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Cytokines That Associate with the Signal Transducer gp130 Activate the Interferon-induced Transcription Factor p91 by Tyrosine Phosphorylation*

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Gerald M. Feldman, Emanuel F. Petricoin III, Michael David‡, Andrew C. Larner, and David S. Finbloom§

From the Division of Cytokine Biology, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892

Interleukin-6, leukemia inhibitory factor, and oncostatin M exert a broad range of similar biological activities through association of their receptors with the signal-transducing component gp130. Although it is known that these cytokines trigger rapid tyrosine phosphorylation of a common set of cellular proteins as well as induction of several of the same early response genes, the mechanisms by which these genes are activated is not well understood. In this report, we show that interleukin-6, leukemia inhibitory factor, and oncostatin M stimulate the assembly of protein complexes that recognize conserved sequences within the enhancers of two genes (interferon regulatory factor 1 and Fcγ receptor type I) that are rapidly activated by these cytokines. These enhancers are known to be required for transcriptional induction of these genes by interferon-γ. Assembly of the DNA-binding protein complexes occurs within minutes after ligand addition and depends upon tyrosine phosphorylation. These complexes contain the p91 transcription factor, which is tyrosine-phosphorylated in response to these cytokines. An additional tyrosine-phosphorylated protein of 93 kDa can be coimmunoprecipitated with antibodies against p91. These findings further expand the network of cytokines known to activate p91 and, in addition, support the concept that sets of tyrosine-phosphorylated proteins may be responsible for the cytokine-regulated expression of early response genes.

The cytokines interleukin-6 (IL-6),[1] leukemia inhibitory factor (LIF), and oncostatin M (OSM) evoke both unique and overlapping responses in a variety of cells. Their receptors share structural homologies, suggesting that they are part of a subfamily of cytokine receptors (1–3). These three cytokines also associate with an identical second component of the receptor complex, termed gp130, which plays a major role in signal transduction. Although it is known that the binding of IL-6, LIF, and OSM to their respective receptors rapidly triggers tyrosine phosphorylation of several cellular proteins (4–9), no identifiable tyrosine kinase domain exists in either component of the receptor. This finding implies that part of the signal transduction pathway most likely consists of class II or III tyrosine kinases such as those of the src or tyk2 family that associate with these receptors (10).

In as much as these cytokines frequently induce a differentiated phenotype that is dependent upon the stimulation of both early and delayed response genes, it becomes important to identify pathways whereby these genes become activated. Until recently, the only example of cytokine-activated gene expression and tyrosine phosphorylation of cellular proteins that function as positive transcriptional regulators has been for interferons (11–16). Both interferon-α (IFN-α) and interferon-γ (IFN-γ) regulate gene transcription by activation of a tyrosine kinase(s) that phosphorylates a 91-kDa transcription factor, p91 (11, 15, 16). Treatment of cells with IFN-α also results in tyrosine phosphorylation of two related proteins, p84 and p113 (11, 15). These SH2 domain-containing proteins translocate from the membrane and cytoplasm to the nucleus, where they bind as multisubunit complexes to enhancers required for transcriptional activation of specific cellular genes. The list of cytokines known to activate p91 has recently been expanded from just interferons to include growth factors such as epidermal growth factor, platelet-derived growth factor, and macrophage colony-stimulating factor in addition to cytokines such as IL-10 and IL-6 (17–21). Recent investigations have also identified other cytokine-induced (IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor) tyrosine-phosphorylated DNA-binding proteins that bind to the same element as p91 but do not contain p91 (21). Therefore, it appears that a common pathway exists for the activation of genes by cytokines that utilize tyrosine-phosphorylated molecules such as p91 and possibly other related proteins.

M1 cells are a murine myeloid leukemia cell line that differentiate and undergo changes in morphology and cell-surface markers in response to IL-6, LIF, or OSM (22, 23). One of these markers is the FcγRI gene product, the expression of which increases as the cell becomes more macrophage-like (24). An enhancer in this gene termed the IFN-γ response region (GRR) shares a common motif (TTC/ACNNNAA) with other early response genes such as IRF-1 that have been reported to be activated by IL-6, LIF, and OSM (25, 26). This same sequence is required for LIF or IL-6 to induce the expression of acute-phase protein genes in hepatocytes, where nuclear factors bind to this motif in response to either IL-6 or LIF (26). This sequence is also similar to the *sis*-inducible element in the c-*fos* promoter. Sadowski *et al.* (18) have shown that IL-6 will activate a DNA-binding complex using a modified *sis*-inducible element probe termed *sis*-inducible element (m67). Although these investigators describe the activation of proteins that rec-

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§ To whom correspondence should be addressed: Food and Drug Administration, Div. of Cytokine Biology, HFM-505, 8800 Rockville Pike, Bethesda, MD 20892. Tel.: 301-496-0864; Fax: 301-402-1659.

[1] The abbreviations used are: IL-6, interleukin-6; LIF, leukemia inhibitory factor; OSM, oncostatin M; IFN, interferon; FcγRI, Fcγ receptor type I; GRR, IFN-γ response region; IRF-1, interferon regulatory factor 1; IRF-1-E, interferon regulatory factor 1 element; EMSA, electrophoretic mobility shift assay.

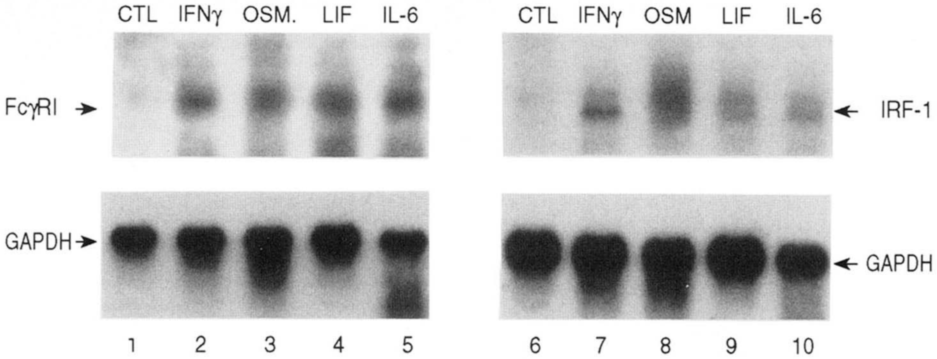

FIG. 1. **IFN-γ, IL-6, LIF, and OSM induce expression of FcγRI and IRF-1 RNAs in M1 myeloid leukemia cells.** M1 cells were either untreated (control (*CTL*); *lanes 1* and *6*) or treated with each individual cytokine for 1 h prior to harvesting total RNA, which was then analyzed by Northern blot hybridization. *Lanes 1–5* were probed with the FcγRI cDNA, and *lanes 6–10* were probed with a cDNA to IRF-1. *Lanes 2* and *7*, IFN-γ; *lanes 3* and *8*, OSM; *lanes 4* and *9*, LIF; *lanes 5* and *10*, IL-6. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

ognize this element, they do not identify any of the proteins that are components of the IL-6-activated DNA-binding complex. Since it has been reported that treatment of cells with cytokines such as IL-6, LIF, and OSM results in tyrosine phosphorylation of several cellular proteins of various molecular sizes (4–9), any of these proteins may be part of a cytokine-activated DNA-binding protein complex. In this report, we investigate whether IL-6, LIF, and OSM activate early response genes by tyrosine phosphorylation of cellular proteins that recognize both the GRR element and the IRF-1 element in these genes.

MATERIALS AND METHODS

Cells and Reagents—Murine M1 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and gentamicin (25 μg/ml). Murine IFN-γ was provided by Genentech and was used at a final concentration of 20 ng/ml. Recombinant IL-6 was obtained from the Genetics Institute and was used at a final concentration of 20 ng/ml. LIF (20 ng/ml) and oncostatin M (30 ng/ml) were purchased from Genzyme Corp.

Measurement of FcγRI and IRF-1 RNAs—RNA was prepared as described (27). Northern blots were hybridized with cDNA probes corresponding to either mouse IRF-1 (28) or FcγRI (29). To ensure that equal concentrations of RNA were present in each sample, we rehybridized the membranes with a cDNA corresponding to glyceraldehyde-3-phosphate dehydrogenase (30).

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 Hepes (pH 7.0), 10 mM KCl, 300 mM NaCl, 0.5 mM dithiothreitol, 0.1% Triton X-100, 200 mM phenylmethylsulfonyl fluoride, 1 mM vanadate, and 20% glycerol). The suspension was gently vortexed for 10 s, incubated at 4 °C for 10 min, and centrifuged at 18,000 × *g*, and the supernatant was transferred to a new tube. Nuclear and cytoplasmic extracts were prepared by Dounce homogenization of cells in extraction buffer without NaCl. The lysate was layered over a sucrose cushion (35% sucrose in 100 mM Hepes (pH 7.0), 20 mM $MgCl_2$), and the nuclei were isolated by centrifugation at 3000 × *g* for 15 min. Nuclei were resuspended in extraction buffer and extracted by vortexing. To the postnuclear supernatant was added 0.3 M NaCl (cytoplasmic extract). Protein concentrations for each type of extract were determined and normalized by the addition of extraction buffer.

Electrophoretic Mobility Shift Assay (EMSA)—EMSAs were performed as previously described (31). The GRR (5′-AGCATGTTTCAAGGATTTGAGATGTATTTCCCAGAAAAG-3′) of the promoter of the FcγRI gene and the IFN-γ-induced element (5′-AGCCTGATTTCCCCGAAATGATGAGGCCGAGTGG-3′) of the mouse IRF-1 gene (32) were 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 GRR, IRF-1, and the IFN-stimulated response element of ISG15 (5′-GATCCATGCCTCGGGAAAGGGAAACCGAAACTGAAGCC-3′) (33).

Immunoprecipitations with Anti-p91—Whole cell extracts were prepared as described above and incubated with anti-p91 for 2 h at 4 °C. The immune complexes were precipitated with protein G-Sepharose, washed, and released in SDS sample buffer. The immunoprecipitates were analyzed by 8% SDS-polyacrylamide gel electrophoresis, followed by protein transfer to Immobilon. The membranes were then probed with biotin-labeled anti-phosphotyrosine antibodies (PY20, ICN) and developed using ECL (Amersham Corp.). The membrane was reprobed with an antibody raised against a peptide common to p91 and its splice variant, p84, followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG. The membrane was then developed using nitro blue tetrazolium chemistry.

RESULTS

IFN-γ, IL-6, LIF, and OSM Activate the Transcription of FcγRI and IRF-1—Several early response genes have been reported to be induced in a variety of cells by treatment with IFN-γ, IL-6, LIF, or OSM (22–24, 26, 32, 34). These genes have included, among others, IRF-1 and FcγRI (22–24, 26, 32, 34). Since murine M1 cells are responsive to IFN-γ, IL-6, LIF, and OSM, we used this cell line to define a set of genes that is activated by all four cytokines. Total cellular RNA was isolated 1 h after treatment with each cytokine, and Northern blot analysis was performed to determine steady-state levels of IRF-1 and FcγRI mRNAs. Incubation of M1 cells with each of these cytokines resulted in a marked increase in the concentrations of both RNAs (Fig. 1). Similar amounts of RNA were applied for each sample as determined by reprobing of the blots with glyceraldehyde-3-phosphate dehydrogenase.

IL-6, LIF, and OSM Treatment of Cells Results in the Activation of Proteins That Specifically Bind to an Enhancer Required for Transcriptional Induction by IFN-γ—The GRR enhancer is required for IFN-γ-induced expression of the FcγRI gene in myeloid cells (35, 36). A similar sequence was identified not only in the promoter of the IRF-1 gene, which is also induced by IFN-γ, but also in the promoters of the interferon consensus sequence-binding protein, guanylate-binding protein, and Ly-6A/E genes (32, 36–38). To determine whether the GRR and the element in the IRF-1 gene (which we have designated IRF-1-E) interacted with specific proteins in response to IL-6, LIF, or OSM, we prepared whole cell extracts after treatment with the individual cytokines at 37 °C for 30 min. EMSAs were done using ^{32}P-labeled oligonucleotide probes corresponding to the GRR or IRF-1-E (Fig. 2*A*). Extracts from cytokine-treated cells contained inducible complexes (indicated by the *arrows*) that interacted with both oligonucleotide probes (compare *lane 1* with *lanes 2–5* and *lane 6* with *lanes 7–10*). The complexes designated IL-6-, LIF-, and OSM-SF (where SF represents stimulated factor) migrated with approximately the same mobility as the previously reported IFN-γ-induced complex. All induced complexes specifically bound the ^{32}P-labeled GRR probe and could be displaced by the addition of excess unlabeled probes corresponding to the GRR (Fig. 2*B*, *lanes 2*

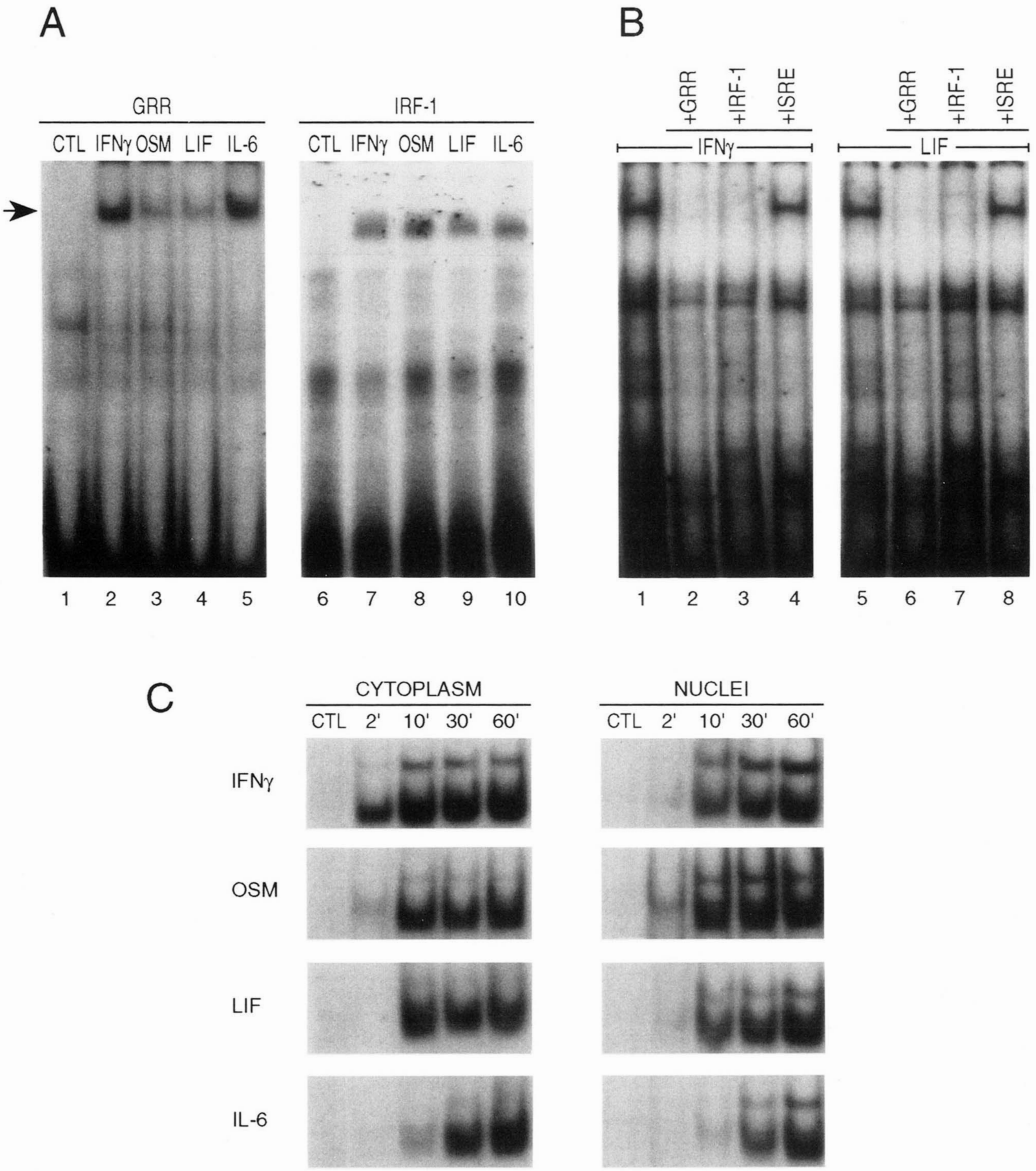

FIG. 2. **IL-6, LIF, and OSM activate a DNA-binding complex that recognizes the GRR and IRF-1 enhancer sequences.** *A*, activation of proteins that bind to the GRR or IRF-1-E. Cells were harvested, resuspended in fresh medium, and either untreated (control (*CTL*); *lanes 1* and *6*) or exposed to IFN-γ (*lanes 2* and *7*), OSM (*lanes 3* and *8*), LIF (*lanes 4* and *9*), or IL-6 (*lanes 5* and *10*) for 30 min at 37 °C prior to preparation of whole cell extracts. DNA-binding complexes were assayed using an EMSA with ^{32}P-labeled probes corresponding to either the GRR (*lanes 1–5*) or to IRF-1-E (*lanes 6–10*). The shifted complexes induced by treatment of the cell with the cytokines are indicated with *arrows*. *B*, competition assays using unlabeled oligonucleotide. Extracts were prepared from cells that had been previously incubated with either IFN-γ (*lanes 1–4*) or LIF (*lanes 5–8*), and competition assays were performed by adding a 50-fold molar excess of unlabeled oligonucleotides corresponding to the GRR (*lanes 2* and *6*), IRF-1-E (*lanes 3* and *7*), or the IFN-simulated response element (*ISRE*) of ISG15 (*lanes 4* and *8*). *C*, cytoplasmic and nuclear localization of cytokine-induced complexes that bind to the GRR. M1 cells were incubated with IFN-γ, IL-6, LIF, and OSM for the indicated times (minutes), and cytoplasmic and nuclear extracts were prepared as described under "Materials and Methods." EMSAs were performed using the GRR oligonucleotide. The appearance of the upper shifted complex was variable.

and *6*) or IRF-1-E (*lanes 3* and *7*), but not by unlabeled oligonucleotide corresponding to the IFN-stimulated response element that is required for induction of genes by IFN-α (*lanes 4* and *8*) (39). The specificity was identical irrespective of the radiolabeled probe (GRR *versus* IRF-1-E) or the cytokine treatment (data not shown).

To determine whether IL-6, LIF, and OSM, like IFN-γ (40–42), activated complexes that could be detected within the

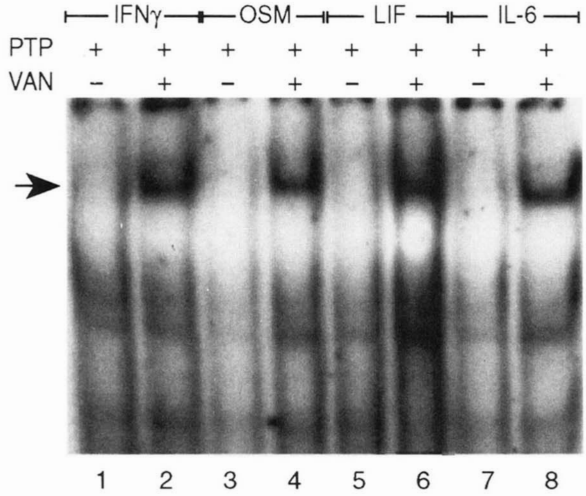

FIG. 3. **Phosphorylated tyrosine is required for assembly of the DNA-binding complexes.** Whole cell extracts were prepared from M1 cells treated with IFN-γ, IL-6, LIF, and OSM for 30 min and from control cells. Control extracts contained no GRR-binding complexes. Extracts from cytokine-treated cells were incubated with 1 mg of purified recombinant *Y. enterocolitica* protein-tyrosine phosphatase (*PTP*) for 30 min at 30 °C in the absence (*lanes 1*, *3*, *5*, and *7*) or presence (*lanes 2*, *4*, *6*, and *8*) of 1 mM orthovanadate (*VAN*; a specific protein-tyrosine phosphatase inhibitor). The extracts were then assayed by EMSA with a ^{32}P-labeled GRR probe.

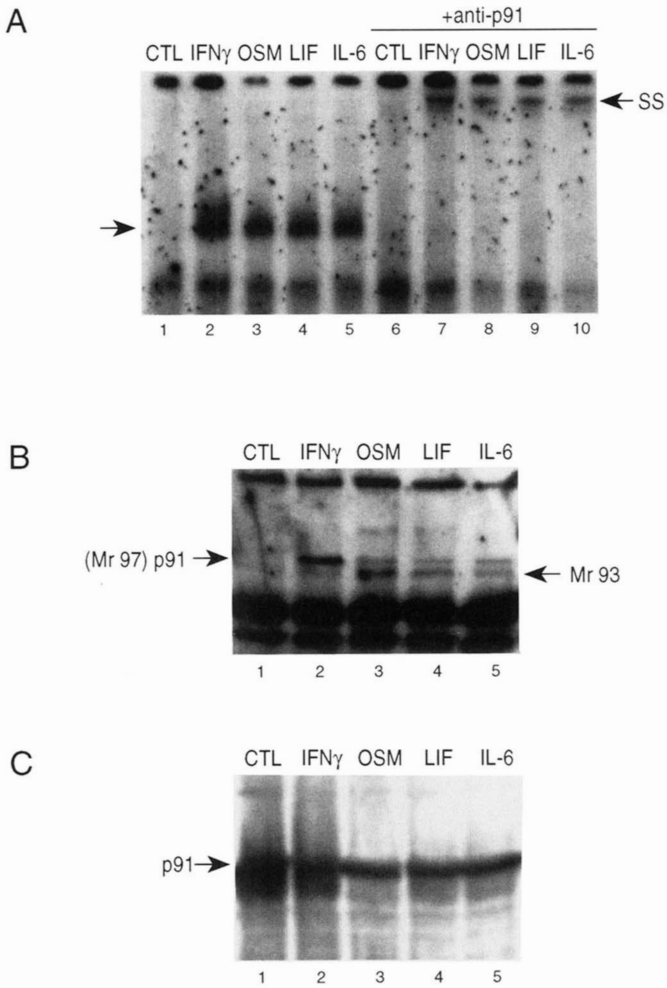

FIG. 4. **Analysis of the DNA-binding complexes activated by IL-6, LIF, and OSM.** *A*, analysis of DNA-binding complexes by antibody supershift assays. Whole cell extracts were prepared from M1 cells treated with the indicated cytokines for 30 min at 37 °C. They were then incubated at 4 °C with anti-p91 antibody for 1 h (*lanes 6–10*) or with an antibody that reacts with the 113-kDa protein (which is a component of the ISGF3 transcription complex) (*lanes 1–5*) and assayed for GRR binding by EMSA. The supershifted (*SS*) complex demonstrating the presence of p91 is indicated with an *arrow*. *CTL*, control. *B*, tyrosine phosphorylation of immunoprecipitated p91 in IFN-γ (*lane 2*)-, OSM (*lane 3*)-, LIF (*lane 4*)-, or IL-6 (*lane 5*)-treated cells. Cells (5×10^7) were treated with cytokines for 30 min at 37 °C prior to preparation of cell extracts. Immunoprecipitated p91 from the extracts was resolved on SDS-polyacrylamide gel electrophoresis and transferred to Immobilon. After probing with an anti-phosphotyrosine antibody, the reactive proteins were visualized with ECL. *C*, the same blot shown in *B* reprobed with an anti-p91/p84 antibody raised against a peptide corresponding to amino acids 607–647 of p91/p84 and developed with an alkaline phosphatase-conjugated secondary antibody.

nucleus, we prepared cytoplasmic and nuclear extracts from M1 cells treated with these cytokines (Fig. 2*C*). Within 2 min of treatment with IFN-γ, the complex was detected within the cell. There was a slightly slower assembly of OSM-SF, LIF-SF, and IL-6-SF. Coincident with the appearance of the complexes in the cytoplasm, there was cytokine-induced GRR binding activity in the nucleus. As with IFN-γ in monocytes (31), there was no clear-cut translocation such that cytoplasmic concentrations of GRR-binding complexes decreased while nuclear concentrations increased. However, there was still a clear nuclear localization of these complexes.

Assembly of the DNA-binding Complexes Requires Tyrosine Phosphorylation—The assembly of all known IFN-activated transcription complexes requires that certain components of the complex be tyrosine-phosphorylated. Tyrosine phosphorylation of these proteins, including p91, is regulated by membrane-associated, IFN-activated tyrosine phosphatase(s) and tyrosine kinase(s) (11, 14, 43). To determine whether the assembly of IL-6-SF, LIF-SF, and OSM-SF also required phosphorylated tyrosine residues, we prepared extracts from cytokine-treated cells and incubated them with recombinant purified phosphotyrosine phosphatase from *Yersinia enterocolitica* (44). Phosphotyrosine phosphatase treatment of each extract disrupted those complexes induced by all four cytokines such that they no longer bound to the GRR element (Fig. 3). The presence of the phosphotyrosine phosphatase inhibitor orthovanadate prevented the effects of the phosphotyrosine phosphatase (compare *lanes 1*, *3*, *5*, and *7* with *lanes 2*, *4*, *6*, and *8*). Although not shown in Fig. 3, the phosphotyrosine phosphatase had no effect on the ^{32}P-labeled oligonucleotide probe (45).

Characterization of Tyrosine-phosphorylated Proteins Activated by IL-6, LIF, and OSM—We next investigated whether the IFN-induced, tyrosine-phosphorylated p91 protein was a component of IL-6-SF, LIF-SF, and OSM-SF. Extracts prepared from cells treated with these cytokines were incubated with anti-p91 prepared against the carboxyl-terminal 39 amino acids unique to the p91 protein. After incubation of the extracts with the antibody, EMSAs were performed with the GRR probe (Fig. 4*A*). The p91 antibodies "supershifted" the IFN-γ-activated complex, which is known to contain p91 (*lane 1 versus 2*) (25, 45, 46), as well as the complexes formed by treatment with OSM, LIF, and IL-6 (*lane 3 versus 8*, *lane 4 versus 9*, and *lane 5 versus 10*). Antiserum against the IFN-α-activated p113 protein, which was used as a control, did not supershift any of the complexes (*lanes 1–5*). Similar results were observed when EMSAs were performed with the IRF-1-E probe (data not shown).

To determine whether the p91 transcription factor was tyrosine-phosphorylated in response to IL-6, LIF, and OSM, we incubated cells with these cytokines or with IFN-γ (as a control). Cell lysates were made and subjected to immunoprecipitation with anti-p91 (Fig. 4*B*). Tyrosine-phosphorylated p91 was detected not only in cells treated with IFN-γ (*lane 2*), but also in cells incubated with the other cytokines (*lanes 3–5*). The phosphorylated form of p91 migrates at 97 kDa. The immunoprecipitates of the cells treated with IL-6, LIF, and OSM also contained another tyrosine-phosphorylated species of ~93 kDa. This lower molecular mass band was of equal or greater intensity when compared to the p91 band. When this phosphotyrosine blot was reprobed with an antibody that recognized both p91 and its 84-kDa spliced variant, the antibody reacted only with tyrosine-phosphorylated p91 and not with the 93-kDa protein (Fig. 4*C*). Since the phosphorylated (*lane 2*) and nonphosphorylated (*lane 1*) p91 proteins migrated similarly, any reactivity of the 93-kDa protein with the anti-p84/p91 antibody would readily be observed. In as much as anti-p84/p91 did not react with the 93-kDa protein, this suggested that the 93-kDa protein was coimmunoprecipitated with p91 during the anti-p91 immunoprecipitation.

DISCUSSION

IL-6, LIF, and OSM affect growth and differentiation in a variety of target cells including hematopoietic, neuronal, and embryonic cells (5, 6, 8, 22, 47). In many cell lines, the activities of these cytokines are similar in that they enhance IL-3-dependent colony formation of primitive blast cells, induce acute-phase proteins in hepatocytes, and induce differentiation of neuronal cells and M1 leukemia cells. Their similar actions can be accounted for by the fact that they are structurally related (48) and that they, along with ciliary neurotrophic factor, use the gp130 signaling protein as a mediator for signal transduction (2, 3). The specificity of the affects of these cytokines resides in the cell-restricted expression of the ligand-specific subunit of the receptor complex to which IL-6, LIF, OSM, or ciliary neurotrophic factor binds (2, 3). Another mechanism that allows cytokines of this family to exert selective effects is that LIF and ciliary neurotrophic factor binding induces the formation of heterodimers of their receptors with gp130, while IL-6 induces homodimerization of gp130 (9, 49). Whether OSM treatment results in homo- or heterodimerization of gp130 has not been determined.

It is evident from this study that a set of early response genes can be activated by IFN-γ, IL-6, LIF, and OSM. Within the promoter for these two genes (FcγRI and IRF-1), there are elements to which cytokine-activated protein complexes bind. We go on to show that these rapidly activated complexes contain tyrosine-phosphorylated proteins of which at least one component is the p91 transcription factor. It has recently been appreciated that p91 is no longer limited to interferon-induced DNA-binding complexes. Cytokines such as IL-10, platelet-derived growth factor, and epidermal growth factor activate DNA-binding protein complexes that contain tyrosine-phosphorylated p91. Although it has been reported that IL-6 induces *sis*-inducible element binding activity, there was no further investigation as to whether or not this activity contains p91 (18). The data from the supershift experiments (Fig. 4*A*) clearly demonstrate the presence of p91 in all cytokine-activated extracts.

In addition to tyrosine phosphorylation of p91, treatment of M1 cells with these cytokines also resulted in tyrosine phosphorylation of a protein of 93 kDa that coimmunoprecipitated with p91 (Fig. 4*B*). This phosphoprotein is not recognized by an antibody raised against a peptide common to both p91 and the p84 splice variant (Fig. 4*C*). This antibody recognizes both p91 and p84 in IFN-α-treated cells.[2] This protein may be similar to the DNA-binding protein identified by Wegenka *et al.* (26) that is IL-6-induced, has a molecular mass of ~110 kDa as determined by Southwestern analysis, and binds to an element similar to the GRR that is found in the promoter of the acute-phase reactant protein α_2-macroglobulin. The 93-kDa protein may also be similar to p88 or p89 recently described by Bonni *et al.* (50). These two proteins are tyrosine-phosphorylated in response to ciliary neurotrophic factor and are coimmunoprecipitated with anti-p91.

The 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 (a related protein) (51). Therefore, it is conceivable that the induction of genes such as FcγRI and IRF-1 by cytokines like IL-6, LIF, and OSM may depend upon the dimerization of p91 with the 93-kDa protein. Other proteins may participate in forming these complexes and may provide a combinatorial mechanism that allows for the specific effects of IL-6, LIF, or OSM. Presumably, the SH2 domains within proteins like p91 may provide a basis by which they can associate. Characterization of the 93-kDa protein as well as the tyrosine kinase(s) that participate in signal transduction will allow a better understanding of the outlines for this new signaling cascade that appears to regulate gene expression by tyrosine phosphorylation.

[2] E. F. Petricoin III and M. David, unpublished observations.

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