Prolactin Modulation of Immune and Inflammatory Responses

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ABSTRACT

Prolactin (PRL), a pituitary peptide hormone, is known to regulate diverse physiological functions via its effects on cellular processes such as proliferation, differentiation, and cell survival. All these activities are mediated by the PRL receptor (PRL-R), a member of the hematopoietin cytokine receptor superfamily. To understand PRL-dependent mitogenic signaling in T cells, we cloned PRL, PRL-R, one mediator of PRL signaling, signal transducer and activator of transcription (Stat) 5b, and a panel of PRL-inducible immediate early-response genes from T cells. We are employing one of these PRL-inducible genes, the transcription factor interferon regulatory factor-1 (IRF-1), a multifunctional immune regulator gene, as a tool to understand how PRL modulates T-cell proliferative responses. In investigating regulatory events along the PRL-R/Janus activating kinase (JAK)/Stat/IRF-1 signaling pathway, we show that Stat factors can activate as well as inhibit IRF-1 promoter activity and that cross talk between Stat and nuclear factor (NF)κB signaling pathways also regulates IRF-1 expression. In understanding how signaling pathways cross talk at the IRF-1 promoter, we obtained insights into how PRL can modulate immune and inflammatory responses. These findings have much broader implications, not only for cells in the immune system but also for other PRL-responsive cells and tissues.

I. Introduction

Prolactin (PRL) is a 23-kDa polypeptide that is synthesized primarily in the pituitary. PRL is also synthesized and secreted by many extrapituitary tissues (Ben-Jonathan *et al.*, 1996). Whether endocrine or autocrine, PRL exerts profound effects on a wide range of tissues, with over 300 effects described in vertebrates (Bole-Feysot *et al.*, 1998). PRL regulates the differentiation of secretory glands, including the mammary gland, ovary, prostate, submaxillary and lacrimal glands, pancreas, and liver (for a review, see Horseman, 2001). PRL also regulates proliferation in different cell types, including mammary epithelium, pancreatic beta cells, astrocytes, anterior pituitary cells, adipocytes, and T lymphocytes (Yu-Lee *et al.*, 1990; DeVito *et al.*, 1992; Nanbu-Wakao *et al.*, 2000; Horseman, 2001). We and others have cloned PRL from T lymphocytes,

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where it has been shown to promote proliferation, protect against apoptosis, and enhance cell survival (LaVoie and Witorsch, 1995; Buckley, 2001). Hence, PRL is also known as a T-cell cytokine (Yu-Lee *et al.*, 1998; Montgomery, 2001). How pituitary or extrapituitary PRL modulates target cell function likely depends on the cell type and its stage of differentiation.

As one approach to understanding how PRL modulates T-cell proliferative responses, we cloned a panel of 26 PRL-responsive immediate early-response genes from a rat T-lymphoma cell line, Nb2, induced to proliferate by PRL (Yu-Lee *et al.*, 1990). Nb2 T cells express a high number of PRL receptor (PRL-R), which is a member of the hematopoietin/cytokine receptor superfamily and is exquisitely sensitive to PRL for growth (Gout *et al.*, 1980). Several PRL-inducible genes have been extensively characterized (Stevens *et al.*, 1995; Morris *et al.*, 1997). We focus here on the transcription factor interferon regulatory factor-1 (IRF-1), which plays a pivotal role in multiple immune functions. In understanding the signaling pathway to the IRF-1 gene, we elucidated not only positive and negative regulation but also how various cytokine signals compete/cross talk at the IRF-1 promoter. We will highlight controversies concerning PRL's role in mediating immune, autoimmune, and inflammatory responses, then summarize the renewed interest in evaluating PRL as a hormone or cytokine involved in maintaining immune system homeostasis (Dorshkind and Horseman, 2001).

II. Prolactin and Immune, Autoimmune, and Inflammatory Responses

A. PROLACTIN AND IMMUNE RESPONSES

A large body of literature dating from the 1930s suggests a role of PRL and other pituitary hormones in modulating the immune system (Smith, 1930; Kooijman *et al.*, 1996). Clinical, animal, and *in vitro* studies combine to suggest that PRL exhibits immunostimulatory properties (Yu-Lee, 1997). PRL has been shown to stimulate T cells, B cells, natural killer (NK) cells, macrophages, neutrophils, CD34+ hematopoietic cells, and antigen-presenting dendritic cells (Kooijman *et al.*, 1996; Dogusan *et al.*, 2001; Matera *et al.*, 2001). However, animals with a targeted disruption of either the PRL (Horseman *et al.*, 1997) or PRL-R (Bouchard *et al.*, 1999) gene (knockout, or KO) suggest that PRL is not essential for normal immune system development or function. The KO animals show normal T-cell, B-cell, and NK-cell development and distribution as well as normal T-cell mitogenic responses, B-cell antibody production, and NK-cell-mediated cytotoxicity (Bouchard *et al.*, 1999). A normal immune response to *Listeria* infection involving innate as well as adaptive immune responses is intact in PRL-R KO mice. However, compensatory actions by other cytokines (redundancy) in these KO mice have not been examined.

In Snell dwarf mice that are deficient in anterior pituitary hormones, normal immune responses were observed in animals housed separately from their wild-type littermates (Dorshkind and Horseman, 2000). In contrast, immune defects were observed only in those dwarf animals housed together with their normal littermates, which resulted in a highly stressful environment. The variable housing conditions apparently contributed to conflicting data on the effects of PRL and growth hormone (GH) on immune responses in the dwarf mice. PRL and other pituitary hormones are suggested to act as stress-adaptation molecules important in maintaining immune system homeostasis (Dorshkind and Horseman, 2001). Under stressful conditions, PRL is needed to balance the negative effects of glucocorticoids and other immune or inflammatory mediators to maintain steady-state homeostasis. This interpretation is supported by *in vitro* studies showing PRL's protective effect in preventing glucocorticoid-induced lymphocyte cell death (apoptosis) (LaVoie and Witorsch, 1995; Buckley, 2001) and by *in vivo* studies showing that PRL improves macrophage and splenocyte functions following trauma-hemorrhage and infections (Zellweger *et al.*, 1996). A concerted effort by many laboratories is underway to evaluate the immunomodulatory activities of PRL in the context of stress, trauma, injury, inflammation, infection, and various autoimmune diseases (Matera *et al.*, 2000; Richards and Murphy, 2000; Dorshkind and Horseman, 2001; Hooghe *et al.*, 2001).

B. PROLACTIN AND AUTOIMMUNE DISEASES

Many autoimmune diseases are prevalent in women of childbearing age, most notably, systemic lupus erythematosus (SLE), which occurs more frequently in females than males by a 9:1 ratio. This female gender bias suggests that female hormones (e.g., PRL, estrogen (E2)) may play a role in the pathogenesis of this autoimmune disease. Pituitary PRL expression is under E2 regulation (Couse and Korach, 1999). PRL, in turn, regulates E2 receptor (ER)α and ERβ expression in the female reproductive tissues and the mammary gland (Tessier *et al.*, 2000). Thus, a positive regulatory loop exists between PRL and E2 action. PRL levels are higher in women than men. Elevated PRL levels have been reported in patients with SLE, multiple sclerosis, rheumatoid arthritis, psoriatic arthritis, AIDS, and prior to transplant rejection (Kanik and Wilder, 2000; Jacobi *et al.*, 2001; Walker, 2001). Bromocriptine (BRC), a dopamine agonist that inhibits PRL release from the pituitary, can suppress autoimmune uveitis and correct T-cell and NK-cell abnormalities in patients with pathological hyperprolactinemia (Vidaller *et al.*, 1992). BRC also suppresses SLE in some patients and reduces the number of lupus flares (Walker, 2001). Although a clear causal relationship is still lacking, these clinical data suggest that altered PRL levels may exacerbate certain autoimmune diseases.

A better correlation between PRL and immune regulation is observed in animal models, where circulating PRL levels can be altered by hypophysectomy, BRC treatment, or genetic deletions. Hypophysectomized animals are deficient in mounting various B- and T-cell-mediated immune responses, which are restored by PRL injections. High PRL levels are found in rats with experimentally induced adjuvant arthritis or encephalomyelitis and in the NZB/NZW F1 lupus mice (Kooijman *et al.*, 1996; McMurray, 2001). BRC treatment reduced disease symptoms and delayed lupus-related death, which results primarily from glomerulonephritis (immunoglobulin deposits) in the kidney (McMurray, 2001). Recent animal studies suggest that E2-treated transgenic animals develop a lupus-like phenotype with an expansion in autoreactive B cells (a breakdown of tolerance) and elevation in antibody production (Peeva *et al.*, 2000; Grimaldi *et al.*, 2001). This E2 effect requires the presence of PRL as BRC treatment reduced antibody production (Peeva *et al.*, 2000). Interestingly, both E2 and PRL can upregulate Bcl-2 expression in B cells (Morales *et al.*, 1999; Peeva *et al.*, 2000; Buckley, 2001) and may account for the enhanced survival of autoreactive B cells. Together, these clinical and animal studies support a role of E2 and PRL in modulating lymphocyte functions in the context of various autoimmune diseases.

C. PROLACTIN AND INFLAMMATORY RESPONSES

PRL and E2 have been shown to be protective against inflammation in the context of severe trauma (Jarrar *et al.*, 2000; Knoferl *et al.*, 2000b). Trauma is the fourth leading cause of death in the United States (Zhu *et al.*, 1997). Gender seems to play a role in the response to trauma. Female patients survive better than male patients in response to severe trauma (Morris *et al.*, 1990), supporting the notion that female hormones may protect against hemorrhage and/or septic complications. In male trauma patients, a greater susceptibility to infections is correlated with a higher serum level of proinflammatory cytokines such as interleukin-6 (IL-6) (Offner *et al.*, 1999; Oberholzer *et al.*, 2000). In animal models, trauma-hemorrhage is associated with depressed immune functions and increased infection, morbidity, and mortality (Zellweger *et al.*, 1996). Under this condition, PRL as well as E2 protects against trauma-hemorrhage by reducing plasma levels of corticosterone and IL-6, enhancing splenocyte proliferation and function, and increasing survival of animals to septic shock (Zellweger *et al.*, 1996; Knoferl *et al.*, 2000a,b). These studies show that both PRL and E2 protect against inflammation and improve dysfunctional immune responses under conditions of severe stress. A reciprocal relationship is also found between high serum corticosterone versus low PRL levels after a burn injury (Thellin *et al.*, 2001). In this model of burn-induced stress, the low level of PRL is correlated with

a significant increase in IL-6 production by gut enterocytes, which is accompanied by a loss of gut integrity, bacterial translocation into the circulation, and septic complications (Ogle *et al.*, 2000).

PRL and E2 also inhibit IL-6 gene expression in female reproductive tissues (Deb *et al.*, 1999) and bone (Manolagas, 2000). During pregnancy, IL-6 expression in the decidua is inhibited by E2 and PRL, as increases in IL-6 can lead to termination of pregnancy. Both PRL and E2 downregulate the expression of the gp130 component of the IL-6 receptor complex (Kurebayashi *et al.*, 1997; Deb *et al.*, 1999). In bone, IL-6 is produced by the osteoblast to regulate osteoclast differentiation and bone resorption. IL-6 is thought to contribute to bone loss during menopause (Manolagas, 2000). E2 prevents bone loss in part by inhibiting IL-6 expression in osteoblasts and bone marrow stromal cells (Girasole *et al.*, 1992). E2 also antagonizes IL-6 function by blocking IL-6-inducible signal transducer and activator of transcription (Stat) 3 activity (Yamamoto *et al.*, 2000). Both osteoblasts and bone marrow stromal cells express the PRL-R (McAveney *et al.*, 1996; Goffin *et al.*, 1999), which suggests that PRL may inhibit IL-6 expression in cells in bone marrow. These studies show that PRL and E2 can inhibit IL-6 function at multiple levels, including blocking IL-6 and IL-6 receptor gp130 expression as well as antagonizing IL-6 signaling potential.

Paradoxically, PRL and E2 contribute to hyperplasia and inflammation in the prostate (Tangbanluekal and Robinette, 1993; Leav *et al.*, 1999; van Coppenolle *et al.*, 2001). Transgenic mice overexpressing PRL develop enlarged prostates (Wennbo *et al.*, 1997). Exposure of rats to PRL and E2 results in prostate inflammation, which is characterized by infiltration of lymphocytes and macrophages into the stromal compartment and of neutrophils into the lumen of the dorsolateral lobe of the prostate gland (Tangbanluekal and Robinette, 1993; Stoker *et al.*, 1999; van Coppenolle *et al.*, 2001). The rat dorsolateral prostate is structurally and functionally most similar to the human prostate. PRL appears to be a survival factor (Ahonen *et al.*, 1999) and induces Bcl-2 expression in prostate epithelial cells (van Coppenolle *et al.*, 2001). Interestingly, E2 upregulates IL-6 gene expression (Harris *et al.*, 2000) and IL-6, in turn, induces androgen receptor (AR) gene expression as well as AR function in prostate epithelial cells (Lin *et al.*, 2001). AR is required for PRL expression in the prostate epithelium *in vivo* (Nevalainen *et al.*, 1997). Thus, a complex positive regulatory loop exists among the hormones, cytokines, and their receptors within the prostate. How these interactions promote prostate inflammation, hyperplasia, and cancer progression remains to be elucidated. Although the mechanisms involved are not known, PRL and E2 can be anti-inflammatory or proinflammatory, depending on the cell type, the tissue, and the physiological state of the organ.

D. PROLACTIN AND HEMATOPOIESIS

PRL and GH play a role in stimulating the hematopoietic system (Bellone *et al.*, 1995; Richards and Murphy, 2000). PRL enhances granulocyte/macrophage-colony stimulating factor (GM-CSF)-mediated maturation of CD34+ human hematopoietic progenitor cells into erythroid precursors in culture (Bellone *et al.*, 1995). Pharmacologic levels of PRL increase the hematopoietic progenitors of the myeloid (colony-forming unit-granulocyte macrophage, or CFU-GM) and erythroid (blast-forming unit-erythocyte, or BFU-E) lineages in the bone marrow and spleen, during myelosuppression following treatment for HIV infection or bone marrow transplantation (Richards and Murphy, 2000). PRL also increases the number of progenitors of other immune cell lineages, including T cells, B cells, and NK cells (Bellone *et al.*, 1995). In various diseases, PRL antagonizes the immunosuppressive effects of transforming growth factor-beta (TGF-β) (Richards *et al.*, 1998), tumor necrosis factor alpha (TNFα) (Luo and Yu-Lee, 2000), or corticosterone (LaVoie and Witorsch, 1995; Buckley, 2001) and thus may enhance recovery of the hematopoietic system. In the pregnant maternal uterus, several PRL-like proteins (PLP) interact with immune function cells. The trophoblast-derived PLP-A binds to and inhibits maternal NK cells to ensure successful fetal development (Muller *et al.*, 1999). The placental-derived PLP-E binds to megakaryocytes and promotes their differentiation and maturation, in preparation for accelerated platelet production during pregnancy (Lin and Linzer, 1999). Thus, placental PRL-like hormones play novel roles in regulating hematopoiesis during pregnancy.

In the following section, we will consider some of the mechanisms by which PRL mediates such diverse biological responses. Our studies on PRL signaling to the master immune regulator gene IRF-1 provide some insight into how PRL activates or inhibits gene transcription. These analyses may help to elucidate how PRL can be anti-inflammatory in one tissue but proinflammatory in another or how PRL can exacerbate autoimmune diseases.

III. Prolactin Receptor Signaling

A. PRL-R STRUCTURE AND FUNCTION

The diverse activities of PRL are mediated by the PRL-R, which is expressed on many cell types. Several receptor forms exist, including the long (85–90 kDa) and short (42 kDa) PRL-R, which result from differential splicing of 3′ end cytoplasmic domain exons from a single gene (Goffin and Kelly, 1997). A naturally occurring intermediate PRL-R form (65 kDa) is found in rat Nb2 T lymphoma cells and results from an in-frame truncation in the cytoplasmic domain. Several intermediate PRL-R forms with varying

cytoplasmic domains have been reported in human mammary as well as prostate tumors but their functional significance remains unclear (Clevenger *et al.*, 1995). The intermediate Nb2 PRL-R is more potent than the long PRL-R in both mitogenic (Yu-Lee *et al.*, 1998) and lactogenic (Goffin *et al.*, 1999) signaling. The short PRL-R is suggested to modulate the activity of the long or Nb2 PRL-R by engaging them in heterodimer complex formation, thereby modulating their signaling capacity (O'Neal and Yu-Lee, 1994; Goffin *et al.*, 1999). Whether the short PRL-R has independent functions is unclear. In Nb2 T cells, where PRL-R is abundant (12,000 PRL-R/cell), only 30% occupancy of surface PRL-R is needed to elicit maximal proliferative response (Gertler, 1997).

1. Receptor Motifs

Several motifs in the PRL-R intracellular domain are important for signal transduction. A proline-rich motif (I-F-P-P-V-P-X-P) proximal to the transmembrane domain is critical for interacting with the protein tyrosine kinase (PTK) Janus activating kinase 2 (JAK2) (Goffin *et al.*, 1999). Upon receptor dimerization and JAK2 activation, several receptor tyrosine residues are phosphorylated, presumably by JAK2. Phosphorylated receptor tyrosine residues provide 'docking sites' for the binding of src homology domain 2 (SH2)-containing proteins, including Stat1, -3, and -5; phosphatases; and other adaptor molecules (Shuai, 2000). In the Nb2 PRL-R, the last tyrosine Y382 or its equivalent Y580 in the long PRL-R is important for signaling via Stat5 (Goffin *et al.*, 1999), while both Y309 and Y382 are needed for signaling via Stat1 to an immediate early-response gene IRF-1 (Wang *et al.*, 1997).

2. PRL-R-interacting Proteins

Using the intracellular domain of the PRL-R in a genetic screen, we isolated an enzyme, 2′5′-oligodenylate synthetase (OAS), as a PRL-R-interacting protein (McAveney *et al.*, 2000). Interestingly, OAS is acting more as an adaptor molecule than as an enzyme involved in regulating RNA metabolism. In this unconventional capacity, OAS interaction with the PRL-R reduces Stat1 phosphorylation and DNA-binding activity, which leads to a reduction in IRF-1 promoter activity. In contrast, OAS increases Stat5 DNA binding and β-casein promoter activity (McAveney *et al.*, 2000). Thus, OAS interaction with the PRL-R enhances PRL-mediated differentiated functions. Consistent with this observation, IFNτ-inducible OAS expression is correlated with increased Stat5-mediated differentiated functions in the pregnant ovine endometrium (Johnson *et al.*, 2001).

B. PROLACTIN-INDUCIBLE KINASE PATHWAYS

1. JAK/Stat Pathway

The best-described signaling pathway activated by PRL is the JAK/Stat pathway (Schindler, 1999) that is commonly used by hematopoietin/cytokine receptors. JAK2 is prebound to the inactive PRL-R monomer, in contrast to other cytokine receptors where JAK PTKs are recruited into the receptor complex upon ligand binding (Yu-Lee and Jeay, 2001). Upon PRL binding and PRL-R homodimerization, JAK2 becomes activated and further phosphorylates downstream targets, including tyrosine residues on the PRL-R itself and Stat factors (Goffin *et al.*, 1999). Stat1, -3, and -5 are activated by tyrosine phosphorylation to form homo- (Stat1/1, Stat3/3, Stat5/5) or heteromeric (Stat1/3) complexes, translocate into the nucleus, bind to conserved DNA elements called interferon (IFN) gamma-activated sequence (GAS), and regulate gene transcription. Since all of the components along the JAK/Stat pathway pre-exist in the cytoplasm, PRL-R signaling is initiated within 1–5 minutes by a series of phosphorylation events. PRL-inducible transcription of target genes is detected in the nucleus within 5–10 minutes.

2. Parallel Kinase Cascades

Other PTKs are activated by PRL stimulation, including Fyn, Src, Ras, and Raf, as well as serine/threonine kinases such as ZAP-70, PI3 kinase, Akt, mitogen-activated protein kinase (MAPK), jun kinase (JNK), and protein kinase C (Clevenger and Kline, 2001). Coordination of parallel kinase cascades with the JAK/Stat signaling pathway likely determines specific patterns of gene expression in various PRL-responsive cells and tissues. The pleiotropic actions of PRL on cellular proliferation, differentiation, apoptosis, or cell survival will depend on the interactions among these parallel kinase cascades.

IV. PRL-inducible Signaling Molecules

A. STAT FACTORS

Stat factors are a family of latent cytoplasmic transcription factors that mediate signaling from cytokine receptors (Horvath, 2000; Shuai, 2000). Seven mammalian Stat genes (Stat1–4, -5a, -5b, and -6) have been identified, each encoding a protein $\approx$ 750–800 amino acids in size with conserved functional domains. These include a coiled-coiled domain; DNA-binding domain; linker domain; SH2, a critical tyrosine residue that is important for dimerization, nuclear translocation, and DNA binding; and a carboxyl-terminus transactivation domain (Horvath, 2000). Additional post-translational modification – such as

serine phosphorylation (Decker and Kovarik, 1999; Kovarik *et al.*, 2001), methylation (Mowen *et al.*, 2001), and acetylation (Shankaranarayanan *et al.*, 2001) – further contribute to the ability of Stat factors to regulate gene transcription. Stat1, -3, -5a, -5b, and -6 have naturally occurring splice variants in the carboxyl terminus, generating dominant-negative β isoforms that can bind DNA but lack intrinsic transactivation activity (Horvath, 2000). Stat factors utilize various domains to interact/cross talk with a diverse set of proteins, to transduce signals from the cytoplasm into the nucleus and to regulate gene transcription.

B. STATS INTERACT WITH CYTOPLASMIC PROTEINS

In addition to interacting with components of the cytokine receptor complex, Stats can interact directly with JAK PTK. The coiled-coil domain of Stats (except Stat2) can interact with the cytoplasmic N-myc interacting protein (Nmi) (Zhu *et al.*, 1999), forming a Stat/Nmi complex that enhances Stat transactivation potentials. Other Stat-interacting proteins include Stat3-interacting protein (StIP1), which interacts with both JAK2 and Stat3 (Collum *et al.*, 2000), and protein inhibitor of activated Stats (PIAS) (Shuai, 2000), which downregulates Stat transcriptional activity. Stats also can interact with Src, in one case as an adaptor molecule (Pfeffer *et al.*, 1997) and in another to potentiate Src-mediated cytoskeletal changes in transiently transfected cells (Kabotyanski and Rosen, 2002). Further, in addition to monomers, dimers, and tetramers, Stats can be found in large (i.e., 1- to 4-MDa) cytoplasmic 'statosome' complexes (Sehgal, 2000), which are thought to contain accessory molecules that facilitate Stat recruitment to the receptor complex as well as Stat translocation into the nucleus. Stat1 also interacts in the cytoplasm with the nuclear transport importin α/β complex for transport into the nucleus (Sekimoto *et al.*, 1997). Thus, in the cytoplasm, Stats interact with numerous proteins and acquire signal-transducing capability.

C. STATS INTERACT WITH NUCLEAR PROTEINS

Activated Stat complexes translocate into the nucleus within minutes (Horvath, 2000). Once in the nucleus, Stats interact with nuclear proteins, bind to cognate DNA elements (interferon-stimulated response elements (ISRE) or GAS), and regulate gene transcription. The transactivation potentials of Stats are modulated by interactions with nuclear proteins such as p48 (a member of the IRF family), IRF-1, c-jun, Sp1, Src, nuclear hormone receptors, MCM5 and BRCA1 (Chatterjee-Kishore *et al.*, 2000; Horvath, 2000; Shuai, 2000), and with various coactivators (Collingwood *et al.*, 1999). Coactivators not only facilitate interactions of transcription factors with components of the basal transcription machinery but many coactivators also exhibit intrinsic histone acetyltransferase (HAT) activities, which modify histones and remodel chromatin at promoters,

resulting in transcriptional activation of genes. Stat1 interacts with three regions within the coactivator protein cyclic AMP response binding protein (CBP)/p300 (Horvath, 2000). Interestingly, one of these regions also interacts with Stat5, leading to the speculation that Stat5 competition with Stat1 for binding to CBP/p300 forms one basis for competitive interactions between these two Stats at target promoters (Collingwood *et al.*, 1999; Luo and Yu-Lee, 2000). Thus, coactivators can integrate the activities of DNA-binding proteins to activate gene transcription or can be a target of competitive binding between nuclear factors, which may inhibit gene transcription.

V. Prolactin Regulation of IRF-1 Transcription

As one approach to understanding PRL action in the immune system, we cloned a panel of immediate early-response genes from a rat T-lymphoma cell line, Nb2. One of these genes is the transcription factor IRF-1 (Yu-Lee *et al.*, 1990). IRF-1 is an important immune response mediator. Its regulation by PRL may elucidate a role for PRL in modulating the immune response.

A. IRF-1 AND IMMUNITY

IRF-1 belongs to a small family of nine IRF proteins (Sato *et al.*, 2000). IRF-1 regulates the expression of a number of genes important for mediating antiviral and antibacterial responses, T-helper 1 immune responses, macrophage and dendritic cell function, NK-cell differentiation, cell-cycle progression, and apoptosis (Taniguchi *et al.*, 2001). Thus, IRF-1 plays an important role in mediating host immune defense. In humans, IRF-1 mutations and/or deletions are correlated with a high incidence of leukemias and myelodysplasia (Taniguchi *et al.*, 2001), suggesting that IRF-1 is a tumor suppressor gene. In view of the diverse functions of IRF-1, its unique response to PRL stimulation (Yu-Lee *et al.*, 1990; Stevens *et al.*, 1995), and the ubiquitous expression of the PRL-R on immune function cells (Goffin *et al.*, 1999; Matera *et al.*, 2001), we suggest that PRL, through the JAK/Stat/IRF-1 pathway, modulates the biological activities of many cell types and tissues as well as aspects of the immune response (Yu-Lee *et al.*, 1998).

B. POSITIVE SIGNALING TO IRF-1

Consistent with its multifunctional role in mediating diverse immunological functions, IRF-1 expression is regulated by a wide variety of signals (Taniguchi *et al.*, 2001). PRL stimulates IRF-1 gene expression in normal rat leukocytes derived from the bone marrow and spleen (Dogusan *et al.*, 2000) and in human granulocytes (Dogusan *et al.*, 2001). In rat Nb2 T cells, PRL stimulates IRF-1 gene transcription in a distinct manner over the cell cycle, with a transient but

dramatic 25-fold induction during early G1 and a second peak of induction at the G1/S transition (Stevens *et al.*, 1995). PRL-inducible G1 transcriptional response is mediated by at least three factors assembled at the IRF-1 promoter: inducible Stat1 binding to a GAS element at −120 bp (Stevens *et al.*, 1995), constitutive Sp1 binding at −200 bp (McAlexander and Yu-Lee, 2001b), and protein-protein interaction between Stat1 and the coactivator CBP/p300 (Luo and Yu-Lee, 2000) (Figure 1A). Our working model is that, upon PRL stimulation, activated Stat1 binds to the IRF-1 GAS. Together with the pre-bound Sp1, it forms an enhance-osome (assembly of transcription factors) (Carey, 1998), which recruits coactivators such as CBP/p300 and cofactor required for Sp1 (CRSP) (Ryu *et al.*, 1999), as well as the general transcription machinery for transcriptional activation of the IRF-1 gene. Additionally, chromatin modification has been shown to play an important role in transcriptional regulation. By using chromatin immunoprecipitation (ChIP) assays, more acetylated histone H4 is found to associate with the IRF-1 promoter, indicating a more 'active' chromatin conformation in response to PRL stimulation, concomitant with the increase in IRF-1 gene transcription during G1 (McAlexander and Yu-Lee, 2001a). Thus, a combination of factors – including PRL-inducible Stat1, constitutively bound Sp1, and coactivators with their associated chromatin remodeling HAT activities – coordinate PRL stimulation of IRF-1 gene transcription *in vivo*.

C. NEGATIVE SIGNALING TO IRF-1

Much less is known about signals that shut off IRF-1 gene transcription. In Nb2 T cells, PRL also activates Stat5 to bind as a minor component in the G1 PRL-inducible IRF-1 GAS complex (Wang and Yu-Lee, 1996). Surprisingly, the functional consequence of Stat5 interaction at the IRF-1 promoter is one of transcriptional repression rather than transcriptional activation (Luo and Yu-Lee, 1997,2000). Stat5 does not interact directly with Stat1 (Greenlund *et al.*, 1995) nor does Stat5 compete with Stat1 for binding to the IRF-1 GAS. In transient transfection studies, Stat5 appears to compete with Stat1 for the coactivator p300/CBP via protein/protein interactions to inhibit PRL signaling to the IRF-1 promoter (Figure 1B) (see Section VI). However, the *in vivo* mechanism involved in Stat5-mediated negative signaling to the IRF-1 gene is unclear. *In vivo*, Stat5 can act as a transcriptional repressor. In Stat5a/Stat5b double KO mice, 'increased expression' of genes, including the IRF-1 gene (T. Teixeira, unpublished results), has been observed. This suggests that Stat5 normally represses these genes *in vivo* (Teglund *et al.*, 1998). In the virgin and early pregnant mammary gland, a high level of Stat1 tyrosine phosphorylation (Liu *et al.*, 1996) correlates with elevated IRF-1 gene expression. In contrast, in the late pregnant and fully differentiated lactating gland, a high level of activated Stat5 is correlated with the complete absence of IRF-1 gene expression (T. Teixeira,

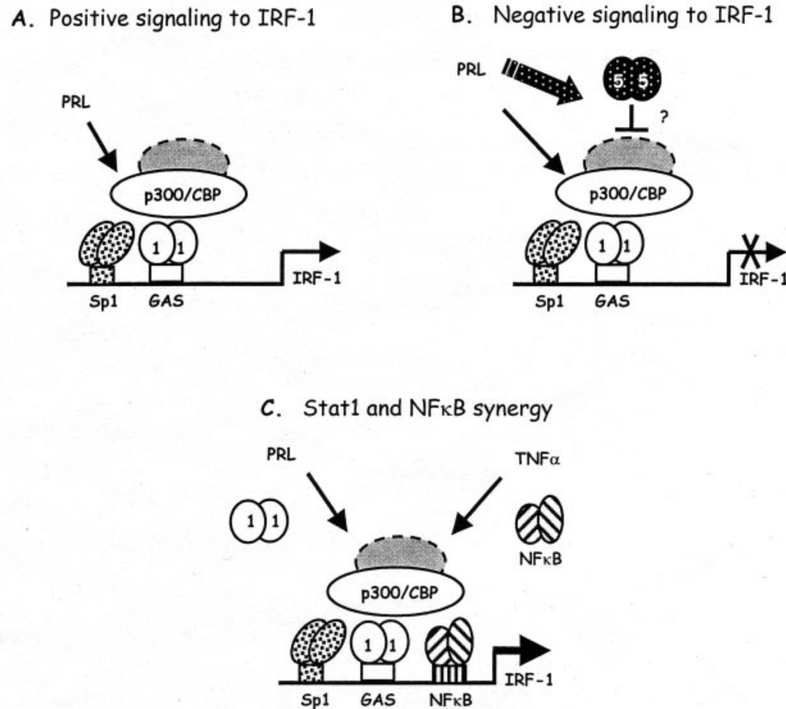

FIG. 1. Model of positive and negative signaling to the interferon regulatory factor-1 (IRF-1) gene. (A) A number of mediators positively regulate prolactin (PRL) stimulation of IRF-1 gene transcription. These include PRL-inducible Stat1, the constitutive factor Sp1, and the coactivator p300/CBP, which enhances Stat1 activation of the IRF-1 promoter. (B) PRL-inducible Stat5 inhibits IRF-1 transcription. Although competition for the coactivator p300/CBP appears to be involved (Luo and Yu-Lee, 1997,2000), the *in vivo* mechanism of inhibition at the IRF-1 promoter is as yet unclear. (C) The IRF-1 promoter can be activated via PRL-inducible Stat1 as well as by tumor necrosis factor alpha (TNFα)-inducible nuclear factor kappa B (NFκB) binding to their respective response elements. In this model of positive and negative cytokine signal cross talk, Stat1 synergizes with NFκB but Stat5 antagonizes NFκB signaling to the IRF-1 promoter. Additional factors that can be recruited by Stats or NFκB to regulate IRF-1 promoter activity are indicated in gray.

unpublished observations). These observations support our model that PRL-inducible Stat5 is involved in negative signaling to the IRF-1 promoter. Other mechanisms for transcriptional shutoff may exist, such as PRL-inducible Stat5 activating a repressor to shut off IRF-1 gene transcription. In this regard, the PRL-inducible suppressors of cytokine signaling (SOCS) proteins can bind to the

PRL-R and turn off signaling at the receptor level in a negative-feedback loop (Naka *et al.*, 1999; Tam *et al.*, 2001).

Interestingly, fewer acetylated histones are associated with the IRF-1 promoter at 4 hours after PRL stimulation, when the transcriptional activity of the IRF-1 gene has returned to baseline (McAlexander and Yu-Lee, 2001a). Thus, a less-active chromatin conformation at the IRF-1 promoter is associated with transcriptional inactivity at the IRF-1 gene. Whether Stat5, co-repressors, and/or histone deacetylase (HDAC) activities are involved in IRF-1 transcriptional shutoff is currently unknown. Our studies show a correlation between the pattern of histone acetylation/deacetylation and biphasic transcription of the IRF-1 gene, implicating histone modification and changes in chromatin structure in PRL regulation of the IRF-1 gene transcription *in vivo*.

VI. Stat5 and NFκB Cross Talk

A. NFκB SIGNALING

The generality of Stat5 acting as a transcriptional inhibitor at the IRF-1 promoter is further illustrated by showing that Stat5 inhibits other signaling molecules that also activate the IRF-1 promoter. One such molecule is NFκB. NFκB initially was identified as a nuclear factor that binds to the immunoglobulin kappa light chain gene enhancer in B cells. It is now known to be widely distributed in all cell types (Israel, 2000; Baldwin, 2001). NFκB was the first transcription factor family shown to reside basally in the cytoplasm but, upon stimulation, translocates into the nucleus to regulate gene transcription. NFκB is comprised of several members, including p65/RelA, RelB, c-Rel, p50, and p52. The most abundant form of NFκB is a heterodimer of p50/p65, which is inducible by a wide variety of signals. In unstimulated cells, NFκB is sequestered in a complex with its inhibitor IκB (Israel, 2000). Upon activation, NFκB is released through IκB turnover, a process that involves IκB phosphorylation, ubiquitination, and degradation via the proteasome pathway. Once in the nucleus, NFκB interacts with multiple factors and the basal transcription machinery to regulate gene transcription.

B. STAT5 ANTAGONIZES NFκB SIGNALING

In addition to the Sp1 and GAS elements that mediate positive PRL signaling, an NFκB site mediates TNFα induction of the IRF-1 promoter (Figure 1C). PRL-inducible Stat1 synergizes with TNFα-inducible NFκB to activate the IRF-1 promoter (Luo and Yu-Lee, 2000). In contrast, PRL-inducible Stat5 inhibits NFκB-mediated signaling to the IRF-1 promoter. Additionally, PRL-inducible Stat5 potently inhibits NFκB-mediated signaling to promoters that

contain only NFκB binding sites. This observation is significant, as it greatly expands potential targets of Stat5 regulation – in particular, Stat5 inhibition. Interestingly, negative cross talk between Stat5 and NFκB is reciprocal in the mammary gland, as NFκB inhibits milk protein β-casein gene expression (Geymayer and Doppler, 2000). This NFκB-dependent inhibition involves a reduction in Stat5 tyrosine phosphorylation in the pregnant gland. We speculate that during mammary gland development, Stat1 and NFκB synergize to activate the IRF-1 gene in the virgin and early pregnant gland, while in the lactating gland, Stat5 coupled with a significant reduction in NFκB levels prevents IRF-1 expression but maximally induces β-casein expression. To confirm our model of positive and negative signaling to the IRF-1 promoter, ChIP assays employing antibodies against Stat1, Stat5, p300 coactivator, and perhaps co-repressors will be used to identify which factors are recruited to the IRF-1 promoter in response to PRL stimulation in a temporally distinct manner to regulate IRF-1 gene transcription *in vivo*.

VII. Positive and Negative Regulation by Stats

In addition to the well-described functions of Stats as positive mediators of cytokine signaling, several lines of evidence now show that Stats can function as transcriptional repressors. Stat1 has been shown to mediate IFNγ-dependent activation or repression of target genes (Ramana *et al.*, 2000). In Stat5a/Stat5b-deficient mice, the expression of some Stat5 target genes is found to be elevated, suggesting a relief of Stat5-mediated repression *in vivo* (Teglund *et al.*, 1998). These findings support the physiological relevance of our observation that Stat5 acts as a transcriptional repressor at the IRF-1 promoter (Luo and Yu-Lee, 2000). Whether Stat5 is acting directly or through a Stat5-inducible factor to repress IRF-1 gene transcription is yet to be determined. We speculate that negative cross talk between Stat5 and NFκB, Smad, or glutocorticoid receptor (GR) could, in part, explain how PRL antagonizes TNFα, TGFβ, or glucocorticoid signaling, respectively, at target genes. It is now known that conformational changes induced by ligand binding to nuclear hormone receptors, coupled with the levels of coactivators or co-repressors present, determine the biological activities of the receptor complex on target gene transcription (e.g., by changing an estrogen antagonist into an agonist) (Lavinsky *et al.*, 1998; McDonnell, 1999). While the mechanistic details are still unclear, transcriptional regulation by Stats is a complex process. Stats can act as transcriptional activators or transcriptional repressors, depending on the promoter context, the concentrations of available coactivators and co-repressors, the presence of other DNA-binding proteins, and the stage of differentiation of the target cell and tissue.

VIII. Concluding Remarks

PRL is a versatile neuroendocrine hormone that also works as a locally produced cytokine. In this capacity, PRL regulates a wide range of physiological responses and a correspondingly wide range of target genes. A large panel of PRL-inducible genes in proliferating Nb2 T cells has been identified (Bole-Feysot *et al.*, 2000). A handful of other genes has been studied to understand PRL-mediated differentiative functions and cell survival responses. Interestingly, a recent study shows fine differences in PRL signaling in different populations of blood leukocytes (Dogusan *et al.*, 2001). PRL activates Stat5 and SOCS3 in peripheral blood mononuclear cells, while PRL activates Stat1 and SOCS2 in human granulocytes. Whether this difference in initial signaling components and target gene activation translates into functional differences in PRL action in these two leukocyte populations remains to be determined. At present, the details of what regulates the qualitative differences in PRL signaling are not understood. It is likely that a combination of steady-state levels, availability and activation of Stats, the levels of coactivators and co-repressors, and even the type of SOCS proteins induced by PRL (Tam *et al.*, 2001) contributes to the tissue-specific or cell-type specific responses to PRL. The challenge is to identify and quantify these differences at the gene and protein levels. A further challenge is to analyze these differences in the context of normal versus pathological states. Future studies will employ a wide variety of approaches to fill in this gap in knowledge. These include DNA microarray; ChIP assays; transgenics overexpressing PRL or mice deficient in PRL, PRL-R, Stat5a, Stat5b or Stat5a/Stat5b; and proteomic technologies. Crossing the PRL-R KO with autoimmune disease strains of mice, for example, will better elucidate how PRL functions as a homeostatic molecule in modulating immune, autoimmune, and inflammatory responses.

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