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Prolactin and growth hormone signal transduction in lymphohaemopoietic cells

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Abstract. The peptide hormones, prolactin (PRL) and growth hormone (GH), are known to regulate numerous target tissues. Among such targets are cells of the immune system, including T cells, B cells, macrophages and natural killer cells. We have cloned a panel of PRL- and GH-inducible T cell genes for use in studies to understand how these hormones through the expression of these genes modulate the biology of immune function cells. This article focuses on the signalling pathways emanating from the PRL receptor (PRL-R) and GH receptor (GH-R), and the expression of PRL-inducible target genes.

Key words. Prolactin; growth hormone; receptor; signal transduction; STAT; IRF-1.

Introduction

Altered prolactin (PRL) levels have been associated with immune dysfunctions in animal models [1] and in certain patients with autoimmune diseases [2]. In parallel, some effects of growth hormone (GH) in the immune system have also been reported. In mice, injections of GH increase thymic size and T cell trafficking into the thymus gland (review in ref. 3). At the cellular level, PRL stimulates T and B cell proliferative responses, T helper 1 [4] and macrophage effector functions [5]. The PRL receptor (PRL-R) appears to be ubiquitously expressed on haematopoietic cells, and its levels can be further increased during T cell activation [6]. Some lymphocyte subsets can synthesize and secrete bioactive PRL, which can act as an autocrine or paracrine factor [7, 8]. GH receptors (GH-Rs) are also present in haematopoietic cells. The human B cell line IM9 has been extensively used as a model system to study GH-R signal transduction [9]. GH-Rs have also been reported in B and T human cells [10].

To understand how PRL and GH act as cytokines, we cloned a panel of genes that are regulated by PRL stimulation from T lymphocytes [11, 12]. Our studies address how PRL regulates target gene expression and what potential functions these genes mediate in T lymphocytes. The transcription factor interferon regulatory factor-1 (*IRF-1*) was cloned by differential screening of PRL-stimulated T lymphoma cell line Nb2 [12]. This multifunctional transcription factor has been used extensively to understand PRL signalling to the *IRF-1* promoter [13–16].

This review first focuses on the general mechanisms of GH and PRL signalling via the JAK/Stat pathway and other signalling molecules. We will then describe in more detail the molecular mechanisms involved in the regulation of the *IRF-1* promoter, in particular positive signalling molecules Stat1 and the coactivator CREB binding protein (CBP) and a negative mediator, Stat5b.

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Structure of the GH and PRL receptors

Both the PRL-R and the GH-R are members of the haematopoietin/cytokine receptor superfamily [17]. All these receptors are single transmembrane receptors sharing homologies in their extracellular part, in particular, two pairs of cysteines and a WSXWS sequence upstream of the transmembrane domain (replaced by the conserved YGE/KFS in the mammalian GH-Rs). No catalytic function is present in their cytoplasmic domains, but two important functional sequences have been identified. Box1 is a proline-rich region located just downstream of the transmembrane domain; it is present in all members of the family. Box2 is a hydrophobic and acidic region located downstream of Box1. Several isoforms of GH and PRL receptors have been described. The soluble circulating form of the GH-R is called growth hormone-binding protein (GHBP). GHBP is identical to the extracellular part of the receptor. It is differently generated among species: alternative splicing in mice and rats [18, 19] or proteolysis of the membrane-bound form of the receptor in other species [20]. Other short but membrane-anchored isoforms of the GH-R have been described in humans [21, 22]. They originate from alternative splicing and their amount is variable among tissues. The PRL-R exhibits three membrane-bound isoforms in rodents, the long form [591 amino acids (aa)], the Nb2 form (393 aa) and the short form (291 aa) [23]. In the thymus, both long and short forms are expressed equally. In addition, the hormonal environment associated with the oestrus cycle, pregnancy or lactation modifies the relative expression of the isoforms of the PRL-R [24].

Signalling pathways activated by GH and PRL

The JAK/Stat pathway

Our understanding of how PRL and GH signal to target cells was aided by studies of the interferon (IFN) receptor signalling pathway [25] (fig. 1). Upon ligand binding, PRL or GH receptors homodimerize, leading to transphosphorylation of receptor-associated JAK2 tyrosine kinase and an increase in its kinase activity [26]. Activated JAK2 in turn phosphorylates tyrosine residues on the receptor cytoplasmic domain, which provide 'docking sites' for the recruitment of SH2-containing signalling molecules [27]. These include a family of latent, preexisting transcription factors called 'signal transducers and activators of transcription' (Stat) [25]. Stat factors are recruited to the receptor, become tyrosine-phosphorylated, form homodimers or hetero-complexes, translocate into the nucleus, bind to cognate DNA elements and regulate gene transcription. Recent studies have shown that the transcriptional activities of Stat factors can be further modulated by serine phosphorylation [28] and by interactions with other DNA-binding proteins [29] and non-DNA-binding coactivator proteins [30, 31].

For GH and PRL receptors, most pathways initiated by ligand binding appear to require as a first step JAK2 activation. Furthermore, this seems to be sufficient to mediate GH effects such as GH-induced proliferation and the activation of molecules involved in this process [32, 33]. The membrane proximal part of GH [34] and PRL receptors [35, 36] are sufficient to induce JAK2 activation. Other GH effects require additional sequences in the GH-R cytoplasmic domain. This is the case for GH-dependent transactivation of the insulin gene [37] or the serine protease inhibitor (*Spi*) 2.1 gene [38]. In contrast, the GH-dependent effect on calcium entry [39] as well as internalization of the receptor [40] appear to involve mechanisms independent of JAK2 activation. This predominant role of JAK2 tyrosine kinase is also true for the PRL receptors in Nb2 cells [41].

GH was reported to activate Stat1 in prolymphoma BaF3 cells [42] but not in IM9 cells [43]. GH-dependent Stat3 activation was also found [44], and both Stat5 isoforms are activated in FDCP1 [45] cells. PRL activates Stat1, Stat3 and Stat5 in Nb2 cells [46].

For the GH-R, the receptor domains which are required for Stat activation differ among Stat proteins. Activation of Stat1 and 3 only required JAK2 activation but no specific tyrosine residue in the GH-R cytoplasmic domain [33]. In contrast, the full activation of Stat5 by

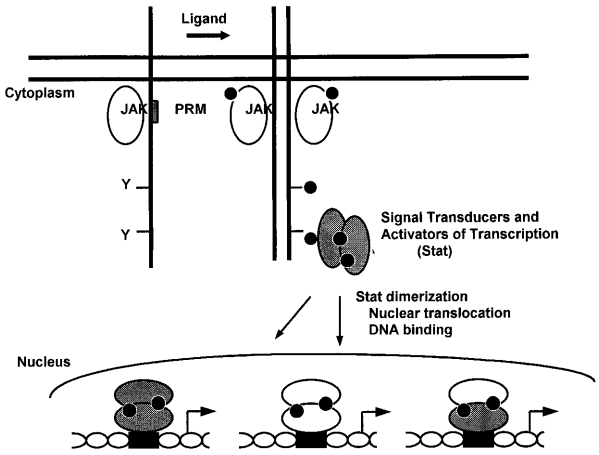

Figure 1. PRL and GH receptor signal transduction through the JAK/Stat pathway. See text for discussion. JAK, Janus kinase; Stat, signal transducer and activator of transcription.

GH is dependent on phosphorylated tyrosine residues in the C-terminal part of the receptor, and a direct association of Stat5 with the C-terminal part of the receptor was shown in vitro [47]. Several tyrosine residues can alternatively bind Stat5a and Stat5b in the cytoplasmic domain of GH-R [48–50]. Several tyrosine residues can also bind Stat5 in the PRL receptor [51]. The predominant role of Stat5 in PRL-mediated effects on mammary gland was also supported by the studies on the phenotype of mice in which Stat5a was inactivated [52]; in these mice, the lactation and breast development defects were similar to what was observed in mice in which the PRL-R was inactivated [53].

Other pathways activated by GH and PRL

GH and PRL receptors have been reported to activate the MAP kinase (MAPK) pathway [34, 54, 55]. This activation could involve SHC, Grb2, Son-of-sevenless (Sos), Ras, Raf and MAP kinase kinase (MEK) [56], but also the PI-3 kinase [57] or PKC activation. The membrane-proximal part of the GH-R cytoplasmic domain including Box1 is sufficient to induce MAPK activation [34]. A recent report suggests that MAPK activation by GH could also be mediated through the EGF receptor, which after phosphorylation by JAK2, would serve as a docking protein for SHC and Grb2 [58]. MAPK substrates include other kinases like Raf-1 or the S6 kinases, the phospholipase A2, some cytoskeletal proteins or transcription factors like c-jun and ternary complex factor or elk1 (see review in ref. 59).

The substrate of the insulin receptor, IRS-1, has been reported to be tyrosine-phosphorylated and associated with GH and PRL receptors [60, 61]. IRS-2 was also shown to be associated with GH-R complexes [62]; the regions of the receptor required for tyrosine phosphorylation of IRS-1/2 are the same as the one required for JAK2 activation. Tyrosine-phosphorylated residues in IRS-1 and IRS-2 provide binding sites for SH2-containing proteins, among them the p85 subunit of PI-3 kinase. PI-3 kinase is implicated in several pathways, including glucose uptake, DNA synthesis, cell cycle regulation via p70 rsk activation [63] and inhibition of apoptosis via Akt serine threonine kinase [64]. PI-3 kinase is activated but not tyrosine-phosphorylated under GH stimulation, whereas it is tyrosine-phosphorylated after stimulation by PRL [61].

Except for JAK2, no other tyrosine kinase has been shown to be involved in GH signal transduction. However, Fyn seems to participate in PRL-R signalling. Fyn is stimulated by PRL in rat Nb2 lymphoma cell line and binds to the PRL-R [65].

PKC may play a role in GH-induced signal transduction [66–68]. Ca^{2+} increase by GH has been reported in IM-9 lymphocytes [69]. This effect seems to be dependent on L-type calcium channel activation by a mechanism that includes phospholipid hydrolysis and PKC activation. Ca^{2+} seems to be important for activation of some GH-induced genes such as *Spi2.1* [39]. Mutagenesis experiments suggest that calcium activation may not require Box1 but only the C-terminal part of GH-R. A possible link between PKC activation and JAK2 activation is not determined. In fact, PI-3 kinase activation is dependent on JAK2, and the byproducts of PI-3 kinase catalytic activity have recently been shown to activate calcium-independent PKC isoforms [70]. A role for PKC isoforms has also been reported in PRL-R signal transduction [71].

Regulation of GH- and PRL-signalling pathways

Several types of mechanisms could be involved in the downregulation of GH and PRL signalling. (i) Ubiquitination of the GH-R is required for internalization and degradation [72]. (ii) The role of phosphatases in GH-R downregulation is not completely demonstrated. We and others have reported that truncation of the GH-R carboxy-terminal part induces persistent phosphorylation of JAK2 [47] and of some JAK2 substrates, in particular Stat3 [44] and IRS-1 [73]. It was therefore speculated that some molecule interacting with this part of the receptor was able to downregulate the signal. SHP-2 interacts with the GH-R, but it could be more a positive rather than a negative regulator of GH-R signalling [74]. SHP-2 was also found to be a positive regulator of PRL signalling [75]. SHP-1 associates with JAK2 [76], and SHP-1 dephosphorylates JAK2 [77]. In mice defective in SHP-1, an increase in the tyrosine phosphorylation of JAK2 as well as of Stat proteins was observed [45]. SHP-1 also migrates to the nucleus under GH stimulation, where it dephosphorylates Stat5b [76]. (iii) Stat inhibitors which were termed SOCS, in particular SOCS-3 and CIS are inducible by GH and seem to inhibit GH-dependent activation of *Spi2.1* [78].

Cloning of new GH-R-associated molecules

In order to identify new molecules involved in GH-R signal transduction, we used the tyrosine-phosphorylated C-terminal part of the GH-R as a probe to screen an expression library. Six independent clones were identified which all encoded for proteins with SH2 domains. Among these, we identified Grb10 as a GH-R-associated protein (J.F., unpublished data). Grb10 was essentially reported as an insulin- and IGF1-associated

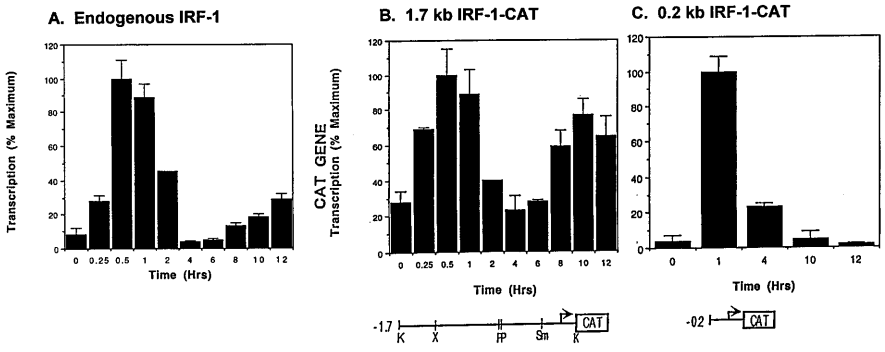

Figure 2. PRL stimulates biphasic transcription of the *IRF-1* gene in Nb2 T cells. (*A*) Transcription rate of the endogenous *IRF-1* gene in response to PRL stimulation across a 24-h cell cycle in Nb2 T cells. (*B*) Transcription rate of a transfected 1.7-kb *IRF-1-CAT* reporter gene across a PRL induction time course in stably transformed Nb2 T cells. The *IRF-1-CAT* reporter gene contains *IRF-1*-promoter/flanking DNA and 180 bp of *IRF-1* exon I sequences which are cloned into the *pBLCAT3* reporter construct as described [13]. (*C*) Transcription rate of the 0.2-kb *IRF-1-CAT* gene as in (*A*). See ref. 14 for details.

protein. It could play a role in growth factor-regulated cell proliferation [79]. Studies are in progress to determine the potential function of this protein in GH-R signal transduction.

Expression of PRL-inducible target genes

IRF-1

The rat Nb2 T cells provide a useful system to study PRL-stimulated early activation genes [11, 80]. These cells represent immature T lymphocytes [81] and express an unusually high number of PRL-Rs [82], which underscores their sensitivity to PRL for growth. Nb2 T cells can be made quiescent by culturing in PRL-deficient medium for 24 h, and then stimulated to grow in a synchronized manner with 1 to 10 ng/ml of PRL. Under these conditions, we cloned *IRF-1* as one of the most PRL-inducible genes in Nb2 T cells [12].

IRF-1 is a multifunctional transcription factor that is expressed in many cell types in response to cytokine [83–85] and developmental cues [86]. In T lymphocytes, *IRF-1* is critical for Th1 development and responses [87, 88] as well as for both proliferation and apoptosis of T cells [89, 90]. In Nb2 T cells, *IRF-1* is induced by PRL stimulation as part of an early G1 activation event (fig. 2). Within 15 min of PRL stimulation, the *IRF-1* transcription rate is rapidly induced, reaches a maximum 20-fold at 1 h and declines to basal levels by 4 h (fig. 2A). This rapid and transient induction is followed by a second rise in the *IRF-1* gene transcription rate between 8 and 12 h during early S phase. Interestingly, the biphasic transcription pattern of the *IRF-1* gene is mimicked by a transfected *CAT* reporter gene that is driven by 1.7-kb 5′-flanking *IRF-1* promoter DNA. This suggests that the 1.7-kb *IRF-1*-flanking DNA contains elements that mediate both G1 and S-phase transcriptional responses (fig. 2B). In contrast, a *CAT* reporter gene containing only 0.2-kb *IRF-1*-promoter DNA only mediated G1 induction by PRL and failed to promote S-phase transcription (fig. 2C). Within the 200-bp *IRF-1*-promoter region is a palindromic sequence, TTTC NNN GAAA, called IFNγ activated sequence, or GAS [25, 91, 92]. Mutations of this single GAS element within the context of the 1.7-kb *IRF-1*-promoter abrogated promoter response to PRL stimulation, as tested by cotransfection of the mutant *IRF-1* promoter with the Nb2 *PRL-R* construct into COS cells (referred to as the COS transfection system) [15]. Electrophoretic mobility shift assays (EMSA) using the GAS oligo as a probe showed that nuclear factors bound to the GAS in a PRL-dependent manner [14]. Antibody supershift experiments showed that Stat1 is a major component and Stat5 is a minor component of the G1 *IRF-1* GAS complex [93]. Thus, GAS is a critical element in mediating PRL signalling to the *IRF-1* promoter, and GAS interacts with Stat1 and Stat5 in a PRL-inducible manner.

Stat1 and CBP: positive regulation

The functional significance of Stat1 and Stat5 interactions at the *IRF-1* promoter was tested by cotransfecting either *Stat1* or *Stat5* into the COS transfection system. COS cells contain endogenous levels of Stat1 but very low to undetectable levels of Stat5. Overexpression of Stat1 further enhanced PRL activation of the *IRF-1* promoter, suggesting that Stat1 is a positive mediator of PRL signalling to the *IRF-1* promoter [15]. Stat factors can interact with other proteins in regulat-

ing gene expression [29–31, 94]. One such factor is the coactivator CBP [95], which does not bind DNA but can enhance the activities of multiple DNA-binding proteins through protein-protein interactions at gene promoters. To address whether CBP can enhance Stat1 activity at the IRF-1 GAS, a heterologous thymidine kinase *TK-CAT* reporter gene containing three copies of the IRF-1 GAS (*3C GAS-TK-CAT*) was tested for its ability to respond to PRL stimulation. Three copies of GAS conferred a 4- to 5-fold PRL inducibility to the *TK* promoter, which was further enhanced to more than 15-fold by CBP (data not shown). Thus, CBP enhances PRL-inducible Stat1 activity at the *IRF-1* GAS.

Stat5: negative regulation

To address the functional significance of Stat5 at the *IRF-1* promoter, *Stat5* was transfected into the COS transfection system. Unexpectedly, Stat5b did not further enhance but instead inhibited PRL activation of the *IRF-1* promoter (fig. 3A). This inhibition is specific, as Stat5b is required to activate another PRL-inducible promoter, the *β-casein* promoter, in our COS transfection system (fig. 3B). Thus, Stat5b can act as a transcriptional repressor or activator, depending on the target promoter. To determine which part of Stat5b might be involved in repressing the *IRF-1* promoter, several Stat5b mutants were examined in the COS transfection system. One is missing the first 46 amino acids at the amino terminus (Stat5b*Δ*46N) [16], one is missing the last 40 amino acids of its carboxy terminus transactivation domain (Stat5b*Δ*40C) [96] and a third contains mutations in the conserved VVVI residues in the DNA-binding domain [16]. The carboxy-terminus Stat5b deletion mutant was no longer able to inhibit the *IRF-1* promoter (fig. 3C). The inhibitory effects of the amino-terminus Stat5b deletion were reduced, allowing reproducibly some recovery of PRL-inducible *IRF-1* promoter activity. Interestingly, the Stat5b DNA-binding mutant can still inhibit the *IRF-1* promoter as effectively as wild-type Stat5b. Thus, both amino and carboxy termini of Stat5b are involved in inhibition of the *IRF-1*, but the DNA-binding property of Stat5b is not required for its inhibitory function at the *IRF-1* promoter. Our interpretation is that Stat5b is not competing with Stat1 for binding to the *IRF-1* GAS. Instead, Stat5b and Stat1 are competing for a factor which is necessary for PRL activation of the *IRF-1* promoter. Our results further suggest that this factor may be necessary for Stat1 to mediate PRL activation of the *IRF-1* promoter.

Positive and negative regulation at the *IRF-1* promoter

A working model of PRL signalling to the *IRF-1* promoter is proposed (fig. 4). PRL-inducible Stat1 acts as a positive mediator of PRL signalling to the GAS element in the *IRF-1* promoter. Stat1 activity is enhanced by interactions with the coactivator CBP. On the other hand, PRL-inducible Stat5b acts negatively to inhibit PRL signalling to the *IRF-1* promoter. We speculate that Stat1 and Stat5 are competing for a factor, such as a coactivator, which is necessary for Stat1 activation of the *IRF-1* promoter. We further suggest that the relative concentration and kinetics of nuclear translocation of Stat1 vs. Stat5 may contribute to the rapid up- and downregulation of *IRF-1* gene transcription in PRL-stimulated Nb2 T cells. Thus, Stat factors can act as transcriptional activators or transcriptional repressors, depending on the target promoter, the presence of coactivators as well as corepressors [97], and promoter-specific DNA-binding proteins.

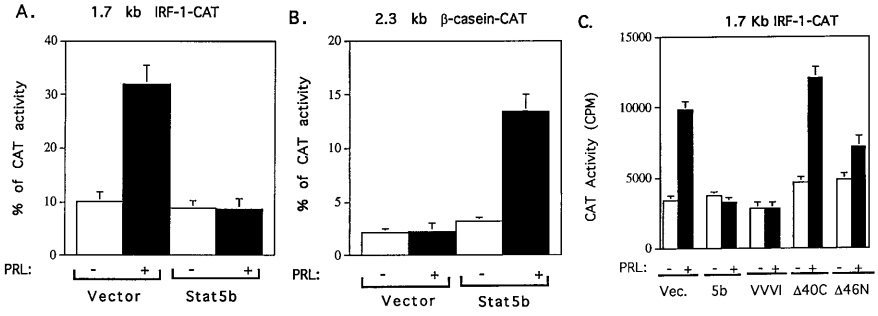

Figure 3. Stat5 inhibits PRL activation of the *IRF-1* promoter. COS cells were transiently cotransfected with the Nb2 *PRL-R* construct, either vector alone or *Stat5b* expression vector and either 1.7-kb *IRF-1-CAT* (*A*) or 2.3-kb *β-casein-CAT* (*C*), stimulated for 24 h with 100 ng/ml of PRL, and assayed for CAT enzyme activity as described [16]. Data are summarized from six independent experiments, each assayed in triplicate.

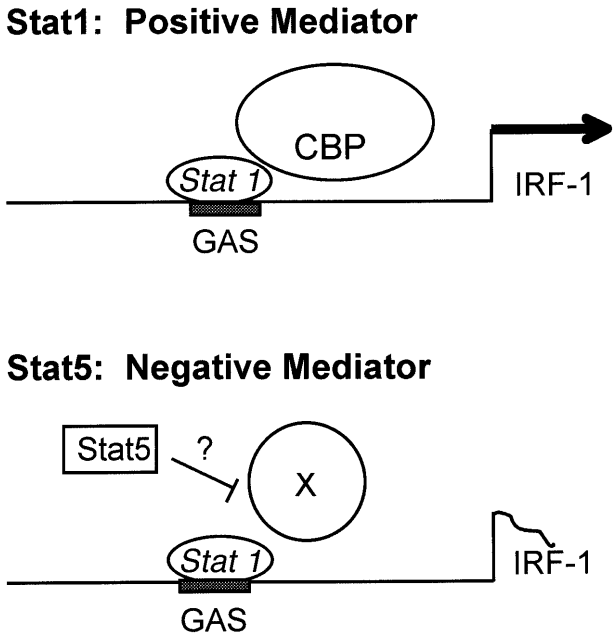

Figure 4. PRL and Stat regulation of the *IRF-1* promoter. Stat1 and the coactivator CBP synergize at the *IRF-1* GAS element to activate *IRF-1* gene expression in response to PRL stimulation. In contrast, Stat5 antagonizes a factor that is critical for PRL activation of the *IRF-1* promoter.

IRF-1 as a mediator of PRL signalling in immune responses

IRF-1 is involved in mediating a number of immune processes (fig. 5) (see references in ref. 98). IRF-1 regulates the expression of the apoptotic gene *ICE* in mature T lymphocytes [90, 99]. IRF-1 also regulates the expression of genes (*TAP1*, *LMP2*) involved in MHC class I expression and thus antigen presentation as well as $CD8^+$ T cell development in the thymus. IRF-1 also regulates interleukin (*IL*)-*7Rα* expression and thus is important for B and T cell differentiation. In macrophages, IRF-1 regulates the expression of inducible NO synthase, which is crucial for mediating macrophage effector functions. IRF-1 is critical for the induction of NK cell-mediated cytotoxicity [100]. Recent *IRF-1* knockout animal studies have shown that *I*RF-1 regulates the expression of the Th1 cytokine IL-12p40, which is critical for the development of Th1 responses [87, 88]. The ubiquitous presence of PRL-R on haematopoietic cells and the robust induction of *IRF-1* gene transcription in response to PRL stimulation provide a means by which to evaluate how PRL, through the JAK/Stat/IRF-1 pathway, may modulate these immune responses. These studies, along with studies of the *PRL* [101] and *PRL-R* knockout animals [53] and hyperprolactinaemic animal models of autoimmune diseases [102], will allow a better understanding of the immunoregulatory properties of PRL.

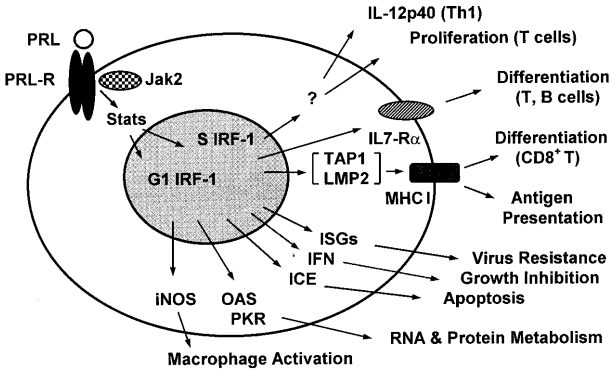

Figure 5. IRF-1 as a central mediator of immune responses. The multiple roles of IRF-1 in modulating the immune response are derived from different cell types, including fibroblasts, T and B lymphocytes, macrophages, and from studies of *IRF-1*$^{-/-}$ knockout mice. See text for discussion.

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