mechanism that ensures apoptosis goes to completion (Cheng et al. 1997; Clem et al. 1998).

To resolve which Bcl-2 family members might be involved in the regulation of programmed cell death that occurs following the withdrawal of trophic factors, we assessed the regulation of steady-state levels of these proteins in both immortal and primary myeloid cells. Here we report that of all the Bcl-2 family members tested, Bcl-X_L protein was unique in its tight regulation by hemopoietins, and that this regulation depends upon a Jak kinase-dependent signaling pathway. Moreover, in screens of murine hematologic malignancies we demonstrate *bcl-X* is selectively activated by retroviral insertions in myeloid and T-cell leukemia. This is the first direct evidence that *Bcl-X* is activated in cancer and underscores the important role of this protein in regulating apoptosis during hematopoiesis and in leukemia.

Results

Bcl-X_L proteins levels are selectively regulated by hemopoietins in immortal and primary myeloid cells

Hemopoietins are required to suppress the PCD of committed hematopoietic progenitors (Williams et al. 1990). In T- and B-lymphoid cells, cytokines have been variably reported to regulate the expression (Reed et al. 1987; Boise et al. 1993; Lin et al. 1993; Broome et al. 1995; Leverrier et al. 1997; Lomo et al. 1997; Yang et al. 1997) and/or cleavage (Cheng et al. 1997; Clem et al. 1998) of the anti-apoptotic family members Bcl-2, Bcl-X_L, Bcl-Xγ, A-1, or Mcl-1, or the activity of pro-apoptotic family members such as Bad (Zha et al. 1996). To determine the family members that play roles in the physiologic PCD observed when myeloid progenitors are deprived of trophic factors, we assessed initially whether regulation of their mRNA or protein levels was dependent upon hemopoietins in immortal and primary myeloid cells. For analyses of immortal myeloid progenitors we used murine 32D.3 and FDC-P1 myeloid cells which, like primary myeloid cells, are diploid, able to differentiate along different lineages in response to specific hemopoietins, and require IL-3 for growth and survival (Dean et al. 1987; Askew et al. 1991). When examined for regulation of Bcl-2 family protein levels by immunoblots following IL-3 withdrawal, only the down-regulation of Bcl-X_L protein correlated with the PCD of either 32D.3 or FDC-P1.2 cells (Fig. 1A,C). Marked down-regulation of Bcl-X_L was coincident with the commitment point for the PCD of these cells (16–20 hr for 32D.3 cells and 24 hr for FDC-P1.2 cells; Fig. 1C). By contrast, when examined for regulation at the RNA level, transcripts for the cell-death antagonists *bcl-2*, *bcl-X*, *A-1*, and *mcl*-1 were all dependent on IL-3, whereas RNA levels of the cell-death agonists *bax*, *bad*, and *bak* were independent of ligand (Fig. 2A; data not shown). Thus, although IL-3 signaling apparently regulates the transcription of all family members that function as cell-death antagonists, only the down-regulation of Bcl-X_L protein was consistent kinetically with a key role in regulating the PCD of myeloid progenitors.

To confirm that the observed regulation of Bcl-X_L in these immortal myeloid cell lines was representative of the response of primary progenitors, we also analyzed the regulation of Bcl-2 family members in primary, fetal-liver-derived, myeloid cell cultures (Pierce et al. 1985) grown in IL-3, IL-6, and stem cell factor. These cells had hallmarks of myeloid stem cells, being $CD34^+$, c-Kit^+, Sca-1^+, and lin^- (data not shown). As observed in the immortal cells, Bcl-X_L was the only cell-death antagonist whose levels were significantly down-regulated following the withdrawal of cytokines from these primary cells (Fig. 1B), and the down-regulation of Bcl-X_L levels was coincident with the commitment point for the PCD of primary myeloid cells (Fig. 1C).

The observations that Bcl-X_L protein was down-regulated in myeloid progenitors following withdrawal of hemopoietins suggested that readdition of cytokines to ligand-starved cells would up-regulate Bcl-X_L protein lev-

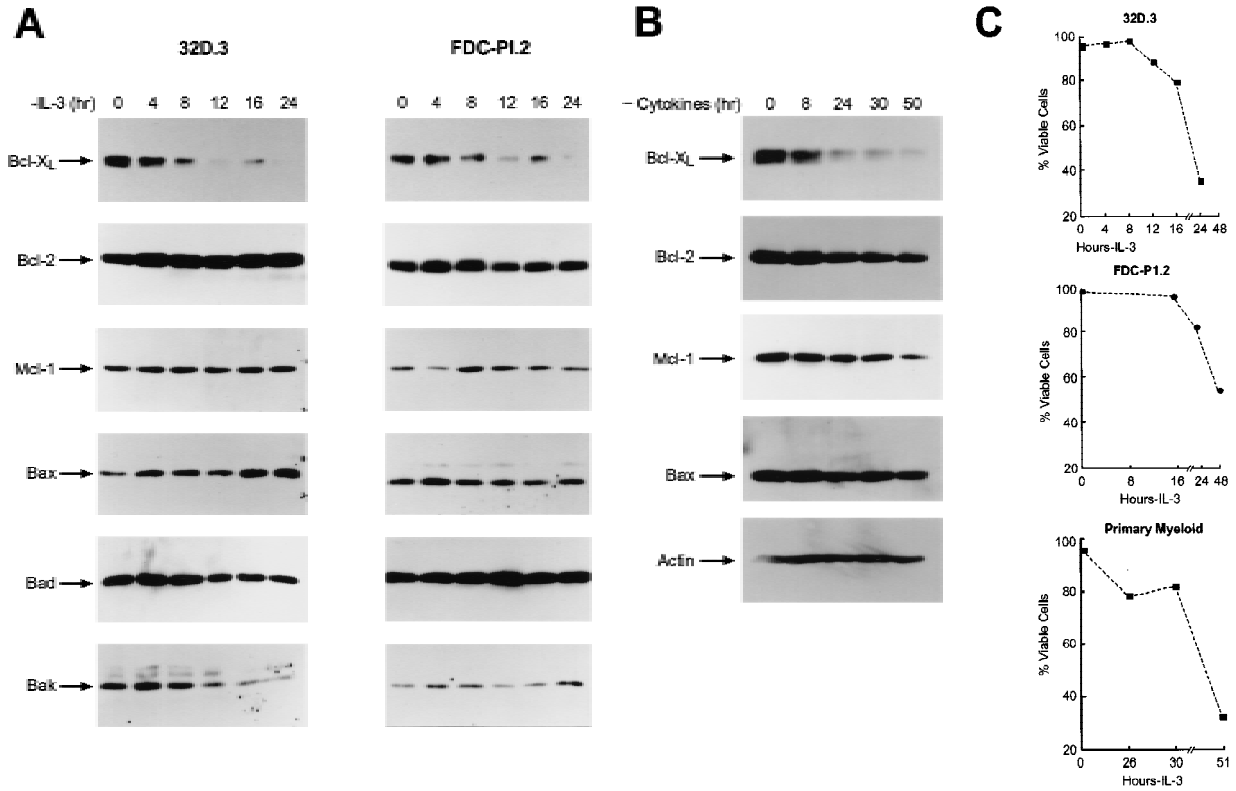

Figure 1. Bcl-X_L protein levels are selectively regulated by hemopoietins. (*A*) Levels of Bcl-2 family members were assessed by immunoblot analyses with antibodies that detect murine Bcl-2, Bcl-X_L, Mcl-1, Bax, Bad, and Bak proteins. Protein extracts (50 µg each) were prepared from 32D.3 (*left*) and FDC-P1.2 (*right*) myeloid cells growing in IL-3 or deprived of IL-3 for the indicated intervals and analyzed by immunoblots. Results shown are representative of six independent experiments. (*B*) Fetal liver-derived myeloid cells were grown in IL-3 (20 U/ml), IL-6 (10 ng/ml), and SCF (10 ng/ml) and then were deprived of all hemopoietins. Protein extracts (40 µg each) were prepared from cells after the indicated times after removal of hemopoietins and analyzed by immunoblot. Results shown are representative of three independent experiments. (*C*) The rates of death of 32D.3, FDC-P1.2, and primary myeloid cells were assessed following withdrawal of cytokines by trypan blue dye exclusion. Cell death was always apoptotic based on morphological criteria, and AnnexinV and TUNEL assays (data not shown). Results shown are representative of 10 independent experiments for 32D.3 and FDC-P1 cells and three for primary myeloid cells.

els, and parallel regulation of *bcl-X* RNA levels. In 32D.3 myeloid cells deprived of ligand, IL-3 induced robust increases of *bcl-X* transcripts, yet the kinetics of *bcl-X* induction were delayed relative to that of immediate early genes like c-*myc* (Dean et al. 1987; Askew et al. 1991), suggesting that induction of *bcl-X* might require de novo protein synthesis. Indeed, pretreatment of ligand-starved cells with cycloheximide blocked the ability of IL-3 to induce *bcl-X* (Fig. 2A), indicating that *bcl-X* is a delayed-early class gene whose induction requires the synthesis of a cytokine-regulated factor. Examination of Bcl-X_L protein levels by immunoblots demonstrated that the kinetics of induction of Bcl-X_L followed temporally the induction of *bcl-X* transcripts. Bcl-X_L levels were increased markedly after 3 hr of addition of IL-3 and continued to accumulate (Fig. 2B). By contrast, the steady-state protein levels of other Bcl-2 family members were not regulated by IL-3 stimulation of ligand-starved cells (Fig. 2B, and data not shown). Thus, unlike other Bcl-2 family members, Bcl-X_L levels are regulated tightly by IL-3, and this regulation parallels that observed at the RNA level.

In contrast to observations in pro-B cells, we failed to detect smaller forms of Bcl-2 or Bcl-X_L in immortal or primary myeloid progenitors following IL-3 withdrawal that were comparable in size to those suggested to be caspase-cleaved forms of Bcl-2 and Bcl-X_L (Cheng et al. 1997; Clem et al. 1998), even when cells were <40% viable as measured by trypan blue dye exclusion (Fig. 1C). Thus, the down-regulation of *bcl-X* mRNA expression and parallel decreases in the steady-state levels of Bcl-X_L protein, rather than cleavage to a pro-apoptotic form (or cleavage of Bcl-2), correlates with the PCDs of these myeloid progenitors. Although the murine *bcl-X* gene expresses three *Bcl-X* isoforms, Bcl-X_L, Bcl-Xβ, and Bcl-Xγ (González-García et al. 1994; Yang et al. 1997), only the expression of Bcl-X_L protein was detected in these cells.

To confirm that the selective down-regulation of Bcl-X_L levels was relevant to PCD following IL-3 withdrawal, we generated 32D.3-derived cells that constitutively overexpressed (SFFV–LTR-driven; Boise et al. 1993) murine Bcl-X_L (Fig. 3A). As expected (Boise et al. 1993; Vaux et al. 1988) pools and clones of 32D.3 my-

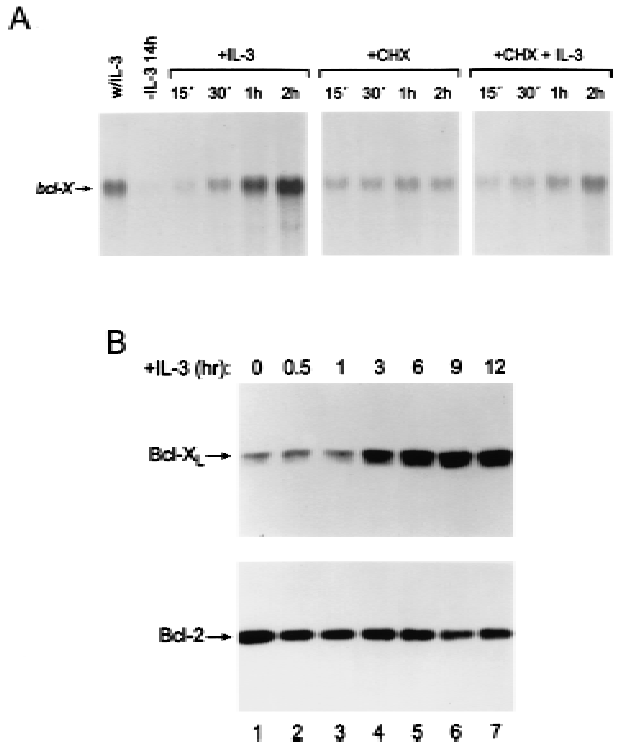

Figure 2. Induction of Bcl-X_L protein parallels the regulation of *bcl-X* gene expression. (*A*) *bcl-X* is a delayed-early class gene. Asynchronously growing 32D.3 cells were deprived of IL-3 for 14 hr and then treated with 100 U/ml of IL-3, 25 μg/ml cycloheximide (CHX), or pretreated with 25 μg/ml CHX before adding IL-3. This dose of CHX blocked ≥95% of protein synthesis within 15 min (data not shown). Total RNA was prepared from cells at the indicated intervals and 20 μg analyzed for *bcl-X* RNA levels using a coding-region probe. Control hybridizations showed equal loading (data not shown). (*B*) Kinetics of Bcl-X_L induction by IL-3. Levels of Bcl-2 and Bcl-X_L were assessed in 32D.3 myeloid cells deprived of IL-3 for 15 hr or following stimulation of ligand-starved cells with IL-3 (20 U/ml) for the indicated intervals. Extracts were prepared and 50 μg of protein was analyzed by immunoblot for changes in Bcl-2 and Bcl-X_L levels.

eloid cells overexpressing Bcl-X_L displayed remarkably extended survival when shifted to medium lacking IL-3 relative to vector-only pools and clones (Fig. 3B). Thus, the selective down-regulation of Bcl-X_L is relevant for PCD observed following the withdrawal of hemopoietins.

The Jak kinase pathway is necessary and sufficient for the regulation of Bcl-X_L protein levels by hemopoietins

Mutational analyses have demonstrated that cytokine receptors utilize multiple signaling pathways following ligand binding, and many of these, including the PI-3′ kinase/Akt kinase, Ras/Raf, and Stat pathways, have been implicated as regulators of cell survival (Cleveland et al. 1994; Kinoshita et al. 1995; Fukada et al. 1996; Canman et al. 1995; Delpeso et al. 1997; Songyang et al. 1997). For cytokines such as IL-3 and Epo, activation of these pathways requires the activity of Jak kinases that associate with and phosphorylate ligated cytokine receptors and their signaling effectors (Witthuhn et al. 1993; Miura et al. 1994a; Damen et al. 1995; Ihle 1995; Quelle et al. 1996).

To identify which hemopoietin-signaling pathways regulate Bcl-X_L protein levels, we took advantage of a series of well-characterized 32D.3-derived transfectants engineered to express different versions of the erythropoietin receptor (EpoR). The EpoR has been very well characterized in terms of its signaling pathways and mutants of the EpoR have been created that abolish selec-

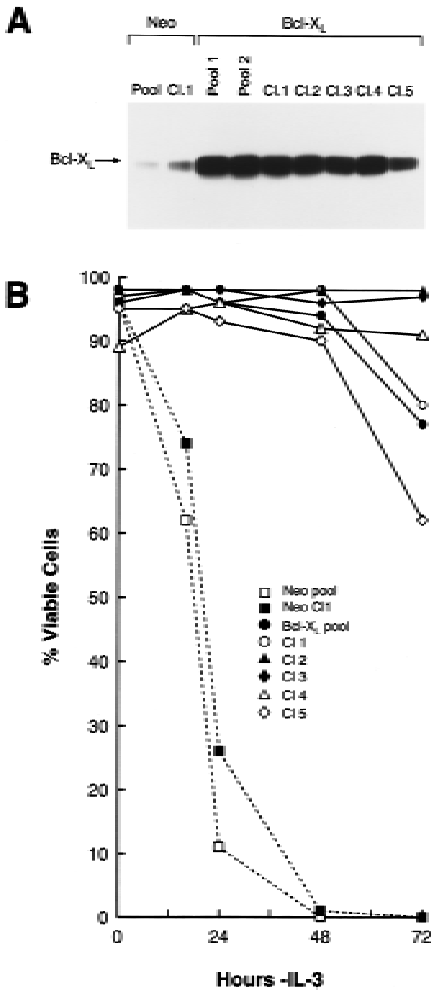

Figure 3. Enforced expression of Bcl-X_L suppresses PCD of myeloid cells in the absence of survival factors. (*A*) Immunoblot analyses of pools and clones of 32D.3-derived, murine Bcl-X_L-overexpressing cells exponentially growing in IL-3 medium. Whole cell extract (50 μg) was analyzed for levels of Bcl-X_L and Bax. (*B*) Overexpression of Bcl-X_L suppresses apoptosis. Control and Bcl-X_L overexpressing pools and clones were deprived of IL-3 and at the indicated interval the percentage of viable cells was assessed by trypan blue dye exclusion. Cells overexpressing Bcl-X_L constitutively expressed Bcl-X_L in the absence of IL-3 and arrested in G_0/G_1 (data not shown). Data shown are representative of four separate experiments.

tively the PI-3′ kinase/Akt, Ras/MAPK, and STAT pathways (Miura et al. 1993, 1994a,b; Quelle et al. 1996). The EpoR carboxy-terminal truncation mutants EpoR-H and EpoR-S cannot activate the PI-3′ kinase and Ras/MAPK pathways, and EpoR-S is defective additionally in activation of the STAT pathway (Ihle 1995; Quelle et al. 1996). In contrast, the internal deletion mutant in homology Box1 of the Epo receptor, EpoR–PB, is defective in activation of Jak2, the only Jak kinase activated by Epo stimulation (Witthuhn et al. 1993). To address which signal was required for maintenance of Bcl-X_L levels, cells expressing these mutated EpoRs were shifted from IL-3 to Epo and expression of Bcl-2 family members was assessed by immunoblots. As expected, EpoR-H and EpoR-S transfectants maintained viability and continued to proliferate when shifted to medium containing Epo, whereas EpoR–PB transfectants began to lose viability at 16 hr following transfer to Epo (Miura et al. 1993 and data not shown). Bcl-X_L levels were maintained in Epo-stimulated cells bearing EpoR-H and EpoR-S receptors, but were down-regulated in cells bearing EpoR-PB at a time coincident with the PCD of these cells (Fig. 4A). By contrast, and as expected, levels of Bcl-2 did not change appreciably in any of the transfectants when they were shifted to Epo. Thus, the selective regulation of Bcl-X_L protein levels is dependent upon a Jak kinase-regulated survival pathway and is independent of activation of PI-3′ kinase/Akt, Ras, and STAT pathways.

Because activation of a Jak kinase pathway was necessary for regulation of Bcl-X_L, we also tested whether activation of Jak kinase was sufficient for the induction of Bcl-X_L. To address this issue we took advantage of 32D.3 transfectants harboring epidermal growth factor receptor (EGFR)–Jak2 kinase domain chimeric receptors that contain the extracellular ligand binding and transmembrane domains of the EGFR fused to the kinase domain of Jak2 (Nakamura et al. 1996). Stimulation of these cells with EGF activates this chimeric kinase and supports survival of these cells in the absence of IL-3. By contrast, stimulation of cells harboring a chimeric receptor containing multiple mutations in the ATP binding site of the Jak2 kinase domain (kinase dead) fails to support cell survival (Quelle et al. 1998). EGF treatment of ligand-starved cells harboring the wild-type, but not the kinase-dead EGFR-Jak2 chimeric receptor induced Bcl-X_L levels (Fig. 4B), and up-regulation in wild-type EGFR–Jak2 transfectants correlated with the extended survival of these cells in EGF (data not shown). By contrast, treatment with IL-3 resulted in a similar up-regulation of Bcl-X_L in both chimeric derivatives. Levels of Bcl-X_L induced by EGF in wild-type EGFR-Jak2 chimeric-receptor-expressing cells were equivalent to those induced by IL-3, albeit with slightly delayed kinetics. Again induction of Bcl-X_L was selective, as Bcl-2 levels remained relatively unchanged after treatment with EGF or IL-3 (Fig. 4B). Thus, activation of Jak kinase pathways is sufficient for up-regulation of Bcl-X_L and is required for cell survival.

Bcl-X is selectively activated by retroviral insertions in murine myeloid and T-cell malignancies

Activation of *BCL-2* and mutational inactivation of *BAX* are observed in only a fraction of hematopoietic malignancies. Given the selective cytokine-dependent nature of Bcl-X_L regulation, and its ability to protect hematopoietic progenitors from apoptosis, we addressed whether *Bcl-X* might contribute to hematopoietic cell transformation. We screened a large panel of spontane-

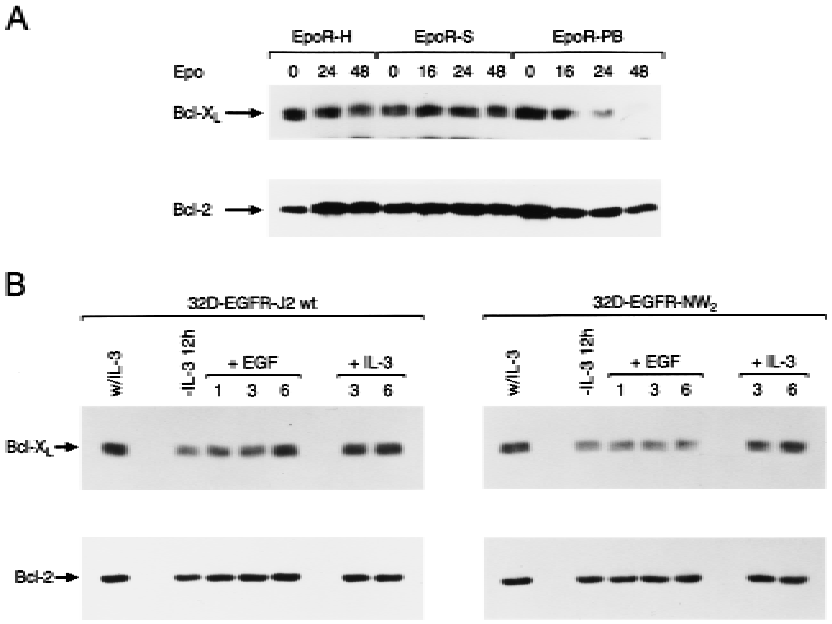

Figure 4. Activation of Jak2 kinase is necessary and sufficient for the induction of Bcl-X_L by hemopoietins. (*A*) Maintenance of Bcl-X_L levels requires activation of the Jak kinase pathway. Exponentially growing cultures of the indicated cell lines in IL-3 (lane *0*) were deprived of IL-3 by washing cells in medium lacking IL-3 and then cultured in medium supplemented with 3 units Epo/ml. At the indicated intervals extracts were prepared and 50 µg of protein was analyzed by immunoblots for changes in Bcl-2 and Bcl-X_L levels. Results shown are representative of three independent experiments. 32D.3 cells engineered to overexpress the wild-type EpoR (Miura et al. 1993) behaved like EpoR-H and EpoR-S cells in their regulation of Bcl-X_L (data not shown). (*B*) Jak2 is sufficient for the induction of Bcl-X_L. 32D.3 cells engineered to overexpress the indicated chimeric EGFR-Jak2 kinase domain receptors have been previously described (Nakamura et al. 1996) and express comparable numbers of chimeric receptors (Quelle et al. 1998). Exponentially growing cultures of the indicated cells in IL-3 were deprived of IL-3 for 12 hr by washing cells in medium lacking IL-3 and then stimulated with 50 ng/ml purified EGF or 20 U/ml IL-3. At the indicated intervals extracts were prepared and 50 µg of protein was analyzed by immunoblots for changes in Bcl-2 and Bcl-X_L levels. Results shown are representative of three independent experiments.

ous, retrovirus- or radiation-induced murine leukemia and lymphoma cell lines [$n = 51$, of myeloid, T-, and B-lymphoid cell lineage (Table 1; Askew et al. 1993)] for changes in the expression of *bcl-X* RNA by Northern blot analyses with a *bcl-X* coding-region probe. Although most tumor lines expressed the expected 2.7-kb *bcl-X* RNA, we detected elevated levels of altered, high-molecular-weight *bcl-X* RNAs in 5 (9.8%) of the tumor-derived lines (14–259, NFS-124, BXH2-4S, WEHI-22, and ABPL-4 cells; Fig. 5A; Table 1). WEHI-22 cells also expressed a smaller *bcl-X* RNA and failed to express the normal 2.7-kb RNA, whereas the other tumor lines also expressed low levels of the normal 2.7-kb transcript (Fig. 5A). Altered *bcl-X* RNA expression was restricted to leukemic cells of myeloid or T-cell lineage that lacked requirements for specific hemopoietins normally essential for cell survival. Altered *bcl-X* transcripts were not evident in any of the B-cell-derived malignancies tested ($n = 11$). Overall, aberrant *bcl-X* RNAs were detected in 4/18 (22%) myeloid and 1/6 (16.6%) T-cell growth-factor-independent leukemias. Although levels of transcripts of some of the other Bcl-2 family members varied,

Table 1. *Expression of Bcl-2 family members in murine myeloid and T-cell hematologic malignancies*

Tumor line	Cytokine dependence[a]	Lineage[b]	*bcl-2*	*bcl-x*	*mcl-1*	*A-1*	*bax*	*bad*	*bak*
DA-1	IL-3	myeloid	−	+	+	+	+	−	+[c]
DA-3	IL-3	myeloid	+	+	+	N.T.[d]	+	N.T.	N.T.
DA-7	IL-3	myeloid	+	+	+	+	+	++	−
DA-13	IL-3	myeloid	+	+	+	+++	+	+	+[e]
DA-22	IL-3	myeloid	+	+	+	N.T.	+	N.T.	N.T.
DA-24	IL-3	myeloid	+	+	+	−	+	+	+++[e]
DA-28	IL-3	myeloid	+	+	+	−	+	+	+[c]
DA-29	IL-3	myeloid	−	+	+	N.T.	+	N.T.	N.T.
DA-31	IL-3	myeloid	−	+	+	N.T.	−	N.T.	N.T.
DA-34	IL-3	myeloid	+	+	+	N.T.	+	N.T.	N.T.
NFS-36	IL-3	myeloid	+	+	+	−	+	++	+[c]
NFS-58	IL-3	myeloid	++	+	+	N.T.	+	N.T.	N.T.
NFS-60	IL-3	myeloid	+	+	+	N.T.	+	N.T.	N.T.
NFS-78	IL-3	myeloid	+	+	++	N.T.	+	N.T.	N.T.
NFS-107	IL-3	myeloid	++	+	+	+	+	+	+[e]
DA-33	−	myeloid	+	+	+	+	+	+	−
NFS-56	−	myeloid	+	+	+	−	+	+	+
NFS-61	−	myeloid	+	++	+	−	+	+	+[c]
NFS-124[f]	−	myeloid	+	+++	+	+++	+	+	+[c]
C6	−	myeloid	++	+	+	−	+	+	+[c]
C10	−	myeloid	+	+	+	N.T.	+	N.T.	N.T.
14-122	−	myeloid	+	+	+	−	+	+	+[c]
14-166	−	myeloid	+	+	+	−	+	+	+[c]
14-259[f]	−	myeloid	+	+++	+	+++	+	+	+[c]
15-299	−	myeloid	+	+	+	−	+	+	+[c]
7M12	−	myeloid	+	+	+	−	+	+	+[c]
M1	−	myeloid	++	+	+	+++	+	+	+[c]
ABPL-4[f]	−	myeloid	+	+++	+	+++	+	+	+[c]
BXH2-4S[f]	−	myeloid	+	++	+	+++	+	+	+[c]
BXH2-11S	−	myeloid	−	+	+	+++	+	+	+[c]
WEHI-3	−	myeloid	+	+	+	++	+	+	+[c]
AFSTL-1	−	mast	+	+	+	+	+	+	+[c]
AFSTL-2	−	mast	+	+	+	−	+	+	+[c]
WEHI-22[f]	−	T-cell	+	+++	+	−	+	+	+[c]
WEHI-164	−	T-cell	+	+	+	−	+	+	+[c]
WEHI-279	−	T-cell	−	+	++	+++	+	+	+[c]
RL-12	−	T-cell	+	+	+	−	+	+	+[c]
EL-4	−	T-cell	+	+	++	+++	+	++	++[c]
DA-2	−	T-cell	+	++	++	−	+	+	+[c]
CTLL	IL-2	T-cell	+	+	+	−	+	+	+[c]

Expression was determined by conventional Northern blotting using coding region probes for each of the indicated family members.
[a]Denotes the specific requirement of the tumor cell lines for cytokines for growth and survival.
[b]Lineage determined by cell surface markers and morphology (Askew et al. 1993).
[c]Two transcript of 2.4 and 3.0 kb detected.
[d](N.T.) Not tested.
[e]One transcript of 2.6 kb detected.
[f]Denotes tumors having rearranged *bcl-X* gene.

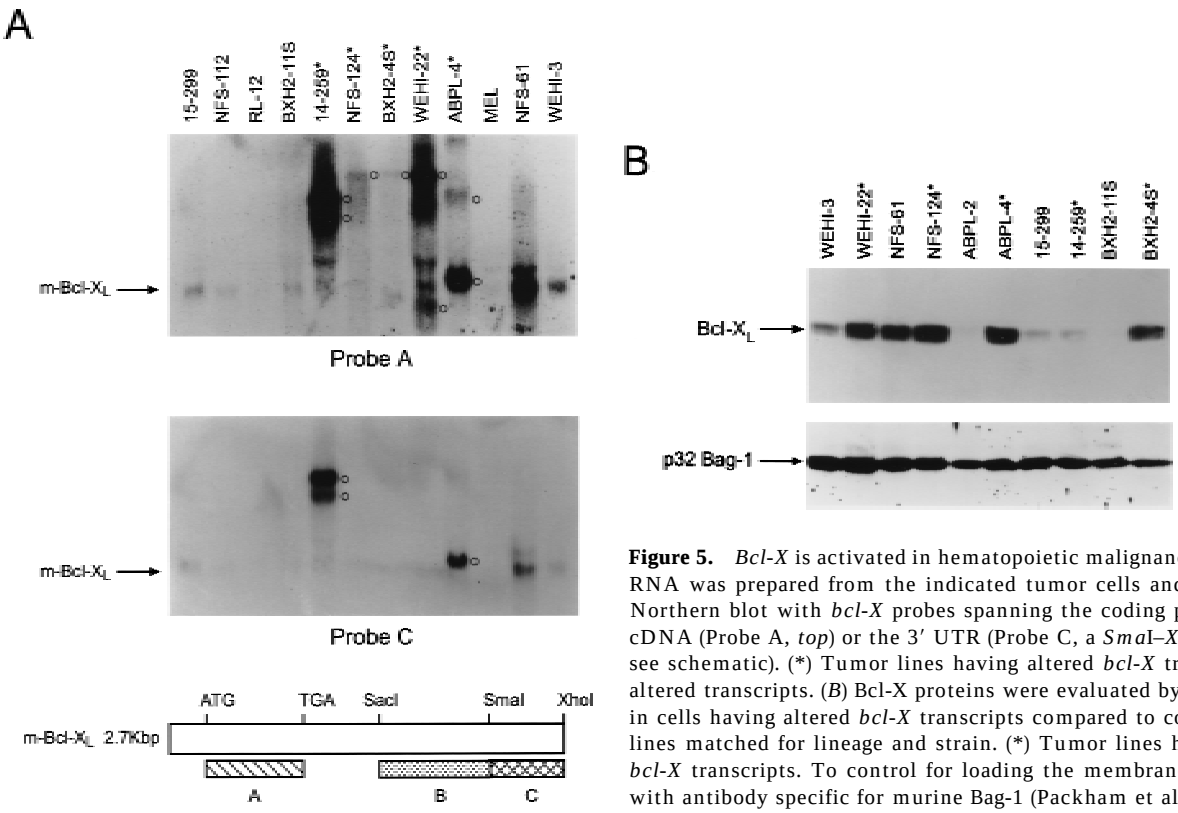

Figure 5. *Bcl-X* is activated in hematopoietic malignancies. (*A*) Total RNA was prepared from the indicated tumor cells and analyzed by Northern blot with *bcl-X* probes spanning the coding portion of the cDNA (Probe A, *top*) or the 3′ UTR (Probe C, a *Sma*I–*Xho*I fragment, see schematic). (*) Tumor lines having altered *bcl-X* transcripts; (○) altered transcripts. (*B*) Bcl-X proteins were evaluated by immunoblot in cells having altered *bcl-X* transcripts compared to control tumors lines matched for lineage and strain. (*) Tumor lines having altered *bcl-X* transcripts. To control for loading the membrane was blotted with antibody specific for murine Bag-1 (Packham et al. 1997).

we failed to detect any tumors having visibly altered RNAs (Table 1; data not shown), demonstrating that the *bcl-X* alterations were selective. Leukemic cells having altered *bcl-X* transcripts constitutively expressed *bcl-X* RNA independent of cytokines or serum (data not shown). Elevated levels of Bcl-X$_L$ protein were observed in four of the tumor lines relative to controls matched for mouse strain and tumor-cell lineage (Fig. 5B). We did not detect Bcl-X proteins with altered mobilities in these leukemias, suggesting that the *bcl-X* open reading frame (ORF) had been preserved.

Many tumor lines with altered *bcl-X* RNAs were induced by retroviral infection (Askew et al. 1993), suggesting that the altered RNAs were caused by fusion with retroviral transcripts generated from insertions in the *bcl-X* gene. Consistent with this interpretation, altered *bcl-X* transcripts also hybridized with an LTR-specific probe (data not shown). The RNA encoding Bcl-X$_L$ contains three exons (Grillot et al. 1997) and to assess the structure of altered *bcl-X* RNAs, we initially used a probe derived from the 3′ untranslated region (UTR) of *bcl-X* exon 3 in Northern blots. This probe failed to detect the altered *bcl-X* RNAs in NFS-124, BXH2-4S, and WEHI-22 cells (Fig. 5A), indicating that the putative insertion site in *bcl-X* in these cells is likely 5′ of the *Sma*I site in the 3′ UTR of exon 3. In contrast, the 3′ *bcl-X* UTR probe detected altered *bcl-X* RNAs in APBL-4 and 14–259 cells. Thus, the putative retroviral insertions in these lines lie either 5′ of the *bcl-X* ORF or within the large intron 2 of *bcl-X* (Grillot et al. 1997).

By Southern blot analysis, *bcl-X* gene rearrangements were evident in WEHI-22, NFS-124, and BXH2-4S cells and mapped within a 793-bp region between the *Sac*I and *Sma*I sites present in the noncoding portion of *bcl-X* exon 3 (Fig. 6A). In WEHI-22 cells, which lacked expression of the normal 2.7-kb *bcl-X* RNA (Fig. 5A), no bands were shared with matched controls (from tumors with normal *bcl-X* transcripts) indicating that, in addition to gene rearrangement, they had lost the normal *bcl-X* allele. Duplication of the altered *bcl-X* allele was also evident in WEHI-22 cells (Fig. 6A). By contrast, NFS-124 and BXH2-4S cells retained an apparently normal *bcl-X* allele in addition to the rearranged allele, consistent with the expression of both normal and altered *bcl-X* RNAs (Fig. 5A). There was no evidence for *bcl-X* gene rearrangements in APBL-4 and 14–259 cells by conventional Southern blot analysis (Fig. 6A), yet alterations of larger DNA fragments were detected by pulse-field gel electrophoresis (Fig. 6B). Therefore, these tumor lines also have retroviral insertions that alter *bcl-X* expression, but the sites of insertion are relatively distant or are within intron 2. In these leukemias retroviral–*bcl-X* fusion transcripts (Fig. 5A) must include splicing into *bcl-X* exon 2, which contains the Bcl-X$_L$ initiator codon.

To pinpoint the retroviral insertion sites in WEHI-22, BXH2-4S, and NFS-124 cells, we performed 3′ rapid amplification of cDNA ends (RACE) and the PCR products were cloned and sequenced. Three unique retrovirus integration sites were identified (Fig. 7A) and, in agreement with Northern and Western blot analyses (Fig. 5), the integrations were outside of the Bcl-X$_L$ ORF in the 3′ UTR of the gene (Fig. 7A). Sequence analysis of the viral

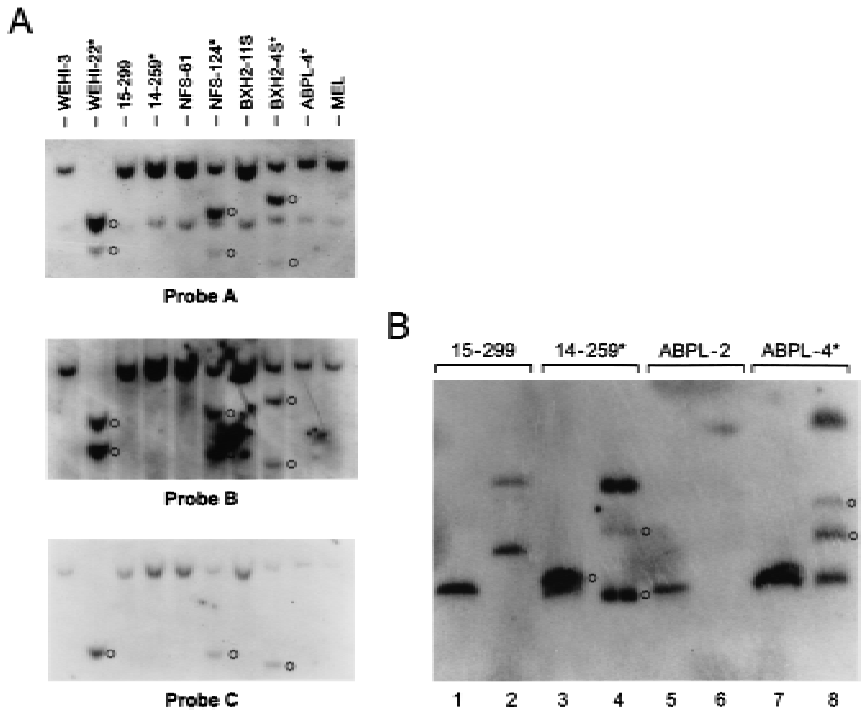

Figure 6. *bcl-X* is rearranged in tumor lines having altered *bcl-X* transcripts. (*A*) Southern blot analyses of *Kpn*I + *Eco*RI-restricted genomic DNA of tumor lines having altered *bcl-X* transcripts (*) relative to tumors matched for lineage and mouse strain. No polymorphisms were detected with five distinct restriction digests of these matched tumor line DNAs (data not shown). Blots were hybridized sequentially to probes A, B, and C (see Fig. 5A). (*B*) Blot analyses of pulsed-field inversion gels of genomic DNA from *bcl-X* rearranged tumors (*) vs. matched controls (15–299 and ABPL-2) using the full-length *bcl-X* cDNA as region probe. (○) Altered bands. Genomic DNA was digested with *Sfi*I (odd numbered lanes) or *Mlu*I (even-numbered lanes).

portion of the *bcl-X*–retrovirus fusion RNAs demonstrated they were identical to those of the virus used to derive the tumors (data not shown). The integration sites were further characterized by performing RT–PCR using *bcl-X*- and LTR-specific primers (Fig. 7B) and sequencing these products (data not shown). Overall these data demonstrate that activation of *bcl-X* in hematopoietic malignancies preserves the ORF and results in enhanced and constitutive expression of the gene, underscoring its important role as a regulator of PCD of hematopoietic progenitors.

Discussion

We demonstrate here that Bcl-X_L protein levels are regulated selectively by hemopoietins in primary and immortal myeloid progenitors, and that the murine *bcl-X* gene is activated in myeloid and T-cell malignancies that lack requirements for hemopoietins. These facts, coupled with observations linking the regulation of Bcl-X_L to Jak kinase-dependent pathways that are required for cytokine-receptor signaling (Ihle 1995), support the hypothesis that Bcl-X_L plays a key role as a cytokine-regulated

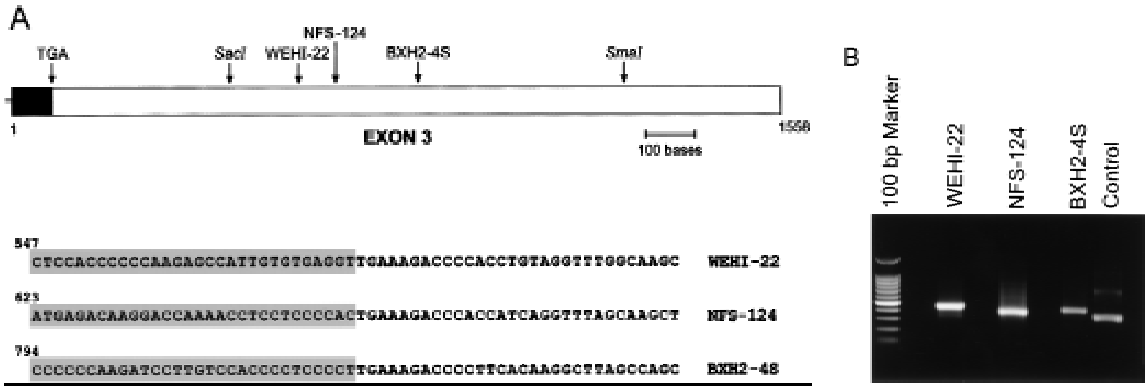

Figure 7. *bcl-X* is activated by retroviral insertions. (*A*) 3′ RACE extended *bcl-X* cDNAs were generated from WEHI-22, NFS-124, and BXH2-4S RNAs and the products amplified by PCR. The PCR products were then cloned and sequenced. (*Top*) Diagrammatic of *bcl-X* exon 3 (Grillot et al. 1997) and the sites of retroviral insertions; (*bottom*) the sequence of these junctions. The shaded region indicates *bcl-X* sequences and number denotes nucleotide number of *bcl-X* exon 3. Identical results were obtained in two additional 3′ RACE analyses. Retroviral integrations occurred at the following position of *bcl-X* exon 3: WEHI-22, nucleotide 576; and NFS-124, nucleotide 652; BXH2-4S, nucleotide 823. (*B*) RT–PCR analyses of *bcl-X*–LTR fusion transcripts. Primers spanning the identified *bcl-X*-retroviral junctions were used to amplify cDNA made from the indicated RNA samples, and the resultant PCR products were analyzed by agarose-gel electrophoresis. Products of the expected lengths were obtained (see Materials and Methods) and identity was confirmed by sequencing (data not shown). Control lane is the positive RT–PCR reaction provided with the kit.

cell-death antagonist. In agreement with this concept, deletion of *bcl-X* in mice leads to embryonic lethality that may in part be caused by the rampant apoptosis of hematopoietic progenitors (Motoyama et al. 1995). Thus, it appears that one function of Bcl-X_L is as an important and limiting mediator of hemopoietin-dependent survival pathways that regulate progenitor cell numbers in vivo. The *bcl-X* activations that we have detected in murine malignancies therefore likely contribute to tumorigenesis by providing constitutive signals for cell survival.

The selective regulation of Bcl-X_L protein levels by hemopoietins, relative to other Bcl-2 family members, was surprising given that the RNA levels of all cell-death antagonists tested were dependent upon cytokines. On face value this suggests that the half-life of Bcl-X_L protein is either shorter than that of other Bcl-2 family members, or that Bcl-X_L is targeted selectively for degradation. The rate of decay of Bcl-X_L protein following the withdrawal of survival factors roughly parallels the drop in *bcl-X* transcripts (data not shown). However, the decreases in steady-state levels of Bcl-X_L were not associated with the appearance of smaller molecular weight forms that would be comparable to those detected in IL-3 deprived pro-B-lymphoid cells and implicated as caspase-generated pro-apoptotic forms of Bcl-X_L and Bcl-2 (Cheng et al. 1997; Clem et al. 1998). This fact may in part explain the apparent increased potential of exogenous Bcl-X_L to protect 32D.3 myeloid cells, relative to that seen in BaF-3 pro-B cells, from PCD following removal of IL-3 (cf. Fig. 3B and Clem et al. 1998). Thus, at least in myeloid cells, the PCD that occurs following the withdrawal of survival factors is associated with the down-regulation of steady-state levels of Bcl-X_L that parallels silencing of *bcl-X* gene expression, rather than caspase cleavage. The concept that this form of regulation of Bcl-X_L is also operational in other survival pathways is supported by the observations that Bcl-X_L protein levels are also regulated by CD40 ligation, IL-3, and IL-2 in lymphocytes (Broome et al. 1995; Tuscano et al. 1996; Leverrier et al. 1997).

Post-translational modification has been proposed to link cytokine signaling and regulation of apoptosis by Bcl-2 family proteins. Phosphorylation of the cell-death agonist Bad in response to IL-3 in pro-B cells prevents the association of Bad with Bcl-X_L, thereby releasing Bcl-X_L to function, and this is thought to be one signaling pathway through which IL-3 suppresses cell death (Zha et al. 1996; Delpeso et al. 1997). Moreover, the Akt serine/threonine kinase, a downstream effector of the PI-3′ kinase pathway (Franke et al. 1995), has been shown to mediate Bad phosphorylation induced by both NGF and IL-3 (Delpeso et al. 1997; Datta et al. 1997), and myeloid cells engineered to overexpress constitutively activated versions of Akt, have delayed rates of death following the withdrawal of survival factors (Songyang et al. 1997). Along with the data demonstrating that treatment of neuronal cells with the PI-3′ kinase inhibitor wortmannin induces apoptosis (Yao and Cooper 1995; Dudek et al. 1997), these data suggest that the PI-3′/Akt kinase pathway plays a key role in regulating cell survival. Although we cannot strictly rule out a contribution of the PI-3′/Akt kinase pathway to myeloid cell survival, several observations are inconsistent with this pathway having an essential role. First, myeloid-cell survival is supported by versions of the EpoR that are defective in activation of PI-3′ kinase (Miura et al. 1993; 1994a; Damen et al. 1995), yet are competent to signal through Jak kinase (Witthuhn et al. 1993) and maintain Bcl-X_L expression (Fig. 4A). Second, myeloid cells are remarkably resistant to the effects of wortmannin. At concentrations of wortmannin that inhibit Akt activity in myeloid cells (20 nM, Songyang et al. 1997), or at concentrations as high as 1 μM, there are no deleterious effects on the survival of primary or immortal myeloid cells (data not shown). Third, we have failed to detect any changes in the phosphorylation status of Bad potein following cytokine treatment of myeloid cells (data not shown). Therefore, at a minimum our data now suggest that regulation of Bcl-X_L levels is also a critical contributor to hemopoietin-dependent survival pathways of myeloid cells.

In addition to the PI-3′/Akt kinase pathway, STAT and Ras/Raf pathways have also been implicated as mediators of cytokine survival pathways. Dominant-negative versions of STAT-3 or activated Ras or Raf-1 have been shown to modulate hematopoietic cell survival (Cleveland et al. 1994; Canman et al. 1995; Fukada et al. 1996). Moreover, forms of the GM-CSF receptor that fail to activate the Ras/Raf pathway are unable to support cell survival under serum-free conditions (Kinoshita et al. 1995). By contrast, the activation of Ras/Raf or STAT pathways in myeloid cells is dispensable for Epo-mediated cell survival (Miura et al. 1993; Quelle et al. 1996) and regulating Bcl-X_L (Fig. 4A). Furthermore, the rather modest effects of Raf-1 on promoting 32D.3 cell survival (Cleveland et al. 1994), relative to Bcl-2 or Bcl-X_L overexpression (Nip et al. 1997, Fig. 3B), are independent of any effects upon Bcl-X_L regulation (G. Packham and J.L. Cleveland, unpubl.). Rather, our results suggest that a Jak-kinase dependent signaling pathway emanating from the membrane proximal domain of cytokine receptors is necessary and sufficient as an upstream signal that regulates cell survival and Bcl-X_L. Interestingly, this same pathway has also been linked to the control of hematopoietic cell survival in response to DNA damage (Quelle et al. 1998). Up-regulation of the Jak kinase pathway has been implicated in leukemia (Lacronique et al. 1997), whereas deletion of Jak2 or Jak3 in mice compromises the development of the myeloid/erythroid or lymphoid compartments, respectively (Nosaka et al. 1995; Parganas et al. 1998). Our findings that Bcl-X_L is downstream of this pathway, and is regulated selectively by hemopoietins and activated in hematopoietic malignancies, underscores the importance of this pathway in the control of PCD and in cancer.

Finally, to our knowledge this is the first direct evidence that *bcl-X* is activated in cancer. Of particular note are the facts that for each tumor bearing alterations of *bcl-X* mRNA, the rearrangements preserved the ORF and led to the constitutive, growth-factor-independent, ex-

pression of the gene, which normally is dependent upon cytokines (Fig. 2A). Furthermore, these observations suggest that components of the Jak kinase survival pathway leading to Bcl-X_L expression may also be targets for activation in human cancer. Finally, these data predict that *BCL-X* contributes directly or indirectly to human malignancies. Although translocations involving *BCL-X* at 20q11 (NCBI STS Data Base, accession no. U72398) have yet to be observed, this region is associated with interstitial deletions common in myeloid malignancies (Kurtin et al. 1996), and it is possible these deletions augment expression of *BCL-X_L*. Moreover, *BCL-X_L* is expressed highly in some human cancers without obvious genetic alterations (Foreman et al. 1996; Pallis et al. 1997; Silva et al. 1998; Tu et al. 1998), suggesting that other mechanisms may lead to BCL-X_L overexpression.

Materials and methods

Cell culture

The tumor cells used in this study were maintained in RPMI-1640/10% FCS + L-glutamine and, where necessary, 20 units murine IL-3/ml (Dean et al. 1987; Askew et al. 1991). Radiation-induced leukemias were provided by David Askew (University of Cincinnati, OH). Others have been described previously (Askew et al. 1993). 32D.3-cell-derived pools and clones that overexpress murine Bcl-X_L were generated using the SFFV–Bcl-X_L expression construct (Boise et al. 1993) as described previously (Packham and Cleveland 1994). Analyses of the affects of IL-3 withdrawal on apoptosis and/or expression of Bcl-2 family members in 32D.3 and FDC-P1.2 cells and stable transfectants were performed as described previously (Packham and Cleveland 1994). Exponentially growing cells were seeded on consecutive days at 5×10^5 cells per ml and on day 3 were deprived of IL-3 by washing cells in RPMI-1640 medium lacking IL-3. Cells were reset at 0.5×10^6 cells per ml in RPMI-1640/10% FCS medium and cell viability determined by trypan blue dye exclusion (Askew et al. 1991). 32D.3-derived cells overexpressing wild type and mutants of the EpoR have been described previously and express equivalent amounts of EpoR on the cell surface (Miura et al. 1993). Exponentially growing cells in IL-3 media were deprived of IL-3 by washing cells twice in RPMI-1640 and then reset at 0.3×10^6 cells/ml in RPMI-1640/10%FCS medium supplemented with 3 units Epo/ml. 32D.3 myeloid cells engineered to overexpress wild-type and mutant EGF receptor–Jak2 kinase domain chimeric receptors have been described previously and express comparable numbers of chimeric receptors on the cell surface (Nakamura et al. 1996; Quelle et al. 1998). Cells growing in IL-3 medium were deprived of IL-3 by washing in RPMI-1640 media/1% FCS and after 12 hr were stimulated with 50 ng/ml purified EGF or 20 units of IL-3/ml.

Fetal liver-derived myeloid cultures were prepared from day 17 mouse embryos by culture of suspension cells in RPMI-1640 media supplemented with IL-3 (20 U/ml), IL-6 (10 ng/ml), and SCF (10 ng/ml), 10% FCS, and L-glutamine (Pierce et al. 1985). After removal of adherent cells by consecutive passages, proliferating populations were $CD34^+$, $Sca1^+$, and c-Kit^+, but were negative for all other more differentiated markers of mature myeloid and lymphoid cells. Antibodies for FACS analyses were purchased from Becton Dickinson. To assess rates of cell death, cells were seeded as above and cultured in RPMI-1640/10% FCS medium lacking cytokines. Cell viability was again determined by trypan blue dye exclusion.

Northern and Southern blotting

Total RNA and genomic DNA isolation and Northern and Southern blotting of agarose and pulsed-field inversion gels were performed using conventional techniques. For Northern blots the coding portions of human *mcl-1* and murine *bcl-2, bax, A-1,* and *bad* cDNAs were used [provided by R. Craig (Dartmouth Medical School, Hanover, NH), D. Askew, J. Reed (The Burnham Institute, La Jolla, CA), C. Kürschner (St. Jude Children's Research Hospital), and S. Korsmeyer (Washington University, St. Louis, MO), respectively]. The *bcl-X* probes were the full-length cDNA probe, a coding region probe, or 3′ UTR-specific probes (Fig. 5A). The human *bak* cDNA was cloned by conventional RT–PCR.

Immunoblotting

Immunoblots were performed using 40 or 50 µg of whole-cell extracts as described previously (Cleveland et al. 1994). To assure that proteins were not subject to artificial cleavage by caspases following cell lysis, cell were collected, washed once in PBS, and the dry cell pellets snap frozen in liquid nitrogen. Frozen pellets were lysed rapidly on ice in RIPA buffer containing a cocktail of protease inhibitors [AEBSF, E-64, pepstatin, aprotinin, leupeptin, and PMSF (Sigma)], clarified by centrifugation, an aliquot removed for protein assays (Bio-Rad) and immediately boiled for 5 min in 2% SDS sample buffer. In the absence of protease inhibitors, Bcl-X_L protein present in lysates from cells undergoing cell death was sometimes processed to forms comparable to putative pro-apoptotic forms observed in pro-B cells (Clem et al. 1998), but was never observed when protease inhibitors were included in the lysis buffer. Antibodies used were as follows: mouse Bcl-2 (15021, PharMingen, 1:250); mouse Bcl-X (B2260, Transduction Labs, 1:250); mouse Bax (13686E, PharMingen, 1:500); Mcl-1 (B54020, Transduction Labs, 1:500); Bak (G-23, Santa Cruz, 1:100); Bad (B36420, Transduction Labs, 1:250) and Bag-1 (Packham et al. 1997). Using Bcl-2, Bcl-X_L, Bak, and Bax-specific antibodies from other sources gave comparable results, including those antisera used to detect proteolytically clipped forms of Bcl-2 (Cheng et al. 1997) or Bcl-X_L (Clem et al. 1998). Bound immunocomplexes were detected by enhanced chemiluminescence (Amersham) or Supersignal (Pierce).

Rapid amplification of cDNA ends and RT–PCR

3′ RACE for amplification of cDNA ends was performed according to the manufacturer's instructions (Life Technologies). 3′ RACE products were generated by converting 1 µg RNA template into first-strand cDNA in a reaction volume of 11 µl containing 1× buffer (20 mM Tris-HCl at pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 500 nM adapter primer, 500 µM dNTPs) and 200 units of Superscript II reverse transcriptase. Two microliters of cDNA from first-strand cDNA were amplified using 200 nM gene-specific primer (*bcl-X*, CCACATCTCAGTTCTCTTGG) and 200 nM abridged universal amplification primer in total reaction volume of 50 µl containing 1× buffer (20 mM Tris-HCl at pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs) and 1.25 units *Taq* polymerase. Samples were cycled 34 times using an annealing temperature of 47°C. 20 microliters of sample were gel purified and a second amplification was done with primer *bcl-X2* (CGCGTCGACTAGTCTAAACCAGCTCCTTGGAG) with the same reaction conditions. 20 microliters of sample was gel purified and subcloned into pGEM-T vector. Clones were cut with *Apa*I to check for inserts and sequenced subsequently on both strands using Taq FS dye terminator chemistry. Retroviral sequences were identified in GenBank by BLAST.

RT–PCR on tumor-derived RNAs having altered *bcl-X* transcripts was performed according to the manufacturers instructions (Perkin Elmer) using primers specific for the retroviral sequences for reverse transcription of RNA to cDNA, followed by amplification using primers for *bcl-X*. Viral and *bcl-X* primers were as follows. For NFS-124: *bcl-X*, CAGGCTGCTTGGGATAATGAG; retrovirus, CAAACAGAAGCGAGAAGCG (346 bp). For WEHI-22: *bcl-X*, GAGCCATTGAGTTGAAAGAC; retrovirus, GCACTGCAAGAGGTTTATTG (446 bp). For BXH2-4S: *bcl-X*, CCACTTGTGGTCTGAATG; retrovirus, GGGAACTTGAGACAATTCTG (412 bp).

cDNA was prepared using 1× buffer [50 mM as above but with 5 mM $MgCl_2$, 50 pmol downstream (retroviral) primer, 1 mM dNTPs, 1 µg RNA template] and 2.5 units of MuLV reverse transcriptase and 1 unit RNase inhibitor in a total reaction volume of 20 µl. Samples were then incubated at 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min. cDNA was then amplified using 1× buffer as above with 50 pmol upstream (*bcl-X*) primer and 1.25 units of Taq DNA polymerase in a reaction volume of 100 µl. Samples were cycled 39 times in a MJ Research PTC-100 PCR machine using an annealing temperature of 47°C. PCR products (20 µl) were run on an agarose gel and remaining product was purified using Centricon 100 columns according to manufacturer's recommendations and sequenced by TaqFS dye terminator chemistry.

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