REPORTS

Association of Transcription Factor APRF and Protein Kinase Jak1 with the IL-6 Signal Transducer gp130

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Interleukin-6, leukemia inhibitory factor, oncostatin M, interleukin-11, and ciliary neurotrophic factor bind to receptor complexes that share the signal transducer gp130. Upon binding, the ligands rapidly activate DNA binding of acute-phase response factor (APRF), a protein antigenically related to the p91 subunit of the interferon-stimulated gene factor–3α (ISGF-3α). These cytokines caused tyrosine phosphorylation of APRF and ISGF-3α p91. Protein kinases of the Jak family were also rapidly tyrosine phosphorylated, and both APRF and Jak1 associated with gp130. These data indicate that Jak family protein kinases may participate in IL-6 signaling and that APRF may be activated in a complex with gp130.

Interleukin-6 (IL-6), leukemia inhibitory factor (LIF), oncostatin M (OSM), and ciliary neurotrophic factor (CNTF) are members of a family of cytokines and neuronal differentiation factors (1) or neurokines (2). These factors exert pleiotropic effects on multiple cell types and bind to composite receptors containing the signal transducer gp130 (3). The neurokine receptors belong to a superfamily of cytokine receptors sharing both structural and functional features (4). Cytokine receptors initiate related signaling pathways characterized by association with and activation of protein tyrosine kinases of the Jak family (5, 6) and the recruitment of latent cytoplasmic transcription factors of the ISGF-3α family by tyrosine phosphorylation and subsequent nuclear translocation (7–10).

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The IL-6 signal transduction pathway has not yet been elucidated, but the involvement of Jak family members has been proposed (11). A latent cytoplasmic transcription factor, APRF, is rapidly activated in response to IL-6, LIF, OSM, IL-11, and CNTF (12, 13). After activation, the 89-kD protein binds to IL-6 response elements identified in the promoter regions of various IL-6–induced plasma-protein and immediate-early genes (12–14). The binding specificity of APRF is shared by the interferon γ (IFN-γ)–activation factor (GAF) (14), which is identical to the ISGF-3α p91 protein (8, 10). Because APRF cross-reacts with an antiserum raised against the ISGF-3α p91 NH_2-terminus, it is likely to be related to p91 (13). Thus, the signaling pathway of IL-6 may be similar to that induced by other cytokines.

Because activation of ISGF-3α by IFN-α involves tyrosine phosphorylation of the p113, p91, and p84 components and GAF activation by IFN-γ requires the tyrosine phosphorylation of p91 (9, 10), we investigated whether APRF becomes tyrosine phosphorylated in response to IL-6. Lysates from untreated or IL-6–treated human hepatoma (HepG2) cells were subjected to immunoprecipitation with an antiserum to the NH_2-terminus of p91 and p84 (anti-p91,p84) and analyzed by an immunoblot with monoclonal antibodies to phosphotyrosine. The IL-6 induced the appearance of two major tyrosine-phosphorylated protein bands of 91 and 89 kD (Fig. 1A). The 91-kD band was also observed after IFN-γ treatment of HepG2 cells (Fig. 1A) and was immunoprecipitated by other antisera to p91, demonstrating its identity with ISGF-3α p91 (15). Tyrosine-phosphorylated p91 has a higher apparent molecular size after SDS–polyacrylamide gel electrophoresis (PAGE) than unphosphorylated p91 (10). In fact, when immunoblotted with antise-

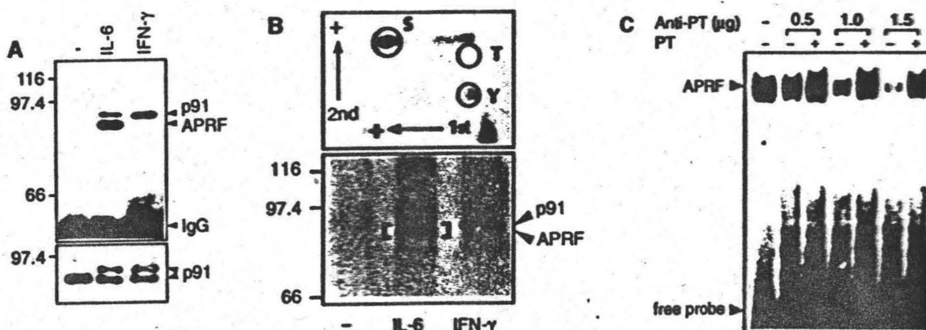

Fig. 1. Tyrosine phosphorylation of APRF and ISGF-3α p91 in response to IL-6 (A) HepG2 cells (10^7) were treated for 20 min without or with human IL-6 (100 units/ml) or IFN-γ (25 ng/ml). Cells were rinsed with cold phosphate-buffered saline and lysed in 0.5-ml lysis buffer (24) for 20 min at 0°C. Proteins in the lysates were immunoprecipitated with anti-p91,p84 (4 μl) (25). Immune complexes were separated by SDS-PAGE (7% gel), transferred to polyvinyldifluoride membrane (Qiabrane, Diagen), and probed with monoclonal antibodies to phosphotyrosine (PY20, ICN). To verify application of equal protein amounts, we stripped and reprobed the blot with antiserum to p91 (25). The unphosphorylated and phosphorylated forms of p91 (lower and upper band, respectively) are indicated by arrowheads (26). IgG, immunoglobulin G. Molecular size markers are indicated on the left in kilodaltons. (B) Phosphoamino acid analysis of APRF. The ^{32}P-labeled HepG2 cells were stimulated with IL-6 or IFN-γ as above. The APRF and p91 were immunoprecipitated with anti-p91,p84, protein immunoblotted, and visualized by autoradiography (lower panel). The phosphorylated APRF band (brackets) was excised and subjected to phosphoamino acid analysis (27) (upper panel). The positions of unlabeled phosphoamino acid standards are indicated. S, serine; T, threonine; and Y, tyrosine. (C) Inhibition of binding of APRF to DNA by antibodies to phosphotyrosine. Nuclear extracts were prepared from HepG2 cells treated for 15 min with IL-6 (100 units/ml) as described (12) and were incubated overnight at 0°C with antibodies to phosphotyrosine (anti-PT) in the absence or presence of phosphotyrosine (1 mM). The DNA binding of APRF was examined in a gel retardation assay (28).

rum to p91, the IL-6– and IFN-γ–induced appearance of a retarded p91 band was observed (Fig. 1A). Activation of p91 DNA binding by IL-6 was also demonstrated by gel retardation assays (*15*).

The 89-kD phosphoprotein band induced by IL-6 corresponds to APRF (*16*). After immunoprecipitation from IL-6–treated, ^{32}P-labeled HepG2 cells, phosphoserine and phosphotyrosine but no phosphothreonine were detected in an APRF protein hydrolysate (Fig. 1B). The presence of phosphotyrosine in the DNA-binding form of APRF was confirmed by the observation that incubation with antiphosphotyrosine antibodies specifically interfered with the formation of the APRF-DNA complex (Fig. 1C). These findings show that both ISGF-3α p91 and APRF are tyrosine phosphorylated in response to IL-6. The time course of this effect (*15*) corresponded closely to that determined for the induction of APRF DNA-binding activity (*12*), indicating that latent APRF is activated by tyrosine phosphorylation.

Tyrosine protein kinase activity coprecipitates with gp130 from IL-6–treated cells (*17*). Antisera to the Jak family members Tyk2 and Jak1 were used to test whether these kinases respond to IL-6 stimulation of HepG2 cells. Both Jak1 and, to a lesser extent, Tyk2 were transiently tyrosine phosphorylated in response to IL-6 (Fig. 2, A and B). Also, LIF, OSM, CNTF, and IL-11 stimulated the tyrosine phosphorylation of Jak1, as well as of APRF and p91 (Fig. 2C). Thus, phosphorylation of these proteins appears to be generally induced upon activation of gp130. The magnitude of the response to different cytokines varied, but the relative extents of APRF and Jak1 phosphorylation changed coordinately. Furthermore, the time course of IL-6–induced Jak1 and Tyk2 tyrosine phosphorylation matched well with the one observed for APRF (*15*). Therefore, Jak family members may be involved in the tyrosine phosphorylation and activation of APRF.

The tyrosine phosphorylation of Jak1 was also induced by IFN-γ (Fig. 2C). In contrast to IL-6, which only transiently activates APRF and p91, IFN-γ induces p91 DNA binding activity for several hours in HepG2 cells (*14*). Similarly, IFN-γ induced the tyrosine phosphorylation of both p91 and JAK1 for at least 1 hour (*15*), confirming a close correlation between tyrosine phosphorylation of members of the Jak and ISGF3α families.

To examine whether Jak family members may be associated with gp130, we studied the possibility of coprecipitations of Jak1 and gp130. When lysates from IL-6–treated HepG2 cells were immunoprecipitated with antiserum to Jak1, coprecipitation of tyrosine-phosphorylated gp130 was observed (Fig. 3) (*18*). Reciprocally, Jak1 was coprecipitated upon immunoprecipitation of gp130. Stimulation by IL-6 was not required for this effect (Fig. 3), indicating that Jak1 constitutively interacts either directly or indirectly with gp130 and hence is

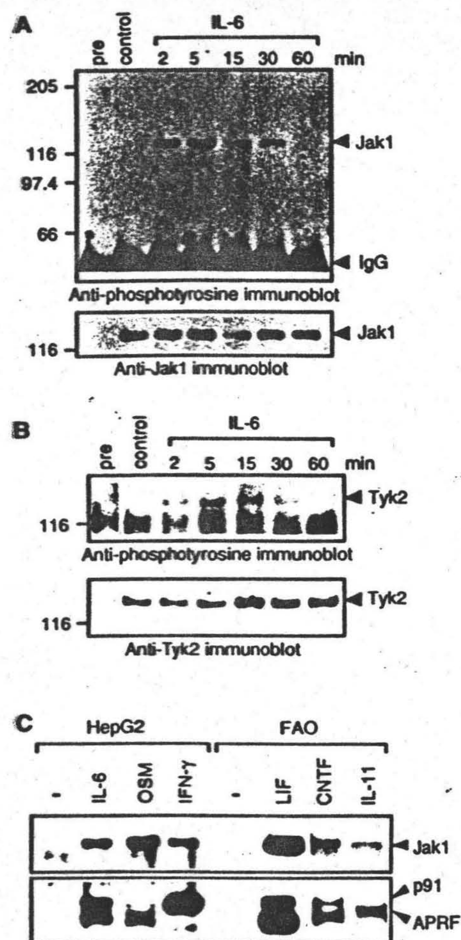

Fig. 2. Tyrosine phosphorylation of Jak family protein kinases in response to IL-6 and other cytokines. **(A)** Time course of Jak1 phosphorylation in response to IL-6. Lysates from HepG2 cells treated with IL-6 (200 units/ml) for various periods (labeled in minutes) were immunoprecipitated with antiserum to Jak1 (*6*) or preimmune serum (pre) and immunoblotted with antibodies to phosphotyrosine (upper panel). The position of Jak1 (130 kD) is indicated. We verified equal protein load by reprobing with antiserum to Jak1 (lower panel). **(B)** Time course of Tyk2 phosphorylation in response to IL-6, analyzed as described above for Jak1 (*29*). The position of Tyk2 (134 kD) is indicated. **(C)** Induction of Jak1, APRF, and ISGF-3α p91 tyrosine phosphorylation by different cytokines. The HepG2 cells were stimulated for 10 min without or with human IL-6 (200 units/ml), OSM (20 ng/ml), or IFN-γ (25 ng/ml), and FAO cells (*30*) were treated for 10 min with human LIF (25 ng/ml), rat CNTF (20 ng/ml), or human IL-11 (50 ng/ml). Cell lysates were immunoprecipitated with antiserum to Jak1 or anti-p91,p84 and immunoblotted with antibodies to phosphotyrosine (*31*).

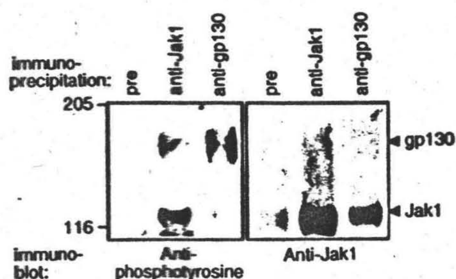

Fig. 3. Association of Jak1 with the IL-6 signal transducer gp130. Untreated HepG2 cells (right panel) or cells treated for 5 min with IL-6 (200 units/ml) (left panel) were washed with ice-cold phosphate-buffered saline and collected. Cells were cross-linked (*32*) and lysed, and proteins were immunoprecipitated with preimmune serum (pre) or antibodies to gp130 or Jak1 as indicated. The cross-linker was then cleaved upon boiling in sample buffer, and proteins were analyzed by immunoblotting with antibodies to phosphotyrosine or Jak1, as indicated.

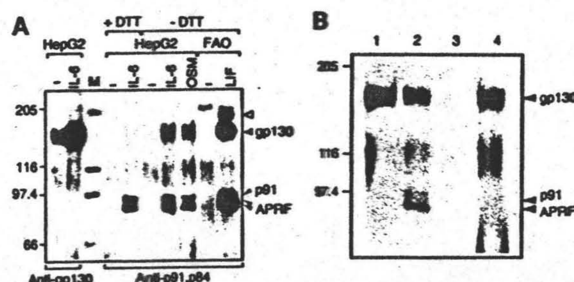

Fig. 4. Coprecipitation of gp130 with APRF from IL-6–stimulated hepatoma cells. **(A)** HepG2 or FAO cells were treated for 10 min with human IL-6 (200 units/ml), OSM (20 ng/ml), or LIF (25 ng/ml). Cell lysates prepared in the presence or absence of dithiothreitol (DTT) were immunoprecipitated with monoclonal antibodies to gp130 or with anti-p91,p84, as indicated, and were immunoblotted with antibodies to phosphotyrosine. The open arrowhead indicates the position of the coprecipitated 190-kD protein after LIF treatment. Lane M shows molecular size markers (labeled on left in kilodaltons). **(B)** The coprecipitated 145-kD phosphoprotein reacts with antibodies to gp130. HepG2 cells treated for 10 min with IL-6 (200 units/ml) were lysed and subjected to immunoprecipitation with antiserum to gp130 (lane 1) or with anti-p91,p84 in the absence of DTT (lane 2). The anti-p91,p84 immunoprecipitates from 2 × 10^7 cells were washed with lysis buffer without DTT (lane 3) or with 1 mM DTT (lane 4). The supernatants of both wash steps were subjected to a second immunoprecipitation with antibodies to gp130 (*33*). All immunoprecipitates were then analysed by immunoblotting with antibodies to phosphotyrosine.

likely to represent at least part of the tyrosine kinase activity coprecipitated with gp130.

Homodimerization of gp130 and activation of its associated protein kinase activity have only been observed in the absence of reducing agents (*17*). Thus, disulfide bridges appear to be important in the formation of an active receptor complex. When immunoprecipitation of hepatoma-cell lysates with anti-p91,p84 was performed under nonreducing conditions, a phosphoprotein similar in size (145 kD) to gp130 was coprecipitated from IL-6–treated HepG2 cells (Fig. 4A). The 145-kD protein could be released from the immune complexes by dithiothreitol and then immunoprecipitated by antibodies to gp130, proving its identity with gp130 (Fig. 4B). Several antisera to p91 that do not recognize APRF failed to coprecipitate gp130 (*15*). These experiments do not show whether the association of APRF and gp130 is direct or mediated by other proteins. However, because coprecipitation of gp130 is observed only in the absence of dithiothreitol, an active gp130 homodimer seems to be required for its interaction with APRF. Therefore, association of APRF with gp130 is likely to be ligand-induced and may direct the factor into a complex with gp130-associated tyrosine kinases. Similarly, a ligand-induced association of p91 with the epidermal growth factor receptor has recently been reported (*19*). After treatment of rat hepatoma (FAO) cells with LIF, gp130 and a 190-kD protein were coprecipitated with anti-p91,p84 (Fig. 4A). This protein probably represents the LIF receptor, which heterodimerizes with gp130 upon LIF treatment (*20*). Although OSM has been proposed to bind to a gp130-LIF receptor heterodimer (*21*), only gp130 was coprecipitated from OSM-treated HepG2 cells (Fig. 4A).

These data indicate that the signaling cascade induced by IL-6 and related factors is similar to that initiated by other cytokines and is characterized by activation and tyrosine phosphorylation of ISGF-3α-related transcription factors and of Jak family protein kinases. The observation that the transcription factor APRF associates with the gp130 signal transducer upon IL-6 stimulation suggests that a transcription factor can be regulated by its physical interaction with a plasma membrane receptor.

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16. The same 89-kD band was immunoprecipitated with anti-p91,p84 from an APRF preparation purified by specific DNA-affinity chromatography, proving its identity with APRF (*13*).
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18. Coprecipitation of Jak1 and gp130 was enhanced by a cleavable cross-linker but could also be observed without cross-linking. Tyk2 also coprecipitated with gp130 from HepG2 lysates, albeit to a smaller extent than Jak1 (*15*).
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24. The lysis buffer contained 50 mM tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM sodium orthovanadate, 1 mM NaF, 0.75 mM phenylmethylsulfonyl fluoride, 15% glycerol, and 10 μg/ml each of aprotinin, pepstatin, and leupeptin.
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26. Because APRF is not detected on immunoblots by anti-p91,p84, only the amount of p91 protein could be analyzed on the blot.
27. The HepG2 cells were labelled with [^{32}P]orthophosphate (1 mCi/ml) for 1 hour in phosphate- and serum-free medium. After stimulation with IL-6 or IFN-γ, the cells were lysed, immunoprecipitated with anti-p91,p84, separated by SDS-PAGE, and blotted to Qiabrane. The APRF band was excised and hydrolysed for 1 hour at 110°C in 6 N HCl. Phosphoamino acid analysis was performed by two-dimensional thin-layer electrophoresis (*22*).
28. Gel retardation assays were done as described (*12*). The ^{32}P-labeled synthetic oligonucleotide 5'-GATCCTTCTGGGAATTCCTA-3' (upper strand) representing the proximal APRF binding site of the rat $α_2$-macroglobulin promoter (*12*) was used as a probe.
29. Antiserum to Tyk2 was raised and affinity-purified against a glutathione-S-transferase fusion protein containing a portion of human Tyk2.
30. We used FAO cells to measure the effect of LIF, CNTF, and IL-11 on Jak1, p91, and APRF tyrosine phosphorylation because HepG2 cells responded poorly to these cytokines (*15*).
31. In several lanes of Fig. 2C, two APRF bands (89 and 87 kD) are observed. These are caused by a different serine phosphorylation status of APRF (*15*).
32. Cross-linking was performed by incubation of pelleted cells in phosphate-buffered saline with 100 μM sodium orthovanadate and the cleavable cross-linker dithiobis-succinimidyl-propionate (DSP, 0.5 mM) for 30 min at 4°C as described (*23*).
33. The monoclonal antibodies to gp130 used for immunoprecipitation did not recognize gp130 in immunoblot experiments. Therefore, direct detection of gp130 on the blot was not possible.

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