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Prolactin activates the interferon-regulated p91 transcription factor and the Jak2 kinase by tyrosine phosphorylation

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ABSTRACT **The prolactin (PRL) receptor is a member of the family of cytokine receptors that lack intrinsic tyrosine kinase activity but contain two conserved cysteines in their N-terminal regions and a WSXWS motif adjacent to their transmembrane domains. In a manner similar to the interferons (IFNs), exposure of cells to PRL results in tyrosine phosphorylation of several cellular proteins and the rapid transcriptional induction of the IFN regulatory factor 1 gene. In this communication, we demonstrate that treatment of rat Nb2 lymphoma cells with PRL activates a latent protein factor so that it binds to an enhancer in the IFN regulatory factor 1 gene. This enhancer has been shown to be required for IFN-γ-activated expression of this gene. PRL-induced assembly of the DNA binding complex, PRL-stimulated factor, required tyrosine phosphorylation. PRL-stimulated factor contained at least one protein that was antigenically similar to the p91 transcription factor, a component of several transcription complexes required for cytokine-activated gene expression. PRL not only induced the tyrosine phosphorylation of p91 but also induced tyrosine phosphorylation of Jak2, a tyrosine kinase required for IFN-γ-activated gene expression. These results provide evidence for a signaling mechanism, some of whose components are shared by both PRL and IFN-γ receptors, that results in the expression of early response genes.**

Prolactin (PRL) is a pituitary hormone responsible for numerous biological effects including regulation of reproduction and lactation, maintenance of salt and water balance, and modulation of growth, differentiation, and immune function (1). The receptors for PRL belong to a family of cytokine receptors that also includes receptors for the interferons (IFNs) (1, 2). Two forms of the PRL receptor have been characterized in mammalian cells and have been found to differ in their cytoplasmic domains (1). Neither form of the receptor contains a tyrosine kinase domain. Although the signal transduction mechanism of PRL remains unclear, several recent reports indicate (3, 4) that a defined set of cellular proteins is rapidly tyrosine-phosphorylated upon exposure of rat Nb2 lymphoma cells to PRL, and one of these phosphotyrosyl proteins copurifies with the activated PRL receptor (4). Another report indicates that several inhibitors of tyrosine kinase activity prevent PRL-induced expression of the milk protein genes (5).

Both IFN-α and IFN-γ regulate gene expression by activation of tyrosine kinase(s) that phosphorylate a 91-kDa transcription factor, p91. This SH2 domain-containing protein then translocates to the nucleus where it is a component of several multisubunit complexes that bind enhancers required for the transcriptional activation of specific cellular genes (6–8). For several cytokines, a common pathway exists that results in activated gene expression and tyrosine phosphorylation of cellular proteins that function as positive transcriptional regulators. These cytokines include among others interleukin (IL)-3, IL-4, IL-5, IL-6, IL-10, granulocyte–macrophage colony-stimulating factor, epidermal growth factor (EGF), platelet-derived growth factor, ciliary neurotrophic factor, growth hormone, and erythropoietin (9–19). Because PRL treatment of cells induces the tyrosine phosphorylation of several cellular proteins (3, 4) and also rapidly activates the expression of the IFN regulatory factor 1 (IRF-1) gene (20) in a similar manner to IFN-γ, experiments were initiated to determine whether any of the tyrosine-phosphorylated transcription factors and tyrosine kinases known to be required for IFN-γ action might also play a role in the rapid signaling events associated with PRL action.

EXPERIMENTAL PROCEDURES

Cells. Rat Nb2 lymphoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 7% (vol/vol) fetal bovine serum, 7% (vol/vol) horse serum, and 20 μM 2-mercaptoethanol. Cells were placed in the same medium without fetal bovine serum for 16–24 hr prior to stimulation with ovine PRL (Sigma) at 100 ng/ml. Mouse M1 cells were cultured in DMEM with 10% fetal bovine serum. Murine IFN-γ was provided by Genentech.

Preparation of Nuclear Extracts. Approximately 5×10^7 cells were collected by centrifugation, washed with phosphate-buffered saline, and resuspended in ice-cold buffer A [1 mM $MgCl_2$/20 mM Hepes, pH 7.0/10 mM KCl/0.5 mM dithiothreitol/0.1% Triton X-100/200 μM phenylmethylsulfonyl fluoride/1 mM vanadate/20% (vol/vol)glycerol]. The suspension was homogenized in a Dounce homogenizer and centrifuged at $3000 \times g$ for 10 min at 4°C. The nuclear pellet was resuspended in buffer A with 0.3 M NaCl, extracted by vortex mixing, and centrifuged at $3000 \times g$ for 10 min. The supernatant (nuclear extract) was transferred to a new tube. Protein concentrations for each extract were determined and normalized by the addition of extraction buffer (9).

Electrophoretic Mobility Shift Assay (EMSA). EMSAs were performed as described (21). The IFN-γ-induced element (5′-AGCCTGATTTCCCCGAAATGATGAGGCCGAGTGG-3′) of the mouse IRF-1 gene (IRF-1E) (22) was end-labeled using polynucleotide kinase and [γ^{32}P]ATP. Competitive inhibition experiments were performed using a 50-fold molar excess of unlabeled oligonucleotides: the γ response region (GRR) (5′-AGCATGTTTCAAGGATTTGAGATGTATTTCCCAGAAAAG-3′) of the promoter of the gene for the high-affinity receptor for IgG (FcγRI), the IFN-stimulated response element (ISRE) of IFN-stimulated gene

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Abbreviations: IFN, interferon; PRL, prolactin; PRLSF, PRL-stimulated factor; IRF-1, IFN regulatory factor 1; IRF-1E, IFN regulatory factor 1 element; ISRE, IFN-stimulated response element; GRR, IFN-γ response element; FcγRI, gene for the high-affinity receptor for IgG; EMSA, electrophoretic mobility shift assay; PTPase, protein tyrosine phosphatase; IL, interleukin; EGF, epidermal growth factor; GAS, IFN-γ-activation sequence.

ISG15 (5′-GATCCATGCCTCGGGAAAGGGAAACCGAAACTGAAGCC-3′) (23), and the IFN-γ activation sequence (GAS) of the guanylate binding protein gene (5′-AGTACTTTCAGTTTCATATTACTCTAAATC-3′) (24).

Affinity Purification of PRL-Activated IRF-1E-Binding Proteins. Nuclear extracts were prepared from either untreated or PRL-treated Nb2 cells, and the extracts were incubated with agarose-conjugated IRF-1E for 2 hr at 4°C. After extensive washing in buffer A, affinity-purified proteins were eluted with SDS sample buffer, resolved by SDS/PAGE in 8% gels, and transferred to an Immobilon membrane. The blots were then probed with biotin-labeled anti-phosphotyrosine antibodies (PY20, ICN), developed using an enhanced chemiluminescence system ECL (Amersham), and then reprobed with anti-p91 serum (25).

Immunoprecipitations. After treatment with PRL, cells were solubilized in 1% Triton X-100 (Pierce) in 0.15 M NaCl/50 mM Tris, pH 8.0/50 mM NaF/5 mM sodium pyrophosphate/1 mM phenylmethylsulfonyl fluoride/1 mM orthovanadate. The postnuclear lysate was then precleared with a suspension of protein G-agarose (Pharmacia) for 1 hr at 4°C. After separation of the protein G-agarose from the lysate by centrifugation, the lysate was incubated with normal rabbit serum or rabbit anti-Jak1 or rabbit anti-Jak2 serum (both from Upstate Biotechnology, Lake Placid, NY) for 2–18 hr at 4°C. Each immunoprecipitation was performed with 150–200 × 10^6 cells. After the incubation with antibody, protein G-agarose was added for 1 hr at 4°C to isolate the immune complexes. The protein G conjugates were then washed several times in lysis buffer and boiled in SDS/sample buffer.

The samples were then analyzed by SDS/PAGE on 8% gels followed by electrophoretic transfer to Immobilon-P membranes (Millipore) by using a semi-dry transfer apparatus (Bio-Rad). The membranes were then blocked using 4% (wt/vol) ovalbumin (Sigma) in Tris-buffered saline (150 mM NaCl/10 mM Tris, pH 8.0) with 0.05% Tween 20 for 1 hr at room temperature followed by incubation with biotin-conjugated mouse anti-phosphotyrosine monoclonal antibody (PY20) (ICN) for at least 2 hr at room temperature. After extensive washing, the membranes were incubated with peroxidase-conjugated streptavidin (Sigma) and developed using enhanced chemiluminescence (Amersham). To analyze those protein(s) specifically immunoprecipitated after chemiluminescence detection of the bound mouse anti-phosphotyrosine antibody, we reexposed the membranes to anti-Jak1 or anti-Jak2 serum for 2–24 hr at room temperature. After washing, the membranes were probed with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin and developed using nitroblue tetrazolium.

RESULTS AND DISCUSSION

An enhancer sequence that we have designated IRF-1E has been defined in the promoter of the IRF-1 gene that is required for IFN-γ to induce its expression (22, 26, 27). Similar sequences, required for IFN-γ induction, have been identified in the promoters of the FcγRI, ICSBP, guanylate binding protein, and Ly-6A/E genes. These sequences also have been shown to bind tyrosine-phosphorylated p91 after treatment of cells with IFN-γ (21, 25–28). To determine whether the IRF-1E also interacted with specific proteins activated in response to PRL, we prepared nuclear extracts from Nb2 cells that had been incubated with PRL for 5, 15, 30, or 60 min and performed EMSAs with a ^{32}P-labeled oligonucleotide probe corresponding to the IRF-1E (Fig. 1*A*). A PRL-stimulated factor (PRLSF) was detected in nuclear extracts from cells after a 5-min incubation with PRL (lanes 1 vs. 2) and was still present in cells after 1 hr. This complex was also seen in cytoplasmic extracts from cells after a 5-min PRL treatment in the same experiment (data not shown). The PRLSF specifically bound to the ^{32}P-labeled IRF-1E. It could be displaced by the addition of excess unlabeled IRF-1E probe (Fig. 1*B*, lane 2) or an oligonucleotide corresponding to the GRR of the FcγRI gene (Fig. 1*B*, lane 3), a sequence similar to the IRF-1E that was required for IFN-γ-induced expression of the FcγRI gene (29). Unlabeled oligonucleotides corresponding to the ISRE, required for induction of genes by IFN-α (30), and an oligonucleotide corresponding to the GAS, required for induction of the guanylate binding protein gene by either IFN-α or IFN-γ (31), were without effect (Fig. 2*B*, lanes 4 and 5).

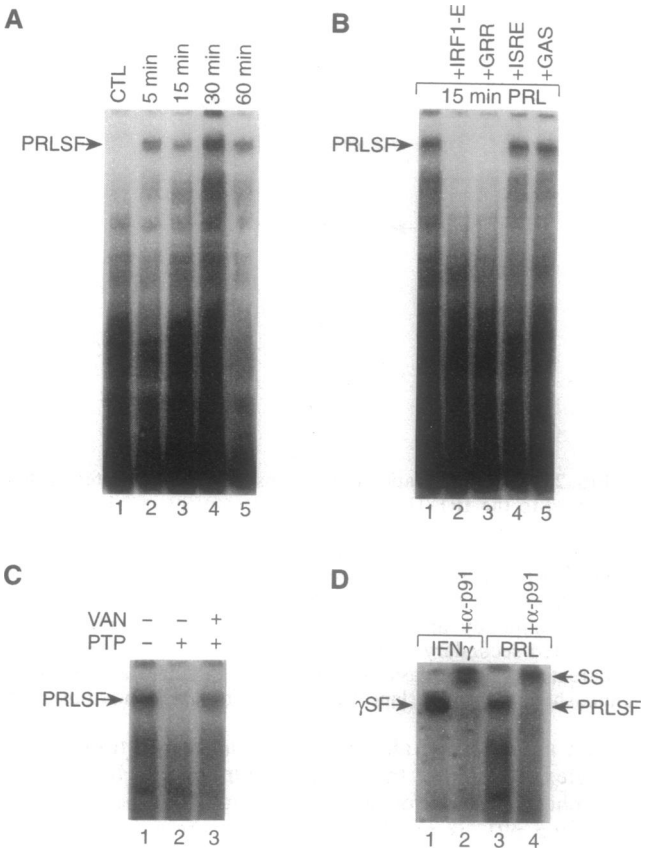

FIG. 1. Characterization of the PRL-activated DNA binding complex that recognizes the IRF-1 enhancer. (*A*) The PRL-induced complex that binds to the IRF-1E localizes to the nucleus. Nb2 cells were incubated with PRL (100 ng/ml) for the indicated times and nuclear extracts were prepared. EMSAs were performed using the IRF-1E oligonucleotide. CTL, control. (*B*) Competition assays using unlabeled oligonucleotide. Extracts were prepared from cells incubated with PRL for 30 min and competition assays were performed by adding a 50-fold molar excess of unlabeled oligonucleotides corresponding to the IRF-1E (lane 2), the GRR (lane 3), the ISRE (lanes 4), or the GAS of the guanylate binding protein gene (lane 5). (*C*) Phosphorylated tyrosine is required for assembly of the DNA binding complexes. Nuclear extracts were prepared from Nb2 cells treated with PRL for 30 min. Extracts were kept at 4°C for 30 min (lane 1) or incubated with 1 μg of purified recombinant *Yersinia enterocolitica* PTPase (PTP) for 30 min at 30°C in the absence (lane 2) or presence (lane 3) of the PTPase and 1 mM orthovanadate (VAN, a specific PTPase inhibitor). The extracts were then assayed by EMSA with the [^{32}P]IRF-1E probe. (*D*) Analysis of the DNA binding complex by antibody "supershift" assays. Nuclear extracts were prepared from M1 cells treated with IFN-γ (10 ng/ml) or from Nb2 cells treated with PRL for 30 min at 37°C. Extracts were then incubated for 1 hr at 4°C with anti-p91 antibody (lanes 2 and 4) and assayed for IRF-1E binding by EMSA. The supershifted (SS) complex demonstrating the presence of p91 is indicated, γSF, IFN-γ-stimulated factor.

PRL treatment in the same experiment (data not shown). The PRLSF specifically bound to the ^{32}P-labeled IRF-1E. It could be displaced by the addition of excess unlabeled IRF-1E probe (Fig. 1*B*, lane 2) or an oligonucleotide corresponding to the GRR of the FcγRI gene (Fig. 1*B*, lane 3), a sequence similar to the IRF-1E that was required for IFN-γ-induced expression of the FcγRI gene (29). Unlabeled oligonucleotides corresponding to the ISRE, required for induction of genes by IFN-α (30), and an oligonucleotide corresponding to the GAS, required for induction of the guanylate binding protein gene by either IFN-α or IFN-γ (31), were without effect (Fig. 2*B*, lanes 4 and 5).

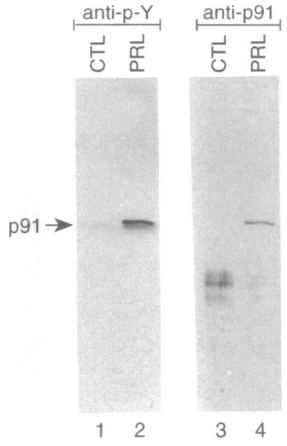

FIG. 2. Characterization of the tyrosine-phosphorylated proteins that bind to the IRF-1E agarose after treatment of Nb2 cells with PRL. Approximately 2×10^8 cells were exposed to PRL as above and IRF-1E binding proteins were isolated using IRF-1E-agarose. (*Left*) Transferred proteins probed with anti-phosphotyrosine (anti-p-Y) antibodies (lanes 1 and 2). (*Right*) The same blot was reprobed with anti-p91 antibody and developed with an alkaline phosphatase-conjugated secondary antibody (lanes 3 and 4). The band with an apparent molecular mass of 50 kDa seen in lane 3 and present in diminished intensity in lane 4 has not been consistently observed.

The assembly of all known IFN-activated transcription complexes requires that certain components be tyrosine-phosphorylated. Tyrosine phosphorylation of these proteins, including p91, is regulated by membrane-associated IFN-activated tyrosine phosphatase(s) and tyrosine kinase(s) (32–35). To determine whether the assembly of PRLSF also depended upon phosphorylated tyrosine residues, we incubated a nuclear extract from PRL-treated cells with recombinant purified phosphotyrosine phosphatase (PTPase) from *Yersinia enterocolitica* (36). PTPase treatment of the nuclear extract disrupted the PRLSF complex, but in the presence of the PTPase inhibitor orthovanadate, the complex was unaffected (Fig. 1*C*, lanes 2 and 3). The PTPase had no effect on the ^{32}P-labeled oligonucleotide probe (data not shown).

Because p91 binds to the GRR in extracts prepared from IFN-γ-treated cells and cells incubated with a variety of other cytokines (21, 25, 26), and the GRR displaced binding of PRLSF from the IRF-1E, experiments were performed to determine whether p91 was a component of the PRLSF complex. Extracts from PRL-treated Nb2 cells or from mouse M1 cells treated with IFN-γ were incubated prior to addition of ^{32}P-labeled IRF-1E with anti-p91 antibody prepared against the 39-amino acid C terminus of p91 (25). (Nb2 cells do not respond to IFN-γ, IFN-α, or EGF under the conditions used for these experiments.) The p91 antibody "supershifted" both the IFN-γ-induced complex (Fig. 1*D*, lanes 1 vs. 2) and the PRLSF (lanes 3 vs. 4), suggesting that both complexes contained p91. An antibody directed against the IFN-α-activated p113 protein, which is a component of the IFN-stimulated gene ISGF3 transcription complex and is structurally related to p91, did not supershift the PRLSF (data not shown).

To further characterize the tyrosine-phosphorylated protein(s) that were components of the PRLSF, we incubated extracts prepared from untreated or PRL-stimulated Nb2 cells with IRF-1E-agarose beads. The material that bound to the IRF-1E beads was resolved by SDS/PAGE, transferred to Immobilon, and probed with an anti-phosphotyrosine antibody (Fig. 2). PRL induced tyrosine phosphorylation of a 97-kDa protein that selectively associated with the IRF-1E beads (lanes 1 vs. 2). A constitutive tyrosine-phosphorylated protein that migrated slightly faster also bound to the affinity beads. Reprobing of the blot with the p91 antibody indicated that the PRL-induced tyrosine-phosphorylated protein was recognized by the antibody (lanes 3 vs. 4), whereas the constitutive tyrosine-phosphorylated protein was not detected with the antibody. These results not only confirmed those shown in Fig. 1*D* but also indicated that the p91 that interacted with the IRF-1E was tyrosine-phosphorylated.

It is now evident that a variety of cytokines, including the IFNs, growth hormone, EGF, erythropoietin, IL-3, and those cytokine receptors that utilize the gp130 signaling protein (17, 19, 37–41), appear to use the tyrosine kinases of the Jak family as components of their signaling cascade. To determine whether PRL might induce the activation of any of the known kinases in this family, NB2 cells were incubated with PRL for 15 min prior to immunoprecipitation of solubilized cells with specific antibodies corresponding to either Jak1 or Jak2. Immunoprecipitates were resolved by SDS/PAGE, transferred to Immobilon-P membranes, and probed with an anti-phosphotyrosine antibody (Fig. 3). PRL treatment of cells caused a selective time-dependent increase in tyrosine phosphorylation of the Jak2 kinase (Fig. 3*A*, lanes 1 vs. 2–5). There was no enhanced tyrosine phosphorylation of either Jak1 (Fig. 3*B*, lanes 1 and 2) or Tyk2 (data not shown). Tyrosine phosphorylation of Jak2 could be detected after a 2-min treatment of cells with PRL and became maximal after

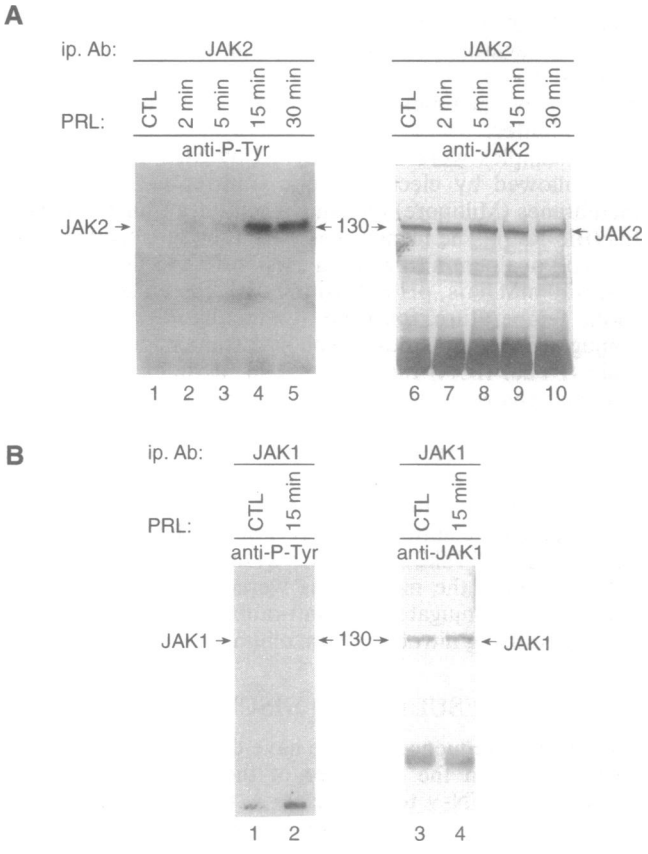

FIG. 3. Jak2 kinase is tyrosine-phosphorylated in Nb2 cells treated with PRL. (*A*) Nb2 cells were incubated with PRL at 100 ng/ml for the indicated times prior to lysis. Lysates were immunoprecipitated with Jak2 antiserum (lanes 1–5). The immunoprecipitates were resolved by SDS/PAGE (8% gels), transferred to Immobilon, and probed with an anti-phosphotyrosine antibody. The tyrosine-phosphorylated Jak2 protein migrates with an apparent molecular mass of 130 kDa as indicated. The blots were reprobed for the presence of Jak2 protein (lanes 6–10). (*B*) Nb2 cells were treated as in *A* for 15 min with PRL and lysates were immunoprecipitated with Jak1 antiserum. The blot was probed with anti-phosphotyrosine antibody (lanes 1 and 2) and then reprobed with anti-Jak1 serum (lanes 3 and 4) for Jak1 protein. CTL, control.

15 min of incubation. The anti-phosphotyrosine blots were reprobed with either Jak2 or Jak1 antiserum to demonstrate that both Jak1 and Jak2 were immunoprecipitated in equal amounts from both untreated cells and cells incubated with PRL (Fig. 3 *A*, lanes 6–10, and *B*, lanes 3 and 4).

In this report we demonstrate that PRL, which is thought to regulate its effects via tyrosine kinase-activated signaling cascades (3–5, 42), can modify proteins by tyrosine phosphorylation so that they bind an enhancer element. This enhancer is present in the promoter of the IRF-1 gene whose transcription is known to be rapidly activated both by IFN-γ and PRL (20, 22). Although it has not yet been determined whether PRL-induced expression of IRF-1 requires the IRF-1E, it has been established that IFN-γ-induced expression of this gene requires this element (22). It therefore seems likely that this element will also be an important component required for PRL-induced IRF-1 expression.

Recent reports have demonstrated that IFN-α-induced phosphorylation of p91 requires the tyrosine kinases Tyk2 and Jak1 (33, 41), IFN-γ requires Jak1 and Jak2, and growth hormone, erythropoietin, and IL-3 mediate signaling through the related kinase, Jak2 (19, 37–40, 43). Since the PRL receptor and the growth hormone receptor are closely related, both activate tyrosine phosphorylation of cellular proteins, and growth hormone enhances Jak2 activity, the observation that PRL also increases tyrosine phosphorylation of Jak2 fits well with the concept of cytokine activation of kinases of the Tyk/Jak family. It is interesting to note that growth hormone treatment of cells also stimulates tyrosine phosphorylation of p91 so that it can bind to the GRR and SIF-E in the c-*fos* promoter (14, 44). Although it has been suggested that the Jak1 enzyme is required for tyrosine phosphorylation of p91 (38), the fact that neither growth hormone nor PRL appear to induce tyrosine phosphorylation of Jak1 would indicate that at least in certain cases tyrosine phosphorylation of p91 does not correlate with tyrosine phosphorylation of this enzyme. To our knowledge, it is not known whether any enzymes of the Jak family directly phosphorylate p91 or whether another as yet to be defined tyrosine kinase is responsible for this event.

Formation of multimers of phosphorylated proteins is being appreciated as a prevalent mechanism by which transcription factors bind DNA. For example, the ISGF3 transcription complex formed in response to IFN-α treatment of cells contains p91 complexed with p84 and p113 (45). In addition, recent evidence has suggested that activation of p91 by EGF, IL-10, ciliary neurotrophic factor, growth hormone, IL-6, leukemia inhibitory factor, or oncostatin M also induces tyrosine phosphorylation of another protein that coimmunoprecipitates with p91 but is antigentically distinct (13, 18, 19). Although no additional phosphotyrosine proteins were immunoprecipitated with p91 after PRL treatment of cells, this does not preclude the possibility that other proteins besides p91 exist in these DNA binding complexes. Alternatively, it has been suggested that p91 alone can bind DNA (8). The mechanisms by which both PRL and IFN-γ can stimulate tyrosine phosphorylation of p91 so that it can bind to the same enhancer yet exert distinct biological actions requires further investigation.

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