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Prolactin-Regulated *pim-1* Transcription

Identification of Critical Promoter Elements and Akt Signaling

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Previously we showed that the distal element (DE) (–427 to –336 bp) within the *pim-1* promoter appeared to regulate its prolactin (PRL)-induced transcription. To determine which specific DE sequences conferred PRL responsiveness, seven 12-bp deletion mutants ligated upstream of the chloramphenicol acetyltransferase gene were transfected into FDC/Nb2 cells. Results from promoter/reporter studies showed that sequential 12-bp deletions of the DE significantly ($p < 0.001$) reduced PRL responsiveness. An additional site, nuclear factor-1 (–224 to –217), was also mutated by deletion or point mutation; both abrogated promoter activation by PRL ($p < 0.0001$). In other experiments, PRL signaling to *pim-1* expression was investigated in FDC/Nb2 cells stably expressing the wild-type (WT) Jak2 cDNA or a carboxy-terminal kinase-deficient Jak2 mutant and in cells infected with adenoviral constructs of WT-Akt or dominant negative Akt. Altered Jak2 did not affect PRL-stimulated *pim-1* expression while inhibition of Akt attenuated its transcription. We conclude that the DE and NF-1 half-site mediate PRL responsiveness of the *pim-1* promoter. Moreover, the accumulated evidence does not support a role for the Jak2/Stat signaling pathway but, instead, implicates that Akt activation was a component of PRL-induced *pim-1* transcription.

Key Words: Prolactin; apoptosis; *pim-1*; Jak2.

Introduction

Over the last two decades, evidence has accumulated suggesting that prolactin (PRL) serves as an immunoregulator. Administration of PRL restored immunocompetence in hypophysectomized rats *(1–3)*; enhanced natural killer and T- and B-cell proliferation in the presence of interleukin-2 (IL-2), *Staphylococcus aureus cowan*, and phytohemagglutinin *(4,5)*; and functions as a cofactor for IL-2-stimulated T-cell proliferation *(6)*.

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Rodent and human lymphocytes synthesize and secrete PRL *(7–11)* and express PRL receptors (PRLRs) on their cell surface *(12,13)*. Based on the amino acid sequence of its extracellular domain, the PRLR has been characterized as a member of the cytokine/hematopoietin receptor superfamily, which includes receptors for numerous interleukins, colony-stimulating factors, interferons, and growth hormone (GH) *(14,15)*. Activation of the PRLR signals through Jak2, a member of the Janus family of tyrosine kinases *(16–18)*. PRL-activated Jak2 phosphorylates STAT (signal transducers and activators of transcription) transcription factors which dimerize, translocate to the nucleus, bind to GAS (γ-interferon activation sequences) sites within the promoter of PRL-responsive genes, and activate gene transcription *(19–22)*.

In addition to Jak2, PRLR signaling has been linked to the activation of other tyrosine kinases, such as $p59^{fyn}$ and ZAP-70 *(23,24)*, and serine-threonine kinases including mitogen-activated protein kinase, protein kinase C (PKC), S6 kinase, and phosphatidylinositol-3-kinase (PI3K) *(25–29)*. Activation of these signaling pathways by PRL may contribute to its co-mitogenic or anti-apoptotic effects in the immune cells.

The PRL-dependent rat Nb2 lymphoma cell line (Nb2-11), developed by Gout et al. *(30)*, is an important paradigm to investigate PRL-PRLR coupling to signaling mechanisms and transcription of survival genes in T-lymphocytes (reviewed in ref. *31*). The parental Nb2 cell line is highly dependent on PRL for proliferation and survival. Culturing the cells in a lactogen-deprived medium for 18–24 h renders them quiescent in the early G1 phase of the cell cycle, whereas prolonged culturing of the cells in the same medium activates apoptosis. Subsequent addition of PRL stimulates cell-cycle progression in a partially synchronous manner and blocks hormone deprivation–induced apoptosis.

We and others have demonstrated that PRL suppresses apoptosis induced by glucocorticoids or PRL deprivation in Nb2-11 cells *(32–35)*. However, the mechanism of antiapoptotic action of the hormone remains to be fully elucidated. To date, PRL has been shown to increase expression of survival genes such as *bcl-2* family members, *XIAP*, and *pim-1*, which likely underlies its suppression of apoptosis *(36–38)*.

The protooncogene *pim-1* encodes a highly conserved serine/threonine kinase that is expressed in myeloid and lymphoid tissues *(39,40)*. Originally identified in malignant

lymphomas, *pim-1* was initially implicated as a regulator of proliferation *(41)*. However, more recent evidence suggests an antiapoptotic role for *pim-1 (42,43)* owing to its effect of blocking mitochondrial dysfunction in a bcl-2-dependent manner during apoptosis *(44)*. Previously, we showed that PRL rapidly stimulates *pim-1* mRNA expression during the early G1 phase of the cell cycle in quiescent Nb2-11 cultures *(36)*. PRL also increased *pim-1* mRNA expression in the presence of dexamethasone, a potent inducer of apoptosis in T-cells *(35)*, suggesting that increased Pim-1 together with enhanced expression of other survival proteins may mediate the antiapoptotic actions of PRL.

In earlier studies, we showed that a distal element (DE) (–427 to –336) and a more proximal element (PE) (–104 to –1) but not GAS or GAS-like sequences were required for PRL-stimulated *pim-1* gene expression *(45)*. We also identified a nuclear factor-1 (NF-1) (–224 to –217) half-site that also appeared to mediate PRL-stimulated *pim-1* expression. The current study was conducted to elucidate PRL response sequences within the DE of the *pim-1* promoter and to investigate whether Jak2 or PI3K participates in PRLR signaling to activation of its transcription.

Results

PRL Signaling Through DE of pim-1 *Promoter*

Our previous studies of the *pim-1* promoter indicated that a DE (–427 to –336) was required for PRL-activated *pim-1* gene expression. To further investigate potential PRL-response elements within the DE, 12-bp deletions were generated (Fig. 1, DE-1 to DE-8) using a –749 bp *pim-1* promoter that contained the DE, PE, and the NF-1 half-site. In addition, identical 12-bp deletion mutants were generated using a DE-100 bp construct, containing only the DE. These constructs were ligated into a chloramphenicol acetyltransferase (CAT) expression vector, cotransfected with a construct containing β-galactosidase (β-gal) into PRL-responsive FDC/Nb2 cells, and stimulated with PRL for 48 h. As shown in Fig. 2A, sequential 12-bp deletions of the DE within the –749 bp construct significantly ($p < 0.001$) reduced PRL responsiveness, illustrating the importance of this sequence in PRL-stimulated *pim-1* gene transcription. Moreover, deletion of the first (DE-1, –427 to –416 bp), third (DE-3, –403 to –392 bp), fifth (DE-5, –379 to –368 bp), or seventh (DE-7, –355 to –344 bp) 12-bp sequences significantly reduced PRL responsiveness compared with the second (DE-2), fourth (DE-4), and sixth (DE-6) deletion constructs. These observations suggest that DE-1, DE-3, DE-5, and DE-7 may represent transcription factor–binding sites that regulate *pim-1* transcription. More important, identical deletions in the DE-100 bp construct did not significantly differ from the wild-type (WT) or each other (Fig. 2B). This suggests that cooperative interactions between the DE and other sites within the –749 bp construct most likely regulate PRL responsiveness of *pim-1* promoter.

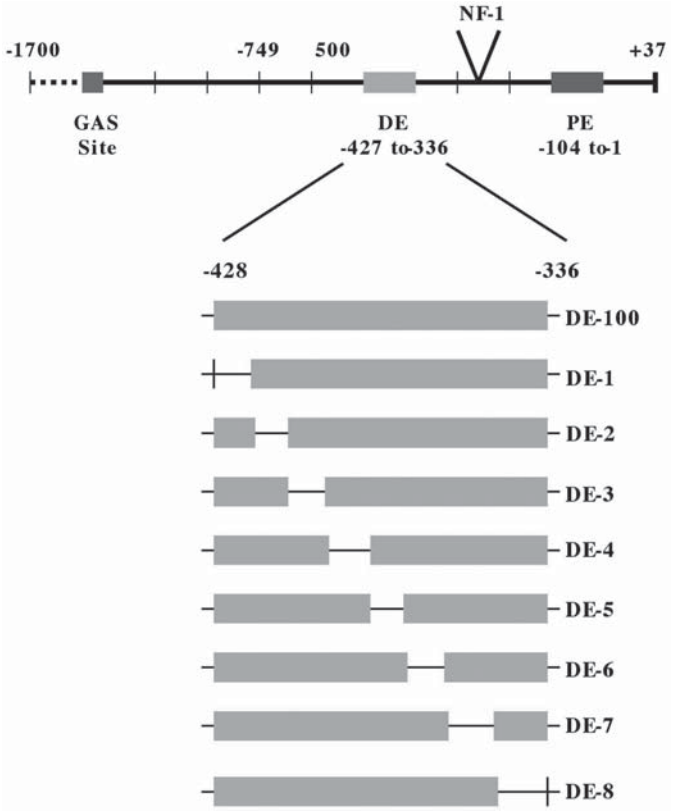

Fig. 1. Schematic diagram of 5' *pim-1* promoter. Deletion mutants of the distal element within the promoter were generated by endonuclease digestion or polymerase chain reaction (PCR) amplification using promoter-specific primers and ligated into the pUC19-CAT expression vector. Nucleotide numbering is relative to the transcription initiation site.

*NF-1: A Component of PRL-Stimulated-*pim-1 *Expression*

Results of DNase I protection analysis of the *pim-1* promoter revealed the presence of an element containing an NF-1 half-site within the *pim-1* promoter (–224 to –217 bp) that appeared to function as a repressor *(45)*. Stimulation with PRL was shown to derepress the NF-1-containing element. To assess further the contribution of this site to PRL stimulation of *pim-1* transcription, mutants of the NF-1 element were generated using –749 or –428 bp constructs, each containing a full-length DE and PE. PRL-stimulated CAT activity was determined following transfection of the constructs into FDC/Nb2 cells. As shown in Fig. 3A, deletion of the NF-1 half-site (GGC, –224 to –222) within the element in the –749 bp construct completely abrogated PRL-stimulated promoter activity ($p < 0.0001$). Similarly, point mutation within the element (G > A, –224 bp) in the –428 bp construct also inhibited the promoter activity ($p < 0.0001$; Fig. 3B), suggesting that the NF-1 site, although a repressor, is required for PRL-stimulated *pim-1* expression.

PRL Signaling to pim-1 *mRNA Expression*

Several reports have suggested that activation of the Jak2/Stat pathway mediates cytokine-stimulated *pim-1* expres-

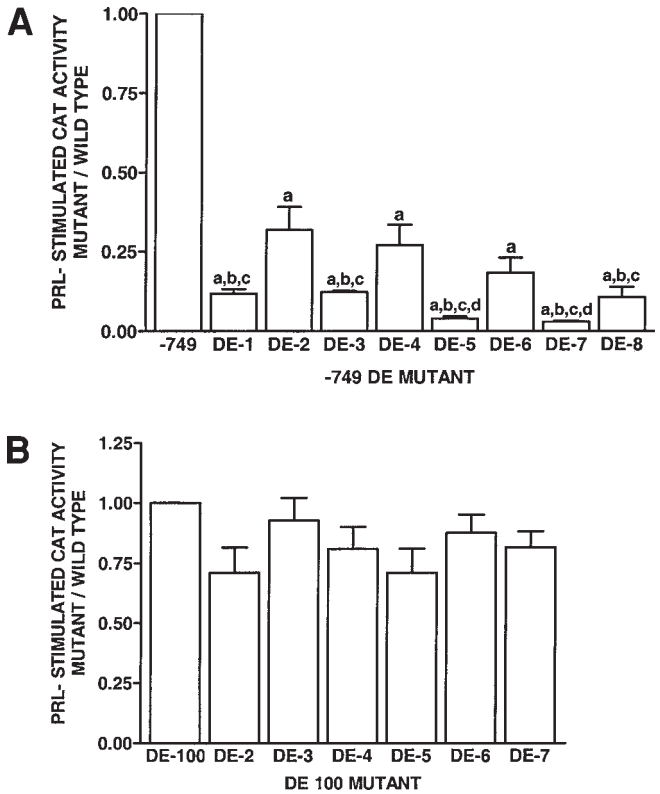

Fig. 2. Effect of PRL on *pim-1* promoter activity. Deletion constructs and a β-gal-containing plasmid were cotransfected into FDC/Nb2 cells. After recovery, cells were cultured in the presence or absence of PRL for 48 h. CAT activity for each of the constructs was determined. (**A**) Sequential 12-bp deletions in the DE were generated in the –749 bp construct of the *pim-1* promoter and ligated into pUC19-CAT expression vector. a, $p < 0.001$ vs –749; b, $p < 0.01$ vs DE-2; c, $p < 0.05$ vs DE-4; d, $p < 0.05$ vs DE-6. (**B**) Sequential 12-bp deletions in the DE were ligated into pUC19-CAT expression vector.

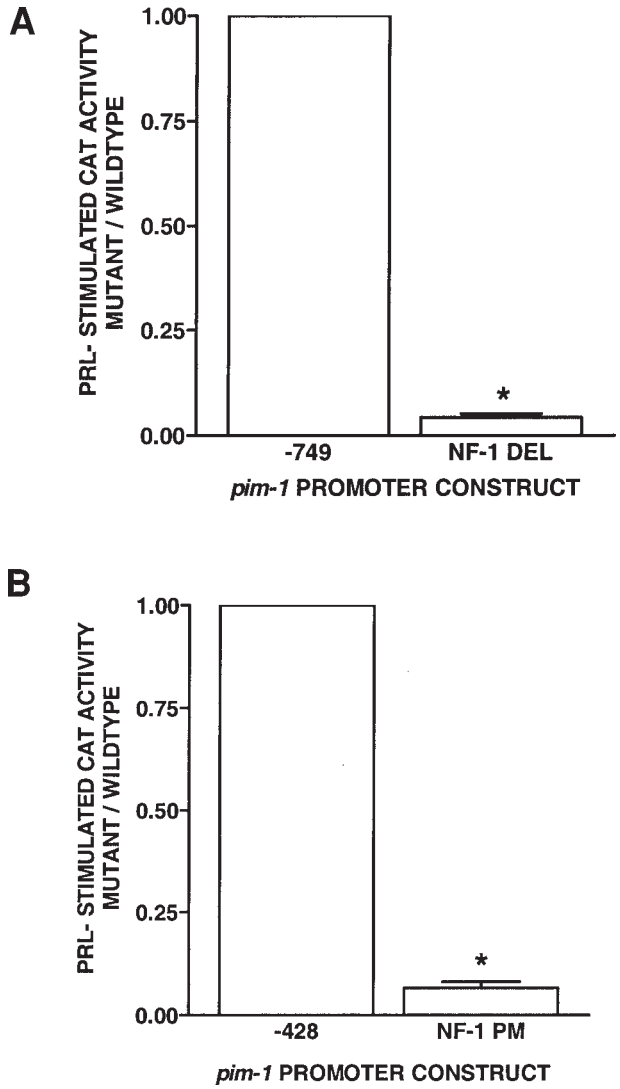

Fig. 3. The NF-1 half-site is required for PRL-stimulated *pim-1* promoter activity. (**A**) Three base pairs were deleted within the NF-1 element (GGC, –224 to –222) in the –749 bp construct using promoter-specific primers. The deletion mutant was ligated into the pUC19-CAT expression vector and co-transfected with a β-gal gene construct into FDC/Nb2 cells. *$p < 0.0001$. (**B**) A point mutation (224 G>A) was introduced into the –428 promoter construct by site-directed mutagenesis, co-transfected with β-gal, and stimulated with PRL, and CAT activity was determined. *$p < 0.0001$.

sion in hematopoietic cells *(46–48)*, although activation of PKC or other downstream transcriptional activators may also be involved *(49)*. Since the Jak2/Stat signaling pathway has been implicated in regulation of several PRL-inducible genes in the mammary gland and in Nb2-T cells, it was of interest to determine whether PRL-activated Jak2 stimulated *pim-1* gene expression. Toward this end, FDC/Nb2 cells were stably transfected with the WT and a kinase-deficient (KD) mutant construct of Jak2, constructs previously shown by Gao et al. *(50)* to effectively block PRL-regulated β–casein gene expression. Stationary transfectants cytotoxic T-lymphocytes (CTLs) were treated with PRL; *pim-1* mRNA expression was determined after 2 h. As shown in Fig. 4A, PRL increased the *pim-1* transcript in untransfected and in FDC/Nb2 cells transfected with the WT construct. Moreover, in cells transfected with the carboxyterminal KD mutant of Jak2, PRL-induced *pim-1* mRNA expression did not differ from that expressed in cells expressing WT or empty-vector constructs. To confirm the fidelity of the constructs, we evaluated PRL-stimulated Jak2 phosphorylation. As shown in Fig. 4C, PRL stimulated Jak2 phosphorylation in empty vector and WT transfected cells. However, phosphorylation of the kinase was reduced in the KD transfectants. Together, these results suggest that Jak2 activation is most likely not a component of PRL-stimulated *pim-1* gene expression.

Previous work, utilizing pharmacologic antagonists of signaling mechanisms, suggested that attenuation of the PI3K reduced PRL-induced *pim-1* expression *(51)*. In addition, Akt (protein kinase B), a downstream effector of activated PI3K, was implicated as a candidate mediator of PRL signaling to *pim-1 (51)*. Thus, to further explore a requirement for the involvement of Akt in PRL-mediated *pim-1* expres-

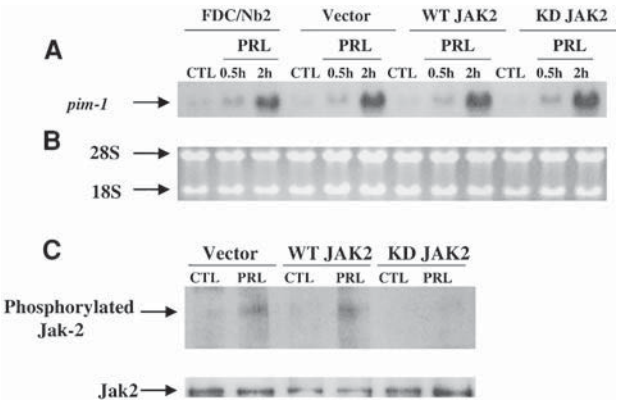

Fig. 4. Effect of a dominant-negative mutant and WT Jak2 on PRL-stimulated *pim-1* mRNA expression in FDC/Nb2 cells. (**A**) Stationary FDC/Nb2 cells transfected with the vector, Jak2 WT or KD mutant cDNAs were stimulated with PRL (20 ng/mL) and cells harvested at times indicated. Northern blot analysis was performed using [^{32}P]-labeled *pim-1* cDNA. (**B**) Ethidium bromide staining of 18 and 28S rRNA photographed under UV illumination. (**C**) Levels of phosphorylated Jak2 in FDC/Nb2 cells transfected with the Jak2 constructs. Stationary FDC/Nb2 cells overexpressing the WT or dominant-negative mutant cDNA of Jak2 were stimulated with PRL (20 ng/mL) and harvested at 10 min. Cell lysates were immunoprecipitated with an anti-Jak2 antibody followed by immunoblotting with an antibody directed toward the phosphorylated form of Jak2.

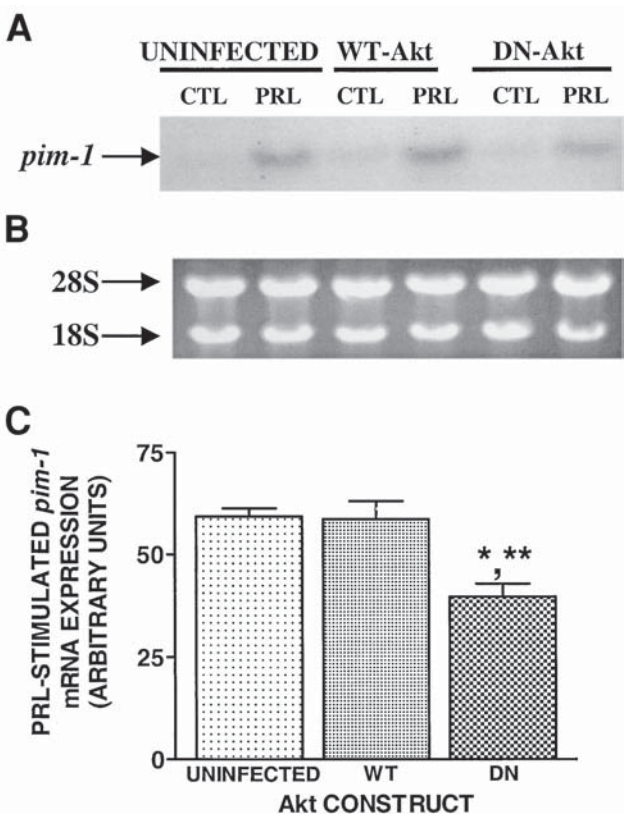

Fig. 5. Role of Akt on PRL-stimulated *pim-1* mRNA expression. FDC/Nb2 cells were infected with conditioned medium containing adenoviral constructs of WT-Akt or DN-Akt. After 60 h, cells were stimulated with PRL (20 ng/mL) for 2 h. Cells incubated in conditioned medium without viral infection were included as controls (uninfected). *Pim-1* expression was determined by Northern blot analysis. (**A**) Representative autoradiograph from an experiment replicated three times; (**B**) ethidium bromide–stained 18 and 28S rRNA; (**C**) densitometric analysis of PRL-stimulated *pim-1* gene expression (*$p < 0.05$, DN vs uninfected; **$p < 0.01$, DN vs WT-Akt).

sion, FDC/Nb2 cells were infected with adenoviral constructs of constitutively active WT and a dominant negative (DN) mutant of Akt. Densitometric analysis of Akt immunoblots was conducted to verify that WT and DN-Akt expression levels were similar. The results presented in Fig. 5 show that expression of DN-Akt significantly reduced PRL-stimulated *pim-1* expression compared to uninfected cells or those infected with the WT enzyme. These results suggest that PRL-stimulated Akt activation is a component of the pathway leading to *pim-1* expression.

Discussion

We previously demonstrated that PRL rapidly elevated *pim-1* mRNA expression and protein translation in Nb2-11 and FDC/Nb2 cells *(36,51)*. We also showed that PRL increased *pim-1* expression in the presence of apoptotic stimuli, suggesting that expression of this kinase contributed to the survival actions of the hormone in hematopoietic cells *(35)*. Moreover, reports by others substantiated the anti-apoptotic effects of *pim-1 (42)*. Therefore, we have employed *pim-1* as a candidate survival gene, to investigate mechanisms underlying PRL signaling to apoptosis suppression.

Previously, the transcriptional regulation of *pim-1* was investigated in the Jurkat T-leukemia cell line by Meeker et al. *(51)*. They identified the DE and PE within the 5' *pim-1* promoter as important regulatory regions for its constitutive expression. Subsequently, we showed that the DE alone was sufficient to drive PRL-stimulated *pim-1* expression *(45)*. Thus, the current study was conducted to determine critical PRL response elements within the DE that may interact with *trans*-acting factors. Our results indicate that sequential 12-bp deletions of the DE within the *pim-1* promoter significantly reduced PRL responsiveness of the promoter, thus suggesting that these 12-bp sequences may be important transcription regulatory elements. Transcription factor recognition sequences within these DE sequences were evaluated using the TRANSFAC database (University of Pennsylvania), which indicated the presence of an Sp1 site within –427 to –416 and –378 to –367 sequences. This is consistent with previous studies in which Sp1 and AP-2 and AP-2 together with a Pim-1 promoter factor –348 were suggested to regulate constitutive *pim-1* expression by binding to the PE and DE *(52)*. Moreover, PRL has been shown to activate Sp1 binding to DNA in Nb2 cells *(53)*. However, in our studies, addition of excess Sp1 oligonucleotides did not compete for protein binding to DE-1 or DE-5 in gel electrophoretic mobility shift assay (EMSA) analysis of the

12-bp sequences, suggesting that additional or alternative transcription factors may be involved (data not shown). It is also interesting to note that the identical deletions in the DE-100 bp construct did not reduce PRL responsiveness, indicating that cooperative interactions most likely exist between the DE and other sites within the *pim-1* promoter.

Our previous promoter-reporter and EMSA studies also identified an NF-1-like element that was derepressed on the addition of PRL *(45)*. The repressive effects of NF-1 on the transcription of myelin basic protein, rat GH, and glutathione transferase genes have been well characterized *(54–56)*. To assess the relevance of this repressor element in PRL-regulated *pim-1* expression, we generated deletion and point mutants of the NF-1 element within the –749- and –428-bp constructs and determined PRL responsiveness of the promoter. Each mutation of the NF-1 element completely abrogated PRL-mediated *pim-1* promoter activation, suggesting that protein binding to this element is most likely required for hormone-stimulated gene expression. This is consistent with a report by Courtois et al. *(57)*, who showed that AP-2 bound to the same site as NF-1 during transactivation of the human GH gene. Thus, it is possible that the NF-1 element, in addition to functioning as a repressor, may regulate *pim-1* transcription by binding alternate transactivators such as AP-2, to facilitate PRL-stimulated *pim-1* mRNA expression. An interaction of AP-2 with the NF-1 element within the *pim-1* promoter warrants further investigation.

The *pim-1* promoter has been shown to contain GAS and several GAS-like sites *(45)*, each representing a potential binding site for Stat transcription factors activated by PRL-stimulated Jak2. However, our earlier results indicated that elimination of the GAS or several GAS-like sequences did not alter PRL-stimulated *pim-1* promoter activation *(45)*, indicating that the Stat family of transcription factors was most likely not required for hormone-induced *pim-1* gene expression. In other studies, pharmacologic inhibition of PI3K, and to a lesser extent Jak2, blocked PRL-stimulated *pim-1* expression *(51)*. To investigate further the potential involvement of Jak2 in this response, we determined PRL-regulated *pim-1* gene expression in cells overexpressing a KD mutant and WT constructs of Jak2. The results of these experiments indicate that hormone-activated Jak2 was not required for *pim-1* transcription, suggesting the involvement of other, additional pathways.

Previously, we showed that PRL activation of PI3K appeared to signal to *pim-1* expression and, thus, suppression of apoptosis *(51)*. Akt (protein kinase B), a downstream kinase effector of PI3K, has been implicated in the anti-apoptotic actions of PI3K *(58)* by a mechanism involving its phosphorylation and inactivation of components of cell death machinery including Bad, caspase-9, and members of the forkhead family of transcription factors *(59–62)*. The results presented here show that inhibition of Akt reduced PRL-stimulated *pim-1* expression by 30% (Fig. 5). The effect of DN-Akt on PRL-induced *pim-1* expression was specific since the expression of other PRL-regulated genes such as *bcl-2* was not affected by the inactive kinase (data not shown). In addition, the relative levels of Akt were verified after transfection and no differences in expression were observed, suggesting that PRL-activated Akt was linked to hormone-stimulated *pim-1* transcription. Since PRL rapidly activates Akt in Nb2 cells *(51)*, we propose that the activated kinase may phosphorylate *trans*-acting factors that subsequently induce *pim-1* expression. Pim-1, in turn, likely phosphorylates its substrates such as suppressors of cytokine signaling, PTP-U2S, cell cycle–associated cdc25A phosphatases, pim-1-associated protein 1, heterochromatin proteins, or NFATc family of transcription factors *(63–68)*. The identification of Pim-1 substrates that interfere with apoptosis in PRL-stimulated cells represents an important avenue for future investigation.

Materials and Methods

Cell Culture

The PRL-dependent premyeloid cell line FDC/Nb2, which stably expresses the intermediate form of the PRLR, was obtained from Li-Yuan Yu-Lee (Houston, TX) and cells were cultured as described *(69)*. Stock cultures were maintained in RPMI-1640 medium containing 25 m*M* HEPES and L-glutamine (Fisher, Pittsburgh, PA) and supplemented with 10% fetal bovine serum and PRL (20 ng/mL) (maintenance medium).

Promoter/Reporter Studies

To identify PRL response elements in the (DE) (–428 to –336) within the *pim-1* promoter, experiments were conducted using a CAT promoter/reporter system. The CAT vector composed of a 1644-bp *Xba*I/*Bam*HI fragment from pCAT-Basic (Promega, Madison, WI) that included the entire CAT gene and SV40 T-antigen sequence was ligated into pUC19 (New England Biolabs, Beverly, MA) using the same restriction enzyme (pUC19-CAT). Deletion mutants of the *pim-1* promoter were generated as previously described *(45)*. Sequential 12-bp deletions in the DE were generated in the –749 DE and DE-100-bp constructs (Fig. 1) of *pim-1* promoter using promoter-specific primers by PCR amplification. To determine the relevance of the NF-1 half-site in PRL-regulated *pim-1* transcription, mutants of the NF-1 element were generated. Three base pairs within the NF-1 element (GGC, –224 to –222) in the –749-bp construct were deleted using promoter-specific primers, and a point mutation (224 G>A) was introduced in the –428-bp construct of the *pim-1* promoter by site-directed mutagenesis. Primers for PCR were designed to include 5'-restriction sites for *Hind*III (+ primer) and *Pst*I (– primer) for subsequent digestion of PCR products and mutants to facilitate ligation into pUC19-CAT vector. All mutants were sequenced using the dideoxy method (University of Cincinnati DNA Core Facility, Cincinnati, OH) to verify sequence and orientation.

CAT constructs (40 μg) and cytomegalovirus (CMV)-β-gal plasmid (30 μg, pCMV β; Clontech, Palo Alto, CA) were co-transfected into FDC/Nb2 cells (1×10^7). Transfection was conducted by electroporation using an ECM 600 electroporator (300 V, 960 μF, 20 s; Genetronics, San Diego, CA). After overnight incubation in RPMI-1640 medium supplemented with 25 m*M* HEPES, L-glutamine, and 1% lactogen-free gelding serum (ICN, Irvine, CA) (assay medium), cells were cultured in the presence of PRL (100 ng/mL). After 48 h, cells were harvested and assayed for CAT and β-gal activities.

Determination of CAT and β-gal

Cell pellets were lysed by three freeze/thaw cycles in ice-cold reporter lysis buffer (Promega), centrifuged (13,000 rpm, 5 min), supernatants assayed for β-gal activities, and heat inactivated (65°C, 10 min) before conducting CAT analysis. To determine β-gal activity, cell lysates were incubated with *o*-nitrophenyl-β-D-galactopyranoside at 37°C for 30 min. Reactions were terminated by the addition of 1 *M* Na_2CO_3 and absorbance at 420 nm was determined. Volumes of cell lysates used for CAT analysis were normalized to the β-gal activities.

CAT activity was determined by incubating the cell lysates for 1 h with [1,2-^{14}C]chloramphenicol (100 μCi/mL, 103 mCi/mmol; ICN, Costa Mesa, CA), acetyl coenzyme A, and 1 *M* Tris-HCl (pH 7.5). Reactions were terminated by the addition of ethyl acetate. CAT activity was determined as previously decribed *(45)*. Data are expressed as CAT activity in PRL-treated cells transfected with deletion mutants normalized to the activity of WT transfectants. Promoterless pUC19-CAT vector ± PRL (negative control), recombinant CAT enzyme (positive control to verify conditions for CAT assay), and transfection with pcDNA 3.1-CAT (positive control for transfection with a constitutive promoter) served as controls.

Northern Blot Analysis

Total RNA was isolated from FDC/Nb2 cells (1.5×10^7) using RNAzol-B (Tel-test, Friendswood, TX), quantitated spectrophotometrically, denatured in formaldehyde, fractionated by agarose gel electrophoresis, and transferred to nitrocellulose membranes (Immobilin Ny+; Millipore). The membranes were hybridized with α-[^{32}P]deoxy-CTP-labeled 743-bp *pim-1* cDNA using methods described previously *(70, 71)*. Equal loading of RNA per lane was verified by ethidium bromide staining of 18S and 28S RNA subunits, which was visualized and photographed under UV illumination.

Immunoprecipitation/Immunoblotting

Subsequent to incubation of stationary FDC/Nb2 cells with PRL (20 ng/mL) for 10 min, cells (1.5×10^7) were washed in ice-cold phosphate-buffered saline (PBS) containing 1 m*M* sodium orthovanadate (Na_3VO_4). Cells were lysed in buffer containing 10 m*M* Tris-HCl (pH 7.4), 0.15 *M* NaCl, 5 m*M* EDTA, 0.1 *M* Na_3VO_4, 1% Triton X-100, 1 m*M* phenylmethylsulfonyl fluoride, and 25 μg/mL each of leupeptin and aprotinin. Total protein concentration in cell lysates was determined by the Bradford method. Cell lysates (285 μg of protein/sample) were immunoprecipitated with α-Jak 2 antibody (Upstate Biotechnology, Lake Placid, NY) at room temperature for 2 h, fractionated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and electrophoretically transferred to a polyvinyl difluoride membrane. Phosphorylated Jak2 was detected using α-phospho Jak2 antibody (Upstate Biotechnology) followed by chemiluminescence detection.

Cell Transfection

Exponentially proliferating FDC/Nb2 cells (1×10^7) were stably transfected with cDNA constructs of WT Jak2 and Jak2-KD (Jak2-VIII) as previously described *(72)* and subjected to antibiotic selection (G418) (CellGro, Herndon, VA). Stable transfectants were maintained in maintenance medium supplemented with 0.1 mg/mL of G418.

To verify the involvement of Akt in PRL signaling to *pim-1* expression, FDC/Nb2 cells were infected with adenoviral constructs of constitutively active Akt (myristylated) or dominant negative mutant (Triple A mutant), generously provided by Dr. Kenneth Walsh (Tufts, University, Boston, MA). Exponentially proliferating FDC/Nb2 cells were incubated with virally conditioned medium (10%) that was previously generated in 293 cells for 48 h. After infection, cells were incubated in lactogen-deprived medium for 12 h and subsequently stimulated with PRL for 2 h, and PRL signaling to *pim-1* expression was determined.

Data Analyses

All experiments were replicated at least three times. CAT activities are represented as mean ± SEM of results obtained from independent experiments. Data are analyzed by analysis of variance followed by student Newman Keuls posttest for multiple comparisons. Representative autoradiographs for Northern blot and immunoblot analysis are presented from experiments replicated four times.

Acknowledgments

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