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Growth Hormone and Erythropoietin Differentially Activate DNA-Binding Proteins by Tyrosine Phosphorylation

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Binding of growth hormone (GH) and erythropoietin (EPO) to their respective receptors results in receptor clustering and activation of tyrosine kinases that initiate a cascade of events resulting not only in the rapid tyrosine phosphorylation of several proteins but also in the induction of early-response genes. In this report, we show that GH and EPO induce the tyrosine phosphorylation of cellular proteins with molecular masses of 93 kDa and of 91 and 84 kDa, respectively, and that these proteins form DNA-binding complexes which recognize an enhancer that has features in common with several rapidly induced genes such as c-*fos*. Assembly of the protein complexes required tyrosine phosphorylation, which occurred within minutes after addition of ligand. The activated complexes translocated from the cytoplasm to the nucleus. The protein activated by GH is antigenically similar to p91, a protein common to several transcription complexes that are activated by interferons and other cytokines. In contrast, the proteins activated by EPO are distinct from p91. These findings establish the outlines for a cytokine-induced intracellular signaling pathway, which begins with ligand-induced receptor clustering that activates one or more tyrosine kinases. These data are the first to demonstrate that GH- and EPO-activated tyrosine-phosphorylated proteins can specifically recognize a well-defined enhancer and therefore provide a mechanism for rapidly transducing signals from the membrane to the nucleus.

The receptors for growth hormone (GH) and erythropoietin (EPO) are members of a large family of cytokine receptors whose structure is characterized by conserved cysteine residues within the extracellular domain (2, 30). However, the intracellular regions of these receptors bear little detectable similarity to each other or to those of other known signaling molecules. Even though these receptors contain no kinase domain, ligand-induced clustering rapidly results in the tyrosine phosphorylation of several cellular proteins. In general, the characterization of these proteins has been limited to size separation on sodium dodecyl sulfate (SDS)-gels, and their biological functions remain unknown (3, 4, 21, 28). Since it has recently been appreciated that besides the interferon (IFN)-induced proteins, other ligand-induced tyrosine-phosphorylated proteins can act as transcriptional regulators, a potential role for some of these EPO- and GH-induced phosphoproteins to act as DNA-binding proteins exists (10, 18, 19, 24, 25, 29). IFNs activate several early-response genes which contain elements in their promoters that recognize SH2 domain-containing proteins that are posttranslationally modified by phosphorylation on tyrosines (6, 8, 13, 26, 27). One such protein is the 91-kDa protein (p91) that is a component of IFN-stimulated gene factor 3 (ISGF3) (9). p91 is rapidly phosphorylated following the addition of either IFN-α or IFN-γ to cells (26, 27). It then assembles into a multisubunit complex(es) that recognizes elements within the promoters of several early-response genes. The proteins that associate with p91 may determine the precise DNA element recognized by the multicomponent complex. One of these elements is the gamma response region (GRR) of the FCγ receptor factor I (FcRFγ) gene. Although GRR-binding activity (of which tyrosine-phosphorylated p91 is a component) can be measured in many cells in response to IFN treatment, the gene itself is transcribed in a cell-specific manner in myeloid cells such as monocytes (15, 22, 23).

Since the receptors for the IFNs, GH, and EPO have no intrinsic tyrosine kinase domains in their intracellular regions, they control signaling by association with intracellular tyrosine kinases. Several cytokine receptors are associated with tyrosine kinases, usually of the Src kinase family. For IFN-α-induced gene expression, the tyrosine kinase tyk2 has been shown to be an integral component in the signaling pathway (31). More recently, a related kinase, JAK2, has been reported to be involved in both GH, EPO, and IFN-γ signal transduction (1, 33, 35). Incubation of cells with GH or EPO permits JAK2 to associate with the receptor and become tyrosine phosphorylated. Indeed, ligand-induced receptor clustering appears to be a commonly used mechanism for the rapid posttranslational modification of proteins by tyrosine phosphorylation (17).

Even though JAK2 kinase activity may be necessary for GH- and EPO-induced signal transduction, it remains to be determined how tyrosine phosphorylation of substrates can alter the cell phenotypically. Since transcriptional activation of genes by cytokines plays a crucial role in dictating the ultimate phenotype expressed, a mechanism for ligand-induced activation of transcription factors might be expected. Since the critical sequence in the GRR element (ATTTCCNNNNAAA) is identical to a similar region of the *sis*-inducible element (SIE) in the c-*fos* promoter (AGTTCCNNNNAAT), an element

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known to be necessary for cytokine (platelet-derived growth factor and epidermal growth factor)-mediated induction of c-*fos*, we used the GRR as a probe to identify and characterize GH- and EPO-activated DNA-binding proteins (10, 32).

MATERIALS AND METHODS

Cells. IM9 cells, a lymphoblastoid cell line (ATTC CCL 159), were cultured in minimal essential medium with 10% fetal bovine serum supplemented with nonessential amino acids. TF1 cells, an erythroleukemia cell line (16), were cultured in RPMI 1640 with 10% fetal bovine serum supplemented with 5 ng of granulocyte-macrophage colony-stimulating factor (GM-CSF; Schering Corp.) per ml. Prior to treatment with EPO (Amgen Inc.), the cells were removed from GM-CSF for 16 h.

Nuclear, cytoplasmic, and whole cell extracts. Cells (5×10^7) were collected by centrifugation, washed with phosphate-buffered saline, and for whole cell extracts resuspended in ice-cold extraction buffer (1 mM $MgCl_2$, 20 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES; pH 7.0], 10 mM KCl, 300 mM NaCl, 0.5 mM dithiothreitol, 1% Triton X-100, 200 μM phenylmethylsulfonyl fluoride, 1 mM vanadate, 20% glycerol). The suspension was gently vortexed for 10 s and allowed to incubate at 4°C for 10 min. The mixture was centrifuged at 18,000 × *g* for 10 min at 4°C, and the supernatant was transferred to a new tube. Nuclear and cytoplasmic extracts were prepared by Dounce homogenization of cells in buffer A (20 mM HEPES [pH 7.0], 10 mM KCl, 10 mM $MgCl_2$, 1 mM orthovanadate, 0.5 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride, 20% glycerol, 0.1% Nonidet P-40). The lysate was layered on a sucrose cushion (35% sucrose in 100 mM HEPES [pH 7.0]–20 mM $MgCl_2$), and the nuclei were isolated by centrifugation at 3,000 × *g* for 15 min. Nuclei were resuspended in buffer A with 0.3 M NaCl and extracted by vortexing. To the postnuclear supernatant, 0.3 M NaCl was added (cytoplasmic extract). Protein concentrations for each type of extract were determined and normalized by the addition of extraction buffer.

EMSA. The electrophoretic mobility shift assay (EMSA) was performed as previously described (34). The GRR (5′ AGC ATGTTTCAAGGATTTGAGATGTATTTCCCAGAAAAG 3′) of the promoter of the Fcgr1 gene was end labeled with polynucleotide kinase and [γ-^{32}P]ATP and used in all EMSAs. Competitive inhibition experiments were performed with a 50-fold molar excess of the following unlabeled oligonucleotides: the GRR, the IFN-stimulated response element (ISRE) of ISG15 (5′ GATCCATGCCTCGGGAAAGGGAAACCGAA ACTGAAGCC 3′), the SIE of the c-*fos* gene (5′ AGCTTAG GGATTTACGGGAAATGA 3′) (32), and the IFN-γ activation sequence (GAS) of the guanylate-binding protein gene (5′ AGTACTTTCAGTTTCATATTACTCTAAATC 3′) (14).

Immunoprecipitations with anti-p91. Whole cell extracts were prepared as described above and incubated with anti-p91 for 2 h at 4°C. Anti-p91 was prepared by immunizing rabbits with a peptide corresponding to the 39 carboxy-terminal amino acids unique to p91. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (8% gel) followed by blotting to Immobilon. The blots were then probed with a biotin-labeled antiphosphotyrosine antibody (PY20; ICN) and developed by using enhanced chemiluminescence (Amersham).

Affinity purification of GH- and EPO-activated GRR-binding proteins. Whole cell extracts were prepared and incubated with agarose-conjugated GRR for 2 h at 4°C. After extensive washing, affinity-purified proteins were eluted with SDS sample buffer, resolved by SDS-PAGE (8% gel), transferred to Immobilon, and probed with an antiphosphotyrosine antibody as described above. Blots were then reprobed with anti-p91/p84. This antibody was prepared by immunizing rabbits with a

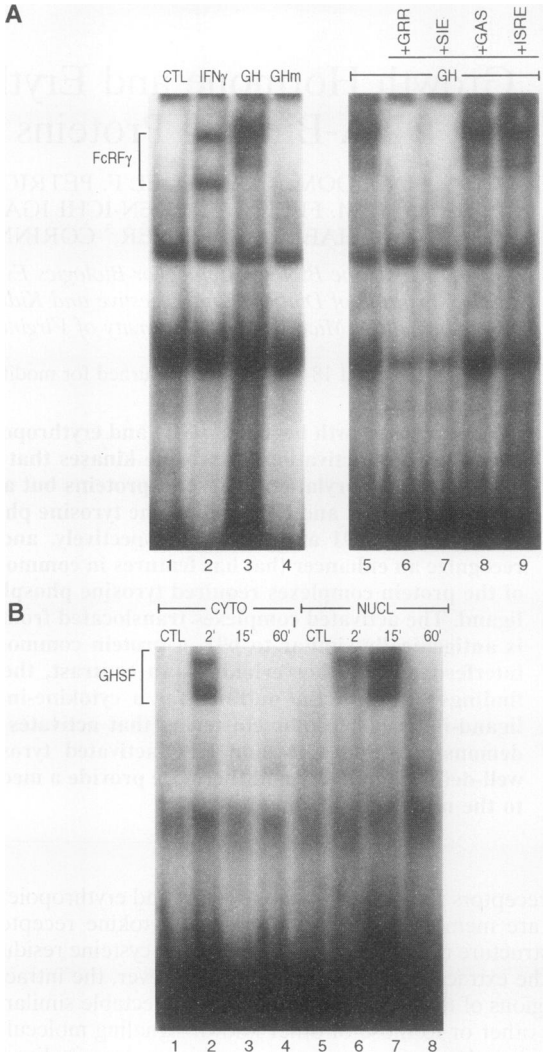

FIG. 1. GH activates a DNA-binding complex that recognizes the GRR sequence. (A) Activation of GHSF or FcRFγ in whole cell extracts prepared from IM9 cells. Cells were harvested, resuspended in fresh medium, and exposed to 200 ng of human recombinant GH (Genentech, Inc.) for 2 to 60 min at 37°C, and then either whole cell or nuclear and cytoplasmic extracts were prepared. DNA-binding complexes were assayed by using an EMSA. For comparison, IM9 cells were treated with IFN-γ (10 ng/ml; Genentech) for 15 min at 37°C, which induced the DNA-binding complex, FcRFγ (34) (lane 2). The shifted complexes marked in lanes 3 to 9 have been termed GHSF. Lanes: 1, untreated cells (control [CTL]); 3 and 5 to 9, cells treated with 200 ng of GH per ml for 15 min at 37°C; 4, cells treated with 400 ng of the mutated GH (GHm) (28) per ml. Competition assays were performed by adding a 50-fold molar excess of an unlabeled oligonucleotide corresponding to the GRR (lane 6), the SIE of c-*fos* (lane 7), the GAS of the guanylate-binding protein gene (lane 8), or the ISRE of ISG15 (lane 9). (B) The GHSF translocates from the cytoplasm to the nucleus. IM9 cells were incubated with GH for the indicated times (minutes), and cytoplasmic (CYTO; lanes 1 to 4) and nuclear (NUCL; lanes 5 to 8) extracts were prepared as described above. GHSF was assayed by EMSA with a GRR probe.

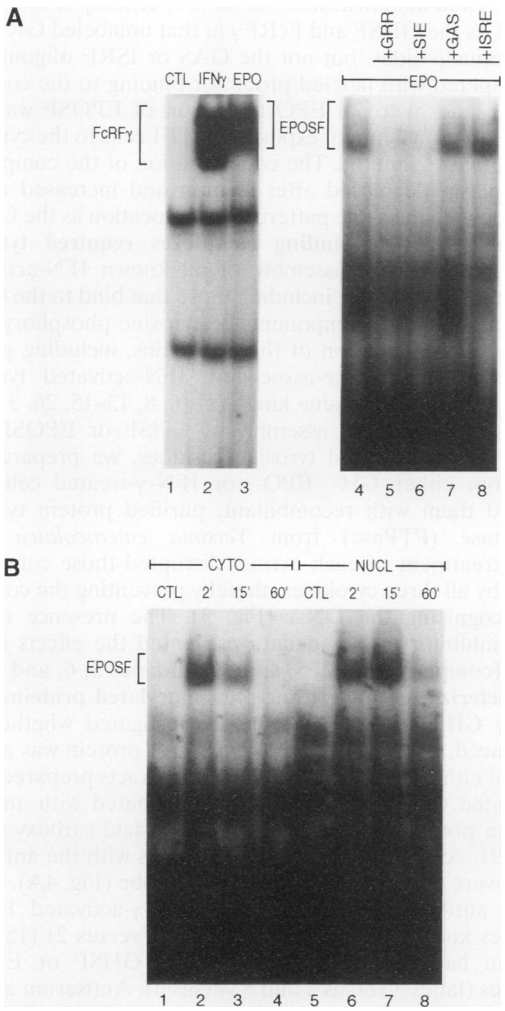

FIG. 2. Incubation of TF1 cells with EPO activates a protein complex which specifically binds to the GRR. After removal from GM-CSF for 18 h, the TF1 cells were harvested, resuspended in fresh medium, and exposed to 20 U of EPO per ml for 2 to 60 min at 37°C. Whole cell, nuclear, and cytoplasmic extracts were prepared as described in Materials and Methods and assayed for GRR binding. (A) EPOSF is present in whole cell extracts prepared from TF1 cells incubated with EPO for 15 min. Lanes: 1, no treatment (control [CTL]); 2, IFN-γ, 15 min; 3, EPO, 15 min; 5 to 8, competitive inhibition assays performed as described for Fig. 1A. (B) EPOSF translocates from the cytoplasm to the nucleus. TF1 cells were incubated with EPO for the indicated times (minutes), and cytoplasmic (CYTO; lanes 1 to 4) and nuclear (NUCL; lanes 5 to 8) extracts were prepared as described for Fig. 1B. EPOSF was assayed by EMSA with a GRR probe.

40-amino-acid peptide (amino acids 607 to 647) common to both p91 and p84.

RESULTS

GH and EPO treatment of cells resulted in activation of proteins which specifically bind to the GRR. We initially investigated whether extracts prepared from GH- or EPO-treated cells contained protein complexes that specifically interacted with the GRR. The human lymphoblast cell line IM9, which is responsive to treatment with GH, and the human erythroleukemia cell line TF1, which is EPO responsive, were

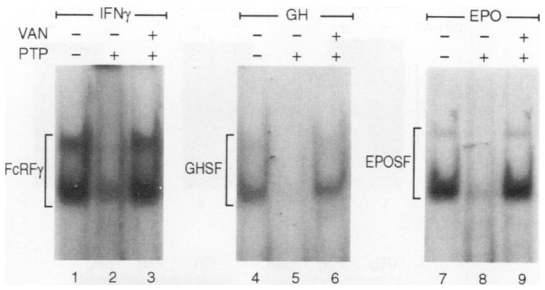

FIG. 3. Phosphorylated tyrosine is required for assembly of the DNA-binding complexes. Whole cell extracts were prepared from either IM9 cells treated with GH (200 ng/ml, 15 min) or IFN-γ (5 ng/ml, 15 min) or TF1 cells treated with EPO (20 U/ml, 15 min). Extracts were incubated with 1 μg of purified recombinant *Y. enterocolitica* PTPase (12) for 30 min at 30°C in the absence (lanes 2, 5, and 8) or presence (lanes 3, 6, and 9) of 1 mM orthovanadate (VAN; a specific PTPase inhibitor). The extracts were then assayed by EMSA, using a ^{32}P-labeled GRR probe. Lanes 1, 4, and 7 were not incubated at 30°C.

incubated without or with GH (Fig. 1) or EPO (Fig. 2) for 15 min prior to preparation of whole cell extracts. EMSAs were performed by the addition of these extracts to a ^{32}P-labeled oligonucleotide corresponding to the GRR sequence followed by nondissociating PAGE. GH-treated IM9 cells contained an inducible complex termed growth hormone-stimulated factor (GHSF) (Fig. 1a; compare lanes 1 and 3) which migrated with a mobility slower than that of the IFN-γ-induced FcRFγ complex (lane 2). This finding suggested that the complex induced by GH differed from that induced by IFN-γ. The GHSF specifically bound to the GRR and could be displaced by the addition of excess unlabeled GRR oligonucleotide (Fig. 1A, lanes 5 versus 6) but not by unlabeled ISRE or GAS oligonucleotide, which binds the IFN-α-induced transcription complex ISGF3 or the IFN-α- and IFN-γ-induced transcription complex IFN-γ-activated factor, respectively (lanes 8 and 9) (5, 7, 14, 20). The GHSF complex was also competed for with an unlabeled oligonucleotide corresponding to the SIE of c-*fos* (lane 7). The IFN-γ-activated FcRFγ complex showed the same binding specificity as GHSF (15, 34). To determine whether GH dimerization was required for activation of GHSF, we examined whether a recombinant form of GH which binds to one GH receptor but does not dimerize the receptor (11, 28) induced the formation of GHSF. Whereas the native recombinant form of GH induced GHSF, the mutated form of GH was ineffective (Fig. 1A, lanes 3 versus 4). Mutated GH did not prevent the formation of FcRFγ by IFN-γ (data not shown).

IFN-induced transcription factors have been shown to translocate from the cytoplasm to the nucleus (5, 7, 20). To determine whether GH-activated GHSF localized to the nucleus, cytoplasmic and nuclear extracts were prepared from IM9 cells treated for 2, 15, or 60 min with GH. After 15 min of treatment, the GHSF localized within the nuclear fraction (Fig. 1B, lane 7) and was not detectable in either fraction after 60 min of incubation (lanes 4 and 8).

Incubation of TF1 cells with EPO resulted in a pattern of response similar to that seen following incubation of IM9 cells with GH (Fig. 1 versus 2). An EPO-induced GRR-binding complex, EPO-stimulated factor (EPOSF), was present in whole cell, cytoplasmic, and nuclear extracts of TF1 cells exposed to the cytokine (Fig. 2). The EPOSF also migrated with a slower mobility than the FcRFγ complex (Fig. 2A, lanes

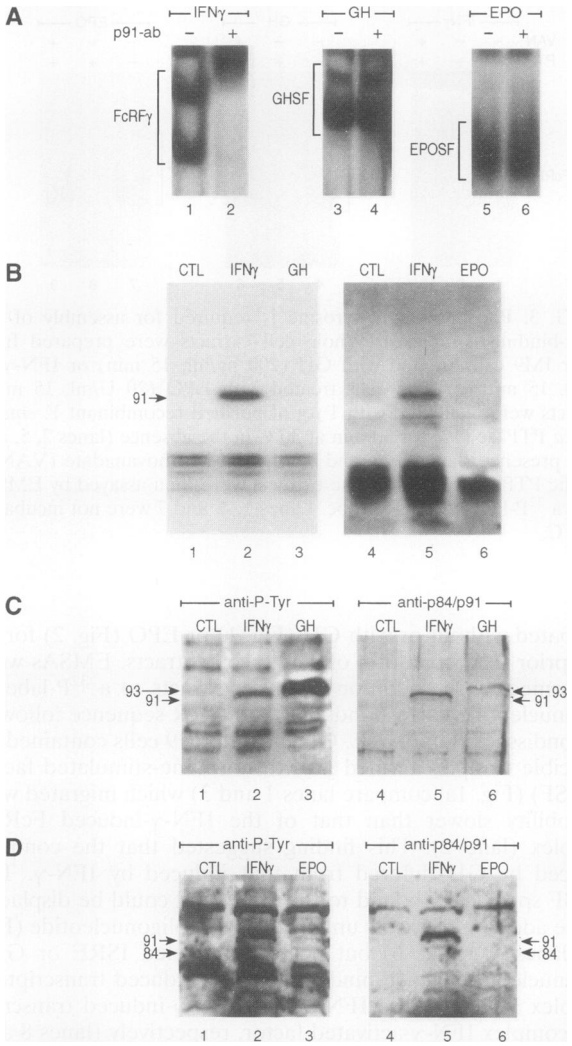

FIG. 4. Analysis of the DNA-binding complexes activated by EPO and GH. (A) Analysis of DNA-binding complexes by antibody supershift assays. Extracts were prepared from either GH-treated IM9 cells (lanes 3 and 4) or EPO-treated TF1 cells (lanes 5 and 6), and extracts from IFN-γ-treated monocytes were used as a control (lanes 1 and 2). Extracts were incubated with an anti-p91 antibody (p91-ab; a tyrosine-phosphorylated protein common to several IFN-activated transcription complexes) and then assayed for the ability to bind the ^{32}P-labeled GRR in an EMSA. (B) Tyrosine phosphorylation of immunoprecipitated p91 in GH-, EPO-, and IFN-γ-treated cells. Cells (5×10^7 to 1×10^8) were treated with IFN-γ (10 ng/ml), GH (200 ng/ml), or EPO (20 U/ml) for 15 min at 37°C. Whole cell extracts were prepared as described in Materials and Methods, and immunoprecipitated p91 was resolved by SDS-PAGE and transferred to Immobilon. The transferred proteins were probed with an antiphosphotyrosine antibody. Lanes: 1 to 3, IM9 cells; 4 to 6, TF1 cells; 1 and 4, no treatment (control [CTL]); 2 and 5, IFN-γ; 3, GH; 6, EPO. (C) Characterization of the tyrosine-phosphorylated proteins that bind to the GRR-agarose after treatment of IM9 cells with IFN-γ or GH. Cells (5×10^7 to 1×10^8) were exposed to either IFN-γ or GH as described above, and GRR-binding proteins were isolated by using GRR-agarose as described in Materials and Methods. Lanes: 1 to 3, the transferred proteins probed with an antiphosphotyrosine (anti-P-Tyr) antibody; 4 to 6, the same blot reprobed with an anti-p91/p84 antibody raised against a peptide corresponding to amino acids 607 to 647 of p91/p84 and developed with an alkaline phosphatase-conjugated secondary antibody. (D) Characterization of the tyrosine-phosphorylated proteins that bind to GRR-agarose after treatment of TF1 cells (200×10^7) with IFN-γ or EPO. The experiment was the same as for panel C except that TF1 cells were treated with either EPO or IFN-γ. Lanes: 1 to 3, GRR-bound proteins resolved by SDS-PAGE were probed with the antiphosphotyrosine antibody; 4 to 6, the antiphosphotyrosine blot (lanes 1 to 3) was reprobed with an antibody which recognized both p91 and its 84-kDa spliced variant and developed with an alkaline phosphatase-conjugated secondary antibody.

2 versus 3) and demonstrated the same specificity of binding to the GRR as the GHSF and FcRFγ in that unlabeled GRR and SIE oligonucleotides, but not the GAS or ISRE oligonucleotide, competed with labeled probe for binding to the complex (Fig. 2A, lanes 4 to 7). EPO activation of EPOSF was also detectable within 2 min of exposure of TF1 cells to the cytokine (Fig. 2B, lanes 2 and 6). The concentration of the complex in the cytoplasm decreased after 15 min and increased in the nuclei, showing the same pattern of translocation as the GHSF.

Assembly of DNA-binding complexes required tyrosine phosphorylation. The assembly of all known IFN-activated transcription complexes, including those that bind to the GRR, 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) (6, 8, 13–15, 26, 31). To determine whether the assembly of GHSF or EPOSF also required phosphorylated tyrosine residues, we prepared extracts from either GH-, EPO-, or IFN-γ-treated cells and incubated them with recombinant, purified protein tyrosine phosphatase (PTPase) from *Yersinia enterocolitica* (12). PTPase treatment of each extract disrupted those complexes induced by all three cytokines, thereby preventing the complex from recognizing the DNA (Fig. 3). The presence of the PTPase inhibitor orthovanadate prevented the effects of the PTPase (compare lanes 2, 5, and 8 with lanes 3, 6, and 9).

Characterization of tyrosine-phosphorylated proteins activated by GH and EPO. We next investigated whether the IFN-induced, tyrosine-phosphorylated p91 protein was a component of either the GHSF or EPOSF. Extracts prepared from cells treated with GH or EPO were incubated with anti-p91 antiserum prepared against the 39-amino-acid carboxy terminus of p91. After incubation of the extracts with the antibody, EMSAs were performed with the GRR probe (Fig. 4A). While the p91 antibody supershifted the IFN-γ-activated FcRFγ complexes known to contain p91 (lanes 1 versus 2) (15), this antiserum had no effect on either the GHSF or EPOSF complexes (lanes 3 versus 4 and 5 versus 6). Antiserum against the IFN-α-activated p113 also did not supershift the GHSF or EPOSF complexes (data not shown). These results suggested that although tyrosine-phosphorylated proteins were required to maintain the assembly of GHSF and EPOSF, the p91 protein was not a component of these complexes or was not in a form recognized by the antibody. To determine directly whether the p91 protein was activated by GH or EPO, cells were incubated with these cytokines or with IFN-γ (as a control) and cell lysates were immunoprecipitated with p91 antiserum. The immunoprecipitates were resolved by SDS-PAGE and probed with an antiphosphotyrosine antibody (Fig. 4B). Immunoprecipitated tyrosine-phosphorylated p91 was detected only when cells were treated with IFN-γ (lanes 2 and 5), not in untreated cells or cells incubated with GH or EPO (lanes 1, 3, 4, and 6).

Because it appeared that neither p91 nor p113 was involved in the assembly of GHSF or EPOSF, and to obtain some information concerning the components of these complexes, extracts from GH- or EPO-treated cells were incubated with

agarose beads coupled with the GRR oligonucleotide. Proteins which bound to the GRR-beads were resolved by SDS-PAGE, transferred to Immobilon, and probed with an antiphosphotyrosine antibody (Fig. 4C). IM9 cells treated with either IFN-γ or GH contained tyrosine-phosphorylated proteins of 91 kDa (lane 2) and 93 kDa (lane 3), respectively, which bound to the GRR-beads. When this phosphotyrosine blot was reprobed with the anti-p91 antibody raised against a peptide corresponding to a different domain of p91 (amino acids 607 to 647), the antibody reacted not only with tyrosine-phosphorylated p91 observed in the immunoprecipitate from IFN-γ-treated cells (lane 5) but also with the GH-induced 93-kDa tyrosine-phosphorylated protein. The antibody raised against the 39-amino-acid carboxy-terminal region of p91 used in Fig. 4B also reacted with the GH-induced tyrosine-phosphorylated protein of 93 kDa on the blot (data not shown). These results suggested that the GH-induced tyrosine-phosphorylated protein of 93 kDa was antigenically related or identical to p91 but could not be recognized in its nondenatured state by anti-p91 antibody when complexed in GHSF. The intense reaction of this protein with PY20 and its slightly slower mobility suggested that it is tyrosine phosphorylated in a manner different from that of IFN-γ-activated p91.

To characterize the tyrosine-phosphorylated proteins which were components of the EPOSF complex, extracts prepared from EPO- or IFN-γ-treated TF1 cells were incubated with GRR-agarose beads. The material which bound to the GRR-beads was resolved by SDS-PAGE, transferred to Immobilon, and probed with the antiphosphotyrosine antibody. Both EPO and IFN-γ induced tyrosine-phosphorylated proteins of 84 and 91 kDa that selectively associated with the GRR-beads. Reprobing of the blot with an antibody which recognized both p91 and the 84-kDa spliced variant of p91 showed that while the IFN-γ-induced tyrosine-phosphorylated proteins corresponding to p91 and p84 reacted, none of the EPO-activated proteins of 84 or 91 kDa reacted with the antibody (Fig. 4D, lane 6).

DISCUSSION

We have shown that both GH and EPO can activate cellular proteins which then recognize an enhancer found in the promoters of several early-response genes. These proteins are posttranslationally modified by tyrosine phosphorylation in a way that is critical for their assembly into a DNA-protein complex. Although the precise stoichiometry of assembly remains to be determined, it is known that many transactivating factors form dimers that interact with enhancers. We show that the GH-activated complex contains a protein that is antigenically related to p91, a transcription factor that has recently been shown to mediate activation of the c-*fos* gene promoter through the SIE (10, 32). Within this element is a core sequence that is virtually identical to that seen in the GRR. Unlike IFN-γ-activated p91, however, GH-activated p91 migrates slightly slower, at about 93 kDa (Fig. 4C). The protein also appears to be more heavily tyrosine phosphoryated in response to GH compared with IFN-γ (Fig. 4C, lanes 2 and 3), and this finding may account for its slower mobility in SDS-polyacrylamide gels. The difference in phosphorylation may also explain why in its native form, GHSF is not recognized by anti-p91. However, denaturation followed by separation on SDS-polyacrylamide gels now exposes epitopes recognizable by the antibody.

Unlike GHSF, EPOSF activates a set of proteins that appear to be unique. Although similar in molecular mass (91 and 84 kDa), these proteins are not recognized by two separate antibodies raised against different domains of the p91 protein. In combination with the inability to supershift the retarded complex on EMSA, these data suggest that for EPO, a distinct set of proteins mediate DNA binding. Conceivably, a dimer is formed by the EPO-activated p91 and p84. The lack of anti-p91 reactivity has also recently been shown for GM-CSF-, interleukin-3 (IL-3)-, and IL-4-induced GRR-binding activity (18, 19). IL-3 and GM-CSF appear to activate the tyrosine phosphorylation of at least one (80 kDa) and possibly other proteins that both bind to the same sequence and can be isolated by their ability to bind to GRR-conjugated agarose (19). Therefore, all of these proteins may be part of what appears to be an emerging family that when activated by ligand receptor-mediated tyrosine phosphorylation assemble into DNA-binding complexes that recognize a critical, core sequence in the promoters of several early-response genes.

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