Prolactin regulation of Bcl-2 family members: increased expression of *bcl-x_L* but not *mcl-1* or *bad* in Nb2-T cells

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Abstract

Prolactin (PRL)-dependent rat pre-T Nb2 (Nb2-11) cell lines serve as a useful model for investigation of mechanisms underlying lactogen-mediated suppression of apoptosis. Glucocorticoids, such as dexamethasone (DEX), induce apoptosis in Nb2-11 cells; the addition of PRL abrogates the cytolytic actions of DEX in this model, presumably because of increased expression of survival genes. In the present study, we investigated whether inhibition of DEX-induced apoptosis by PRL in Nb2-T cells was accompanied by altered expression of Bcl-2 family members, *mcl-1*, *bad* or *bcl-x_L* determined by Northern and immunoblot analysis. The results indicated that a 0.9 kb *bcl-x_L* transcript was rapidly induced by PRL. It reached maximal levels within 2 to 4 h (>20-fold) before declining toward basal values. Similar results were obtained in primary cultures of mouse thymocytes exposed to DEX in combination with PRL. In addition to increasing its mRNA expression, PRL also increased Bcl-xL protein levels by 6 h. Moreover, the effect of PRL to

increase *bcl-x_L* appeared to reflect direct and indirect mechanisms, since it was attenuated by the inhibition of protein synthesis. Results from other experiments suggest that PRL signaling to *bcl-x_L* expression was independent of the Jak2/Stat pathway but appeared to require activation of a Src tyrosine kinase. In contrast, while a 1.1 kb *mcl-1* transcript was detected in proliferating and quiescent cells, PRL did not alter its expression at either mRNA or protein levels. Moreover, neither *bad* mRNA nor its protein product were detectable under any of the experimental conditions evaluated. We have concluded that *bad* and *mcl-1* are unlikely candidates for apoptosis regulatory genes modulated by PRL. However, the kinetic pattern of PRL-provoked *bcl-x_L* expression is consistent with its playing a role as an apoptosis suppressor in Nb2-T cells and primary cultures of mouse thymocytes exposed to glucocorticoids.

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Introduction

In addition to playing a central role in mammary gland development and lactation, prolactin (PRL) has been also implicated as an important modulator of immune cell function. Numerous laboratories have reported stimulatory effects of PRL on lymphocyte mitogenesis (reviewed by Hooghe-Peters *et al.* 2001), secretion of the hormone by stimulated immune cells (Montgomery 2001), and expression of PRL receptors on numerous hematopoietic cell types (Russell *et al.* 1985, Gagnerault *et al.* 1993). However, despite the preponderance of data supporting a role for PRL in immune cell actions, recent studies demonstrating a lack of an immunologic phenotype in mice in which PRL (Horseman *et al.* 1997) or its receptor

(Bouchard *et al.* 1999) were eliminated by targeted gene disruption have cast doubt upon whether the hormone is necessary for mitogen (or antigen)-provoked proliferative responses or antibody production in immune cells under basal conditions. Notably, recent work by ourselves (Dugan *et al.* 2002, Krishnan *et al.* 2003) and others (Zellweger *et al.* 1996a,b) suggests that the immunological actions of PRL are primarily manifest when glucocorticoids are elevated, such as during stress.

Using the rat Nb2 lymphoma T-cell model, which is dependent upon lactogens for growth and survival, Witorsch and co-workers (Fletcher-Chiappini *et al.* 1993, Witorsch *et al.* 1993) were the first to show that PRL may increase lymphocyte population density by suppression of apoptosis. These investigators demonstrated that Nb2 cell

death and its accompanying DNA fragmentation following exposure to the potent glucocorticoid, dexamethasone (DEX), was inhibited by the addition of PRL to the cultures. Since fragmentation of DNA is viewed as a hallmark of apoptosis, this observation suggested that the hormone functioned in this system to suppress cell death.

In previous studies conducted to investigate the mechanism by which PRL inhibited apoptosis in Nb2 cells, we demonstrated that expression of several suppressors of cell death were induced by hormone stimulation. In initial studies, the proto-oncogene, *pim-1*, a highly conserved ser/thr kinase, was rapidly induced by PRL (Buckley *et al.* 1995). Since overexpression of *pim-1* promotes cell survival (Lilly & Kraft 1997), this observation suggested that it may similarly protect Nb2 cells challenged with DEX or other inducers of apoptosis.

In addition to *pim-1*, results from other studies showed that expression of *bcl-2*, the founding member of the Bcl-2 family of apoptosis-associated genes, was rapidly induced by PRL in this T-cell model. Bcl-2 family members are known regulators of apoptosis stimulated by a variety of activators (reviewed by Huang 2000, Zimmermann & Green 2001). Family member proteins can be subdivided into those that facilitate apoptosis and those with anti-cell death properties. Bcl-x_L and Mcl-1 are important anti-apoptotic proteins. In contradistinction, Bax facilitates apoptosis. We have shown that *bcl-2* expression was stimulated by PRL. Interestingly, *bax* was modestly augmented by PRL at the mRNA level in Nb2 cells; however, its protein product was reduced by hormone treatment (Leff *et al.* 1996). In the present study, we have investigated the effects of PRL on the expression of the anti-apoptotic genes, *bcl-x_L* and *mcl-1*. We also assessed whether PRL affected the expression of Bad, an additional pro-apoptotic Bcl-2 family member. The results revealed that while each of the anti-apoptotic genes was expressed in Nb2 cells, only Bcl-x_L was responsive to PRL suggesting that, in addition to Bcl-2, apoptosis suppression induced by hormone exposure likely includes a contribution by this suppressor of apoptosis.

Materials and Methods

Hormones, antibodies and other supplies

Ovine PRL (NIDDK oPRL 20; AFP10677C) was obtained from the National Hormone and Pituitary Program (Bethesda, MD, USA). *mcl-1* and *bcl-x_L* cDNAs and antibodies were kindly provided by John C Reed (The Burnham Institute, La Jolla, CA, USA). The *bad* cDNA was generously provided by Stanley Korsmeyer (Washington University, St Louis, MO, USA). All other chemicals and reagents, unless otherwise indicated, were of molecular biological grade and obtained from Sigma Chemical Company (St Louis, MO, USA).

Cell culture

The PRL-dependent rat pre-T lymphoma cell line, Nb2-11, originally cloned by Dr H G Friesen (Winnipeg, Manitoba, Canada) was obtained from P W Gout (Vancouver, British Columbia, Canada). Nb2-11 cells were maintained at 37 °C in Fischer's medium containing 10% fetal bovine serum (Summit Biotechnology, Ft Collins, CO, USA) as a source of lactogen, 10% horse serum (Summit Biotechnology), 2-mercaptoethanol (2-ME; 10⁻⁴M), penicillin (50 units/ml), and streptomycin (50 µg/ml), as originally described (Gout *et al.* 1980). Cells were rendered stationary by an 18- to 24-h incubation in lactogen-free medium, i.e. Fischer's medium supplemented with 2-ME, antibiotics, and 10% non-mitogenic gelding serum (ICN, Irvine, CA, USA; assay medium).

To determine whether PRL-induced *bcl-x_L* expression reflected a direct effect of the hormone, stationary Nb2-11 cells were preincubated with cycloheximide (CHX; 10 µg/ml) for 15 min prior to hormone stimulation. The effect of protein synthesis inhibition on *bcl-x_L* mRNA was evaluated at 2 h by Northern blotting followed by autoradiography.

To determine whether *bcl-x_L* mRNA was increased in the presence of DEX in combination with PRL, quiescent Nb2-11 cells (2 × 10⁷ cells/ml) were treated with PRL (20 ng/ml) followed immediately by the addition of DEX (100 nM). Cells were harvested at varying time-points through 12 h and evaluated by Northern blotting followed by autoradiography.

Preparation of primary mouse thymocytes

Mice (C57BL/6/J) were killed by rapid decapitation. Thymi were harvested and isolated thymocytes cultured in RPMI-1640 medium supplemented with 1% ITS+ (BD Biosciences, Bedford, MA, USA). Following a 12-h period for stabilization, thymocytes were treated with DEX (100 nM) or PRL (20 ng/ml), then harvested after 2, 4, or 6 h. Expression of *bcl-x_L* mRNA was evaluated by Northern blot analysis.

Northern blotting procedures

Total RNA was isolated from Nb2-11 cells (2 × 10⁷) and quantitated spectrophotometrically. Total RNA was denatured in formaldehyde, fractionated on 1% agarose gels, then transferred to nitrocellulose membranes obtained from Micron Separation Incorporation (Westborough, MA, USA). Equal loading per lane was verified by ethidium bromide staining of 18S and 28S ribosomal RNA which was visualized and photographed under UV illumination. The membranes were hybridized with *bad*, *mcl-1*, or *bcl-x_L* cDNAs labeled with [³²P]deoxy-CTP (New England Nuclear, Boston, MA, USA).

Immunoblot analysis of Bcl-x_L

Stationary Nb2-11 cultures (2×10^7 cells/time point) were incubated with PRL through 24 h. The cells were rapidly cooled and centrifuged, then resuspended in a lysis buffer containing 10 mM Tris HCl (pH 7.4), 0.15 M NaCl, 5 mM EDTA, 1% triton X-100, and 25 µg/ml each of aprotinin and leupeptin. The lysates were centrifuged for 30 min at 14 000 g at 4 °C. Total protein content was determined using the Bradford reagent (BioRad, Richmond, CA, USA). Lysates were fractionated by SDS-PAGE using 10% gels (100 µg/lane) and electrophoretically transferred to Immobilon blotting membrane (BioRad). Membranes were blocked overnight at 4 °C in 5% non-fat dried milk in Tris-buffered saline and then incubated with anti-Bcl-xL (1/1500). Proteins were visualized by chemiluminescence detection using secondary antibodies coupled to alkaline phosphatase followed by exposure to X-ray film for 1–20 min.

Transfection of Jak2 constructs

FDC/Nb2 cells (obtained from L-Yuan Yu-Lee, Baylor College of Medicine, Houston, TX, USA), a pre-myeloid cell line that expresses the intermediate form of the rat PRL receptor, were stably transfected with wild-type Jak2, a Jak2-kinase deficient mutant construct (JK2 VIII; Zhuang *et al.* 1994) or the empty vector (pcDNA3.1; Invitrogen, San Diego, CA, USA, which contains a cytomegalovirus promoter for constitutive expression and a neomycin (G418) resistance gene for selection). Cell transfection was conducted by electroporation using an ECM 600 instrument (300 V/950 F, 20 s; Genetronics Inc., San Diego, CA, USA). The antibiotic selected transfectants were maintained at a low concentration of G418 (100 µg/ml) prior to use in experiments.

To confirm the fidelity of the constructs, exponentially proliferating transfectants were rendered quiescent by lactogen deprivation for 18 h, then stimulated with PRL (20 ng/ml) for 10 min. Cell lysates were quantitatively immunoprecipitated with anti-Jak2 and immunoblotted with anti-phospho Jak2 antibodies (Upstate Biotechnology, New York, NY, USA) to detect the activated Jak2 protein.

Data analysis

Data are presented from experiments replicated at least three times. For all Northern blotting procedures, equal loading per lane was verified by ethidium bromide staining of 18S and 28S ribosomal RNA photographed under UV illumination and subsequently evaluated by densitometry. Where applicable, data are presented as the mean values $\pm$ S.E. Statistical evaluation was performed by ANOVA followed by the Student–Newman–Keuls post-test for multiple comparisons.

Results

PRL induces bcl-x_L but not mcl-1 or bad expression

The effect of PRL on *bcl-x_L* expression was evaluated in Nb2-11 cells in initial experiments. Stationary Nb2 cells were treated with 20 ng/ml PRL, which maximally stimulates proliferation and, in the presence of DEX, inhibits apoptosis (Krumenacker *et al.* 1998). Cells were harvested over a 24-h time-course; the level of the *bcl-x_L* transcript was assessed by Northern blot analysis (Fig. 1). In cells arrested early in the G1 phase of cell cycle (time 0; Fig. 1A), *bcl-x_L* mRNA was nearly undetectable. However, addition of PRL increased the expression of the 0.9 kb *bcl-x_L* transcript within 2 to 4 h to a level more than 20-fold above that observed in untreated cells (Fig. 1B). Following its peak accumulation, the level of its transcript rapidly declined toward control values after 6 h.

To determine whether PRL also increased accumulation of the *bcl-x_L* transcript in the presence of an apoptotic stimulus, stationary cells were treated with DEX (100 nM), a concentration of the steroid that induces profound cell death in this paradigm, in combination with PRL (20 ng/ml) prior to evaluation of gene expression. As shown in Fig. 1C, PRL, in the presence of DEX, augmented *bcl-x_L* expression with a kinetic pattern nearly identical to that observed in cells exposed to PRL alone. Treatment with DEX alone did not increase *bcl-x_L* expression (results not shown). These results therefore demonstrated that PRL, in the presence or absence of an apoptotic stimulus, increases expression of this survival gene.

Since PRL was found to increase the level of *bcl-x_L* mRNA in a time-dependent manner in Nb2-T cells, it was important to determine whether the hormone similarly augmented its protein product. Expression of Bcl-xL was determined in exponentially proliferating and in PRL-treated, stationary Nb2 cells. As shown in Fig. 1D, exponentially proliferating (LOG) cells expressed detectable Bcl-xL as did stationary cultures. The addition of PRL increased its accumulation through 6 h after which it remained unchanged through 12 h. Densitometric analysis of immunoblots revealed that the level of Bcl-xL at 6 h was sixfold ($P < 0.01$) above that determined in untreated controls. Thus, the PRL-stimulated increase in the *bcl-x_L* transcript was coupled to an increase in its protein product. Interestingly, this pattern of mRNA and protein expression of *bcl-x_L* is essentially identical to that observed for *bcl-2* in PRL-treated Nb2 cells (Leff *et al.* 1996).

Despite the observed effects of PRL on *bcl-2* and *bcl-x_L* expression, not all anti-apoptotic Bcl-2 family members are regulated by the hormone. As demonstrated in Fig. 2B, while the Mcl-1 protein was detected in proliferating (LOG), stationary, and PRL-treated cells, its level of expression was not significantly altered by exposure to the hormone at any of the time-points evaluated. Similarly,

Figure 1 PRL increases *bcl-x_L* mRNA expression and protein levels in Nb2-T cells. Quiescent Nb2-11 cells were treated with PRL (20 ng/ml) alone or PRL DEX (100 nM) and harvested at the times indicated. Expression of *bcl-x_L* was evaluated by Northern blotting (20 µg total RNA/lane) as described in Materials and Methods. (A) Upper panel, *bcl-x_L* mRNA; lower panel, ethidium bromide-stained 28S and 18S rRNA. (B) Densitometric analysis of autoradiographs from at least three experiments. Results are presented as means ± s.e. (C) Upper panel, autoradiograph showing effect of PRL+DEX on *bcl-x_L* mRNA expression; lower panel, ethidium bromide-stained 28S and 18S rRNA. (D) Levels of Bcl-xL protein.

the level of its mRNA expression was not significantly influenced by PRL (Fig. 2A).

Since we previously showed that *bax* expression was altered by PRL in Nb2 cells, the effect of PRL on the expression of *bad*, a pro-apoptotic Bcl-2 family member, was determined in other experiments. In contrast to *bcl-2*,

Figure 2 *mcl-1* mRNA expression and protein levels in Nb2-T cells. Proliferating (LOG) or quiescent Nb2-11 cells were treated with PRL (20 ng/ml) and harvested at the times indicated. Isolated total RNA and protein were evaluated by Northern blot analysis (20 µg total RNA/lane) and immunoblotting respectively as described in Materials and Methods. (A) Upper panel, *mcl-1* mRNA; lower panel, 28S and 18S rRNA. (B) Mcl-1 protein.

bcl-x_L and *mcl-1*, *bad* was not detectable at the mRNA level by Northern analysis nor was its protein product observed by immunoblotting under any of the conditions evaluated (results not shown). The reagents utilized in these experiments have been extensively characterized (Andreeff *et al.* 1999). Therefore, of the Bcl-2 family members investigated, PRL augmented expression of the survival proteins Bcl-2 and Bcl-xL (Fig. 1D), and reduced Bax (Leff *et al.* 1996).

Effect of protein synthesis inhibition on PRL-stimulated *bcl-x_L* expression

A characteristic of growth factor-stimulated immediate early gene expression is rapid and protein synthesis-independent activation of transcription. To determine whether *bcl-x_L* represents an immediate early gene stimulated by PRL, its mRNA level was compared between cells treated with PRL alone and those in which CHX (10 µg/ml) was added 15 min prior to PRL stimulation. The cells were harvested after 3 h. As shown in Fig. 3A, *bcl-x_L* mRNA was nearly undetectable in control (stationary; CTL) and in cells treated with CHX alone. PRL significantly increased *bcl-x_L* mRNA expression after 3 h. However, CHX-mediated inhibition of protein synthesis reduced the level of the *bcl-x_L* transcript by more than 50% (Fig. 3B; $P < 0.01$) in cultures treated with PRL. These results indicate that in contrast to the previously demonstrated effects of the hormone on *bcl-2* and *pim-1*

Figure 3 Effect of protein synthesis inhibition on PRL-stimulated *bcl-x_L* expression. Proliferating (LOG) or quiescent (CTL) Nb2-11 cells were treated with PRL (20 ng/ml), CHX (10 µg/ml) or PRL+CHX. Northern blot analysis of *bcl-x_L* expression was conducted as described in Materials and Methods. (A) Upper panel, representative Northern blot; lower panel, ethidium bromide-stained 28S and 18S rRNA. (B) Densitometric analysis of Northern blots from at least three experiments. Results are presented as means ± S.E. **P* < 0.01 vs PRL alone.

expression (Buckley *et al.* 1995, Leff *et al.* 1996), PRL-stimulated *bcl-x_L* reflects direct protein synthesis-independent actions coupled with mechanisms that require *de novo* protein synthesis.

Effect of PRL on *bcl-x_L* mRNA expression in primary thymocytes

Having demonstrated that PRL increased *bcl-x_L* expression in Nb2 cells, it was important to determine whether the hormone stimulated a similar response in primary immune cell cultures. Toward this end, *bcl-x_L* mRNA expression was determined in primary mouse thymocytes treated with PRL (20 ng/ml) or DEX (100 nM). As shown in Fig. 4, *bcl-x_L* mRNA was essentially undetectable in control thymocytes. The addition of PRL increased the *bcl-x_L* transcript through 6 h, while DEX had no effect on its expression. Therefore, similar to the Nb2 cell line,

Figure 4 Effect of PRL on *bcl-x_L* mRNA expression in normal immune cells. Primary cultures of mice thymocytes were prepared from thymi isolated from C57BL6/J mice. After a 12-h incubation period, the thymocytes were treated with PRL or DEX and harvested at the times indicated. Expression of *bcl-x_L* was evaluated by Northern blot analysis (20 µg total RNA/lane). Upper panel, *bcl-x_L* mRNA; lower panel, 28S and 18S rRNA.

PRL-stimulated *bcl-x_L* mRNA may contribute to survival actions of the hormone in untransformed immune cells.

PRL signaling to *bcl-x_L* expression

Signaling of the PRL receptor to activation of Jak2 tyrosine kinase is an important mechanism for gene regulation by the hormone in Nb2 cells and in the mammary gland (Campbell *et al.* 1994, Rui *et al.* 1994). We therefore investigated whether PRL-stimulated *bcl-x_L* expression required activation of the Jak2 tyrosine kinase. For these experiments, the FDC/Nb2 cell line was utilized. This murine pre-myeloid cell line stably expresses the intermediate form of the rat PRL receptor. In these cells, PRL stimulates mitogenesis and the rapid expression of *pim-1* (Borg *et al.* 1999). Cells were stably transfected with wild-type or kinase-deficient Jak2 cDNA constructs. Following selection of the transfectants, stationary cells were treated with PRL and the relative levels of *bcl-x_L* mRNA were determined after 2 and 4 h by Northern blot analysis. As shown in Fig. 5A, PRL (20 ng/ml) increased *bcl-x_L* mRNA at 2 and 4 h in untransfected (FDC/Nb2) and empty vector-transfected (Vector) control cultures. A similar pattern of increased *bcl-x_L* mRNA expression was detected in cells expressing wild-type (WT JAK2) and kinase-deficient Jak2 (KD JAK2) cDNA mutants. To ensure fidelity of the transfections, we also determined the relative levels of activated Jak2 in each of the cell lines. As demonstrated in Fig. 5C, Jak2 was observed to be tyrosyl phosphorylated in empty vector and wild-type transfectants in response to PRL. In contrast, in cells transfected with the kinase-deficient Jak2 mutant, PRL failed to augment Jak2 activation. The effect of AG490 (20 µM), an antagonist of Jak2, was also assessed on PRL-stimulated *bcl-x_L* expression. Pharmacological inhibition of Jak2 did

Figure 5 Effect of Jak2 constructs on PRL-stimulated *bcl-x_L* mRNA expression. Quiescent FDC/Nb2 cells stably expressing Vector, WT JAK2, or KD JAK2 were treated with PRL (20 ng/ml) and harvested after 2 and 4 h. Expression of *bcl-x_L* was evaluated by Northern blot analysis (20 µg total RNA/lane) as described in Materials and Methods. (A) Representative autoradiograph, (B) ethidium bromide-stained 28S and 18S rRNA, and (C) quiescent FDC/Nb2 transfectants were stimulated with PRL (20 ng/ml) and harvested after 10 min. Cell lysates were immunoprecipitated with α -Jak2 and immunoblotted with α -phospho Jak2 or α -Jak2.

not alter *bcl-x_L* levels in cells treated with PRL (not shown). Together, these results suggest that signaling through Jak2 is not likely to be required for increased *bcl-x_L* observed in PRL-treated cultures.

Other experiments were conducted to determine whether alternative PRL-stimulated signaling pathways participated in *bcl-x_L* expression. Activation of p59^{fyn}, mitogen-activated protein kinase (MAPK), c-Jun N-terminal Kinase (JNK), and phosphatidylinositol 3-kinase (PI3K) have also been implicated in the survival actions of PRL, in addition to Jak2 (Clevenger & Medaglia 1994, Buckley *et al.* 1994, Al-Sakkaf *et al.* 1996, Schwertfeger *et al.* 2000, Krumenacker *et al.* 2001). Stationary Nb2-11 cells were treated with pharmacological inhibitors of PI3K (LY294002, 10 µM), JNK (SP600125, 25 µM), p38 MAPK (SB203580, 15 µM), or Src (PP1, 10 µM) for 1 h prior to the addition of PRL. The effect of PRL on *bcl-x_L* expression was subsequently assessed after 4 h. Inhibition of JNK or p38 MAPK signaling did not interfere with PRL-stimulated *bcl-x_L* expression (Fig. 6A). In addition, inhibition of PI3K also did not alter PRL-stimulated *bcl-x_L* expression (data not shown). However, pharmacological antagonism of Src with PP1 significantly reduced *bcl-x_L* levels in PRL-treated cultures (Fig. 6A and B).

Discussion

We have previously shown that expression of Bcl-2 (Leff *et al.* 1996), the ser/thr kinase, Pim-1 (Buckley *et al.*

Figure 6 PRL signaling to *bcl-x_L* mRNA expression. Quiescent Nb2-11 cells were treated with PP1 (10 µM), Sp600125 (25 µM), or SB203580 (15 µM) for 1 h prior to treatment with PRL (20 ng/ml). After 4 h, isolated total RNA was assessed by Northern blot analysis (20 µg total RNA/lane) as described in Materials and Methods. (A) Upper panel, representative autoradiograph; lower panel, 28S and 18S rRNA. (B) Densitometric analysis of four autoradiographs. **P* < 0.05.

1995), and a caspase antagonist, X-linked inhibitor of apoptosis (Krishnan *et al.* 2001) were each increased by PRL during hormone-mediated inhibition of glucocorticoid-induced apoptosis in Nb2-T cells. These observations suggested that survival afforded by PRL most likely reflected contributions of each anti-apoptotic mediator. In the present study, we evaluated whether PRL similarly altered the expression of other Bcl-2 family members, including Bcl-x_L, Mcl-1, or Bad. We have demonstrated for the first time that, similar to its effect on *bcl-2* expression, PRL rapidly increases *bcl-x_L* at the mRNA and protein levels, but not *mcl-1* or the pro-apoptotic family member, *bad*. These results suggest further complexity to the mechanism by which PRL

promotes T-cell survival by implicating additional components of apoptosis regulation.

To date, at least 15 Bcl-2 family members have been identified; each has at least one BH domain (Bcl-2 homology 1–4). Both Bcl-2 and Bcl-x_L can form homo- and heterodimers with other family members including pro-apoptotic Bax and Bad to regulate apoptosis by interactions that involve their BH motifs (Huang 2000, Zimmermann & Green 2001, Martinou & Green 2001). In addition, most Bcl-2 members also express a hydrophobic C-terminal motif that is thought to target the proteins to the outer mitochondrial membrane (Desagher & Martinou 2000). The effects of Bcl-2 family members on mitochondrial cytochrome C release are instrumental in determining cellular fate. Thus, transfection of pro-apoptotic Bax triggers cytochrome C release and subsequent apoptosis (Pastorino *et al.* 1998, Finucane *et al.* 1999), while overexpression of Bcl-2 or Bcl-x_L blocks its release from mitochondria and inhibits caspase activation (Kluck *et al.* 1997, Yang *et al.* 1997). A delicate balance between survival-promoting Bcl-2 family proteins and those that provoke death is therefore required to maintain cell viability.

Deprivation of PRL in Nb2-T cells, similar to the effect of serum starvation in thymocytes, induces apoptosis (Krumenacker *et al.* 1998). In this setting, the level of Bcl-x_L was reduced (Fig. 2A). However, it was significantly increased by the addition of PRL within 4 h. This observation is consistent with the supposition that increased Bcl-x_L expression plays a central role in suppression of cell death by PRL since this time-period coincides precisely with that previously determined for hormonal suppression of apoptosis (Krumenacker *et al.* 1998). Moreover, the glucocorticoid, DEX, is a potent inducer of apoptosis in Nb2 cultures and other T cells (reviewed by Ashwell *et al.* 2000). In DEX-exposed Nb2 cells, PRL again rapidly increased expression of bcl-x_L (Fig. 1C). Thus, in Nb2 cells challenged with two distinct apoptotic stimuli, PRL increased bcl-x_L suggesting that its augmentation contributes to cell survival in hormone-treated cells. A role for bcl-x_L as an anti-apoptotic target for PRL in hematopoietic cells is further supported by the observation that the hormone induced bcl-x_L expression in primary thymocytes under serum-free conditions (Fig. 4).

The mechanism by which PRL receptor activation signals to bcl-x_L expression is not known. However, activation of the tyrosine kinase, Jak2, and its downstream stimulation of one or more signal transducers and activators of transcription (Stat) proteins is a well-characterized mechanism that appears to regulate transcription of several PRL-inducible genes in Nb2 and mammary epithelial cells. Moreover, signaling by this mechanism has been linked to Bcl-x_L regulation in other systems. For example, the Jak2/Stat5 pathway culminating in bcl-x_L expression was implicated in interleukin (IL)-2-mediated lymphocyte

survival (Moriggl *et al.* 1999, Lord *et al.* 2000), while erythropoietin has been shown to transcriptionally up-regulate its expression in erythroleukemia cells via a Stat5 mechanism (Socolovsky *et al.* 1999). In addition, IL-3 caused Stat5 activation leading to increased bcl-x_L expression which was linked to survival of bone marrow cells (Rosa-Santos *et al.* 2000).

To determine whether Jak2 signaling was required for PRL-stimulated bcl-x_L expression, the PRL-responsive myeloid cell line, FDC/Nb2, was transfected with a kinase-deficient Jak2 cDNA mutant, acting as a dominant negative reagent. The results indicated that while PRL-stimulated kinase phosphorylation on tyrosyl residues was reduced by this construct, its expression did not alter the effect of PRL to increase bcl-x_L mRNA accumulation. This observation suggests that a mechanism distinct from the Jak2/Stat pathway is likely coupled to PRL-stimulated bcl-x_L expression. We recently demonstrated that PRL-induced *pim-1* expression appeared to require activation of PI3K and its downstream intermediate, Akt (Krumenacker *et al.* 2001). Since IL-3 signaling through PI3K and Akt have been reported to regulate expression of bcl-x_L in Baf-3 cells (Leverrier *et al.* 1999), it was tempting to speculate that a similar mechanism may be responsible for regulation of its expression by PRL. However, pharmacological inhibition of PI3K did not antagonize the effect of PRL. Instead, inhibition of Src signaling attenuated PRL-stimulated bcl-x_L expression. Therefore, we conclude that PRL suppression of apoptosis reflects its effects on activation of multiple signaling pathways leading to the enhanced expression of survival proteins.

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