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The Interleukin-6-Activated Acute-Phase Response Factor Is Antigenically and Functionally Related to Members of the Signal Transducer and Activator of Transcription (STAT) Family

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Interleukin-6 (IL-6), leukemia inhibitory factor, oncostatin M, IL-11, and ciliary neurotropic factor are a family of cytokines and neuronal differentiation factors which bind to composite plasma membrane receptors sharing the signal transducing subunit gp130. We have shown recently that IL-6 and leukemia inhibitory factor rapidly activate a latent cytoplasmic transcription factor, acute-phase response factor (APRF), by tyrosine phosphorylation, which then binds to IL-6 response elements of various IL-6 target genes. Here we demonstrate that APRF is activated by all cytokines acting through gp130 and is detected in a wide variety of cell types, indicating a central role of this transcription factor in gp130-mediated signaling. APRF activation is also observed in vitro upon addition of IL-6 to cell homogenates. Protein tyrosine kinase inhibitors block both the tyrosine phosphorylation and DNA binding of APRF. The factor was purified to homogeneity from rat liver and shown to consist of a single 87-kDa polypeptide, while two forms (89 and 87 kDa) are isolated from human hepatoma cells. As reported earlier, the binding sequence specificity of APRF is shared by gamma interferon (IFN-γ) activation factor, which is formed by the Stat91 protein. Partial amino acid sequence obtained from purified rat APRF demonstrated that it is likely to be related to Stat91. In fact, an antiserum raised against the amino-terminal portion of Stat91 cross-reacted with APRF, suggesting the relatedness of APRF and Stat91. Altogether, these data indicate that APRF belongs to a growing family of Stat-related proteins and that IFN-γ and IL-6 use similar signaling pathways to activate IFN-γ activation factor and APRF, respectively.

Communication between cells interacting in the immune and hematopoietic systems is mediated by a class of soluble polypeptides generally referred to as cytokines. Most cytokines exert multiple effects on different cell types, a typical example being interleukin-6 (IL-6), which during injuries and infections is released by monocytes, endothelial cells, fibroblasts, and other cells. IL-6 is involved in the differentiation of B and T cells, acts as myeloma growth factor, and is the main mediator of the acute-phase response in the liver (reviewed in references 23 and 33). IL-6 specifically binds to a cell surface receptor which consists of two types of subunits, the ligand-binding glycoprotein gp80 and the signal transducer gp130 (24, 67). Binding of IL-6 to gp80 induces homodimerization and tyrosine phosphorylation of gp130 (42) and results in activation and tyrosine phosphorylation of gp130-associated members of the JAK (janus kinase) family of protein tyrosine kinases (39, 59). Several immediate-early genes (e.g., the *junB*, interferon [IFN] regulatory factor 1, and intercellular adhesion molecule 1 [ICAM-1] genes) are induced via gp130 in various target cells (2, 37, 43). In liver, IL-6 is known to induce the synthesis of the acute-phase plasma proteins which play a protective role during the acute-phase reaction (23). Two types of IL-6-responsive enhancer elements (IL-6REs) have been identified in target genes. One occurs in most acute-phase protein genes and contains binding sites for NF-IL6 and its rat homolog IL-6DBP/LAP, nuclear factors of the C/EBP family which are posttranslationally activated in response to IL-6 (3, 17, 47). We have previously described another transcription factor, acute-phase response factor (APRF), which is present in liver and hepatoma cells and binds to a second type of IL-6RE which is also found in several acute-phase protein genes (62). Moreover, we identified IL-6REs binding APRF in the promoters of several IL-6-induced immediate-early genes, indicating a more general involvement of this factor in the transcriptional control of IL-6 target genes (38, 70). APRF is rapidly activated by IL-6 and leukemia inhibitory factor (LIF) at the posttranslational level (62). Furthermore, we recently demonstrated that IL-6 induces the phosphorylation of APRF at tyrosine residues (39).

The DNA-binding sequence specificity of APRF is very similar to that of IFN-γ activation factor (GAF), suggesting a relatedness of the two factors (70). GAF is member of a family of cytoplasmic transcription factors activated by tyrosine phosphorylation (16, 56) which is referred to as the STAT (signal transducer and activator of transcription) family of proteins

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(57). IFN-γ induces the tyrosine phosphorylation of Stat91, which then homodimerizes to form GAF (56), and IFN-α induces that of Stat91, Stat84, and Stat113, which form the IFN-stimulated gene factor 3-α (ISGF3α) (54). Members of the Stat family have recently also been implicated in the signaling pathways of several other cytokines (e.g., IL-3, IL-5, IL-10, and granulocyte macrophage colony-stimulating factor [GM-CSF]) as well as of platelet-derived growth factor and epidermal growth factor (35, 50, 51).

In the present study, we provide evidence that APRF and GAF are related proteins and are activated by similar signaling events. APRF, although different from known Stat proteins, is likely to represent a novel member of the Stat family. In addition, we demonstrate that APRF is rapidly activated by all cytokines acting through the IL-6 receptor signal transducer gp130 and is present in a variety of nonhepatic cell types. These data lead to the conclusion that a ubiquitous pathway of gp130-mediated signaling involves, via the activation of a protein tyrosine kinase, the tyrosine phosphorylation of APRF, which in turn carries the signal to the nucleus.

MATERIALS AND METHODS

Cytokines, antibodies, and reagents. Cytokines used for stimulation of cells were recombinant human IFN-γ (a gift from Bioferon, Laupheim, Germany), recombinant rat ciliary neurotropic factor (CNTF), recombinant human oncostatin M (OSM), recombinant human LIF (generously provided by N. Nicola, Melbourne, Australia), recombinant human IL-11 (a gift from Genetics Institute, Cambridge, Mass.), and recombinant human IL-6 (a gift from T. Kishimoto and T. Hirano, Osaka, Japan). IL-6 activities are given in B-cell stimulatory factor 2 units. Antisera raised in rabbits against an amino-terminal portion (amino acids 2 to 66) of the p84 and p91 subunits of ISGF3α (anti-p91/84-NH_2), against another fragment (amino acids 598 to 705) of these proteins (anti-Stat91/84), against the Stat91 carboxy terminus (anti-p91), and specific for Stat113 (anti-p113) have been described previously (18, 53). Antiphosphotyrosine monoclonal antibody PY20 was purchased from ICN. An APRF peptide antiserum (anti-APRF) was obtained by immunizing rabbits with the synthetic peptide MLEQHLQNVRKRVQDLE coupled to keyhole limpet hemocyanin. The protein kinase inhibitors staurosporine and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) were purchased from Sigma, and genistein, tyrphostin, sphingosine, and *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W7) were purchased from Gibco-BRL.

Cell culture. Human hepatoma (HepG2) cells were grown in Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (1:1, vol/vol), supplemented with 10% fetal calf serum (FCS) and 100 U each of penicillin and streptomycin per ml. For stimulation with cytokines, cells were grown to confluency and the cytokines were added to the medium. Primary rat hepatocytes were prepared and cultured as described previously (48). NIH 3T3 cells were cultured in DMEM–10% FCS, MonoMac 6 cells (a generous gift from H. Ziegler-Heitbrock and G. Riethmüller, Munich, Germany) were cultured in RPMI 1640–10% FCS (71), and P3X63 Ag8.653 cells (32) were cultured in RPMI 1640–20% FCS. B9 murine plasmacytoma cells (1) were grown in Iscove's modified DMEM–5% FCS with 2-mercaptoethanol (3.5 μl/liter of medium) and IL-6 (10 U/ml); 18 h prior to stimulation with IL-6, B9 cells were washed and transferred to medium without IL-6.

Extract preparation. Nuclear extracts from cultured cells and rat livers were prepared as described by Shapiro et al. (55) and Gorski et al. (20), respectively, with the modifications previously described (62).

HepG2 whole-cell extracts were prepared as follows. Cells were rinsed twice with ice-cold phosphate-buffered saline (PBS), scraped off the plates in PBS, pelleted, and then lysed by incubation in lysis buffer {50 mM Tris HCl [pH 8.0], 10 mM 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), 2 mM EDTA, 100 μM sodium orthovanadate, 5 mM NaF, 1 mM dithiothreitol, 750 μM phenylmethylsulfonyl fluoride, 10 μg each of aprotinin, pepstatin, and leupeptin per ml} for 20 min at 0°C. Insoluble material was spun down for 5 min in a microcentrifuge.

APRF purification and peptide microsequencing. Sixty rats (Sprague-Dawley, 300 to 450 g) were injected intraperitoneally with bacterial lipopolysaccharide (LPS) (10 mg/kg of body weight; Sigma). After 1 h, the animals were killed by asphyxiation, and the livers were removed. Nuclear extract prepared from these livers was used as starting material for APRF purification, which was performed by a previously described procedure (28), with the following modifications. Nuclear protein (100 mg) was loaded onto a heparin-Sepharose (Pharmacia) column equilibrated with 25 mM KCl in column buffer (28). After washing with 10 volumes of the same buffer, the column was eluted by a linear gradient of KCl (25 to 400 mM) in column buffer. The collected fractions containing APRF as assessed by gel retardation analysis were pooled. For the preparation of an affinity resin, the oligonucleotides 5′-GATC CTTCCGGGAAAGTCCTTAATCCTTCCGGGAATA-3′ (top strand) and 5′-GATCTATTCCCGGAAGGATTAAG GACTTTCCCGGAAG-3′ (bottom strand) were synthesized (Gene Assembler; Pharmacia). After annealing, this double-stranded oligonucleotide contained two high-affinity APRF binding sites (underlined) as identified in the ICAM-1 promoter (38, 70) separated by the sequence between the two APRF binding sites of the rat α_2-macroglobulin IL-6RE (62). This arrangement enables APRF to bind cooperatively and with high affinity to adjacent binding sites as shown before for the α_2-macroglobulin IL-6RE (62). Oligonucleotides were highly ligated, biotinylated by filling in 5′ overhangs with dATP, dGTP, dCTP, and biotinylated dUTP (bio-11-dUTP; Sigma) by using Klenow enzyme, and coupled to streptavidin-agarose (Sigma). The resin was then used to affinity purify APRF from the heparin-Sepharose eluate as described previously (28). Shortly, the pooled active fractions from the heparin-Sepharose were adjusted to 100 mM KCl by diluting them with column buffer without KCl, supplemented with 2 μg of poly(dI-dC) (Pharmacia) per ml, and loaded onto the affinity column. After washing with 10 volumes of 100 mM KCl and 4 volumes of 250 mM KCl, each in column buffer, APRF was eluted at 1 M KCl. The second affinity chromatography was carried out in the same manner except that the poly(dI-dC) concentration added to the APRF preparation was 0.1 μg/ml.

From whole-cell extracts of HepG2 cells, APRF was purified by a modification of the procedure described above. Extracts were applied to DEAE-Sepharose, washed with 25 mM KCl in column buffer, and batch eluted with 100 mM KCl in column buffer. The eluate was supplemented with 5 μg of poly(dI-dC) per ml and applied to the affinity column. Two rounds of affinity chromatography were then performed as described above.

To obtain partial amino acid sequence data, 10 μg of APRF purified from rat liver was cleaved with cyanogen bromide, and the peptides generated were separated by reverse-phase high-pressure liquid chromatography. Peptide separation, detection, and collection were carried out as described previously

(8). Two major peptides of the chromatogram were selected for automated amino acid sequence analysis using an Applied Biosystems sequenator.

Gel retardation assays. APRF DNA-binding activity was analyzed by gel retardation assays using a ^{32}P-labeled oligonucleotide containing the rat α_2-macroglobulin IL-6RE core binding site as described previously (62).

Immunoprecipitation and immunoblotting. HepG2 cells were rinsed with cold PBS and lysed with lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM NaF, 0.75 mM phenylmethylsulfonyl fluoride, 15% glycerol, 10 μg each of aprotinin, pepstatin, and leupeptin per ml) for 20 min on ice. Immunoprecipitation of Stat proteins from the lysates and immunoblotting were performed as described earlier (39).

In situ labeling. HepG2 cells were prelabeled for 1 h in phosphate- and serum-free medium containing $^{32}P_i$ (3,000 Ci/mmol) at 0.5 mCi/ml. Then IL-6 (100 U/ml) was added, and the incubation was continued for 15 min. The cells were lysed for 20 min at 0°C with lysis buffer (see above), and the lysate was subjected to APRF purification as described above.

Cell-free activation of APRF. HepG2 cells were rinsed twice with ice-cold PBS and once with extraction buffer (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.8], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.45 mM spermidine, 0.15 mM spermine, 100 μM sodium orthovanadate, 750 μM phenylmethylsulfonyl fluoride, 10 μg each of aprotinin, pepstatin, and leupeptin per ml). The cells were homogenized in 250 μl extraction buffer per 5×10^6 cells in a Dounce homogenizer (pestle B, 20 strokes). Homogenization of the cells was verified by staining nuclei under a microscope. The homogenates (50 μl) were incubated for 15 min at 30°C without or with 200 μM ATP and 100 U of IL-6 in the presence of 4 mM $MgCl_2$. Then the homogenates were subjected to three freeze-thaw cycles to release APRF activity from the membrane fraction and centrifuged, and supernatants were analyzed for APRF DNA-binding activity by gel retardation assays.

RESULTS

APRF is activated by all cytokines acting through the gp130 signal transducer and occurs in various cell types. The gp130 transmembrane protein is an integral part of the receptors for IL-6, LIF, OSM, CNTF, and probably IL-11 and is known to be required for the intracellular signal transduction of these cytokines (15, 19, 24, 30, 69). As a consequence, the early intracellular responses evoked by these cytokines, including protein tyrosine phosphorylation and immediate-early gene induction, are very similar if not identical (37, 52, 68). We recently reported on the activation of the transcription factor APRF as an early event induced by IL-6 and LIF in hepatoma cells (62). We now investigated whether APRF activation is evoked by other cytokines acting through gp130 as well. Human hepatoma HepG2 cells were treated with either IL-6, LIF, IL-11, or OSM for 15 min, a period after which maximal APRF activation in response to IL-6 had been observed (62). After preparation of nuclear extracts, the DNA-binding activity of APRF was examined by gel retardation assays. As a probe, a ^{32}P-labeled synthetic oligonucleotide containing the proximal APRF binding site of the rat α_2-macroglobulin IL-6RE was used (62). OSM activated APRF to a similar extent as did IL-6, while IL-11 did not cause detectable APRF activation in these cells (Fig. 1). HepG2 cells are known to only weakly respond to IL-11 (7), probably because of low IL-11 receptor levels in these cells. Rat hepatocytes in primary

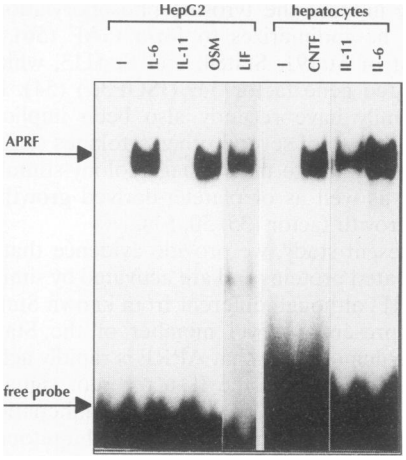

FIG. 1. APRF is activated by all cytokines acting through the signal transducer gp130. Human hepatoma (HepG2) cells or primary rat hepatocytes were treated with IL-6 (100 B-cell stimulatory factor 2 units/ml), IL-11 (100 U/ml), LIF (10 U/ml), OSM (25 ng/ml), or CNTF (10 ng/ml) for 15 min, and nuclear proteins (5 μg) prepared from these cells were analyzed for APRF activity in a gel retardation assay. After incubation with a radiolabeled synthetic oligonucleotide containing the proximal APRF binding site of the rat α_2-macroglobulin promoter, DNA-protein complexes were separated by electrophoresis through a native 4% polyacrylamide gel as described previously (62) and visualized by autoradiography.

culture, however, respond to this cytokine with the induction of acute-phase protein genes (48). As shown in Fig. 1, IL-11 rapidly induced APRF activity in rat hepatocytes. Furthermore, CNTF also activated APRF in primary hepatocytes (Fig. 1) but, like IL-11, was found to be far less potent when tested with HepG2 cells (data not shown). We conclude that IL-6, LIF, OSM, CNTF, and IL-11 all trigger, most likely through the gp130 receptor subunit, a common signaling pathway leading to the rapid activation of APRF.

IL-6 and related cytokines regulate gene expression in a broad spectrum of cell types, and gp130 is ubiquitously expressed on almost all cell types, but the mechanisms by which IL-6 controls transcription in nonhepatic cells are poorly understood. We have recently found that APRF binds to the IL-6REs of several immediate-early genes induced by IL-6 in various cell types (70), suggesting that APRF may be ubiquitously involved in the transcriptional control of such genes. However, the presence of APRF in nonhepatic cells has not yet been tested. We therefore treated cells from various origins with IL-6. In nuclear extracts from all cell lines tested, the IL-6-induced appearance of a protein forming specific complexes with APRF binding sites was observed (Fig. 2). Since these complexes exhibited the same mobility as the one formed by APRF from HepG2 cells, we conclude that either APRF or closely related factors occur in most if not all cell types and tissues. The levels of APRF activation, however, varied considerably among different cell lines. Hepatoma (HepG2) and plasmacytoma (B9) cells contained high amounts of active APRF after IL-6 treatment, whereas intermediate levels were found in NIH 3T3 fibroblasts and the monocytic cell line MonoMac 6. In MonoMac 6 cells, some active APRF was already present in control cells, suggesting that these cells may release a cytokine(s) capable of activating APRF into the medium. The myeloma cell line P3XG3 Ag8.653 exhibited only very low APRF DNA-binding activity (Fig. 2). In addition to the cell lines shown in Fig. 2, low to intermediate levels of

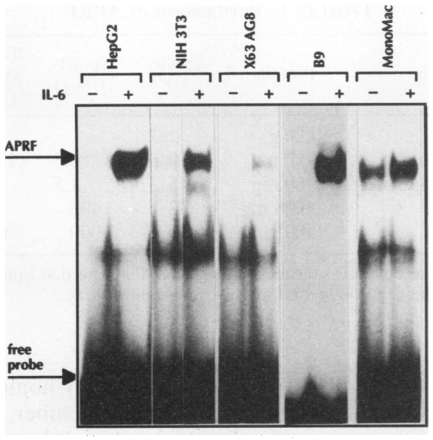

FIG. 2. IL-6 activates APRF in various cell types. The cell lines indicated were cultured as described in Materials and Methods and treated with IL-6 (100 U/ml) for 15 min. Five micrograms of nuclear protein of each cell line was analyzed in a gel retardation assay using a radiolabeled APRF binding site as described in the legend to Fig. 1.

APRF were also observed after IL-6 treatment of primary human monocytes, COS7 cells, MDCK canine kidney cells, and C6 rat astrocytoma cells (data not shown). From these results, we conclude that APRF is likely to be ubiquitously expressed in cells capable of responding to IL-6.

APRF activation is inhibited by protein tyrosine kinase inhibitors. The induction of immediate-early genes by IL-6 is inhibited by both the protein tyrosine kinase inhibitor tyrphostin and H7, a serine/threonine kinase inhibitor (37, 43). To analyze the type of protein kinases involved in APRF activation, we preincubated HepG2 cells with various protein kinase inhibitors prior to the addition of IL-6 (Fig. 3). Neither H7, W7, nor sphingosine, which exhibit different spectra of inhibitory activity toward cyclic nucleotide- or calcium/calmodulin-dependent protein kinases, protein kinase C, and other serine/threonine kinases (21, 25), prevented APRF activation (Fig. 3A). Staurosporine, however, completely inhibited APRF activation at high concentrations. This substance rather specifically inhibits protein kinase C at low concentrations, while at high concentrations it inhibits many kinases, including protein tyrosine kinases (60). Two specific inhibitors of protein tyrosine kinase activity, genistein and tyrphostin (4, 36), also prevented APRF activation (Fig. 3B). We conclude that the pathway leading to APRF activation involves the action of a protein tyrosine kinase(s). This is in accordance with our recent observation that APRF is rapidly tyrosine phosphorylated in response to IL-6 (39). However, the observation that H7 did not prevent APRF activation as measured in a gel retardation assay demonstrates that stimulation of APRF DNA-binding activity alone is not sufficient for the induction of immediate-early genes by IL-6. Rather, an additional, H7-sensitive event is required.

Cell-free activation of APRF. Activation of ISFG3α and GAF by IFN-α and IFN-γ, respectively, was demonstrated to occur in vitro upon addition of IFNs to cell homogenates or plasma membrane fractions, indicating that activation of the transcription factors occurs in close association with the plasma membrane (13, 14). Intrigued by the similarities between APRF and GAF activation, we examined whether APRF is also activated by IL-6 in a cell-free system. HepG2 cells were homogenized, and the homogenates were incubated in the presence or absence of IL-6. To verify that homogenization was complete and no intact cells remained in the homogenate, incubation was first carried out without the addition of ATP. Only marginal APRF activation was observed under these conditions, showing that no intact cells remained in the homogenate (Fig. 4A). However, when ATP was included in the incubation mixture, APRF activation was observed upon incubation of the homogenate with IL-6 (Fig. 4A). Similarly, OSM and LIF were able to stimulate APRF activation in the cell-free system, while IFN-γ, under the same conditions, activated GAF in HepG2 homogenates (data not shown). Cell-free activation of APRF was found to be inhibited by the same inhibitors, i.e., staurosporine and genistein, as was its activation by IL-6 in intact HepG2 cells, indicating the involvement of the same pathway under both conditions (Fig. 4B).

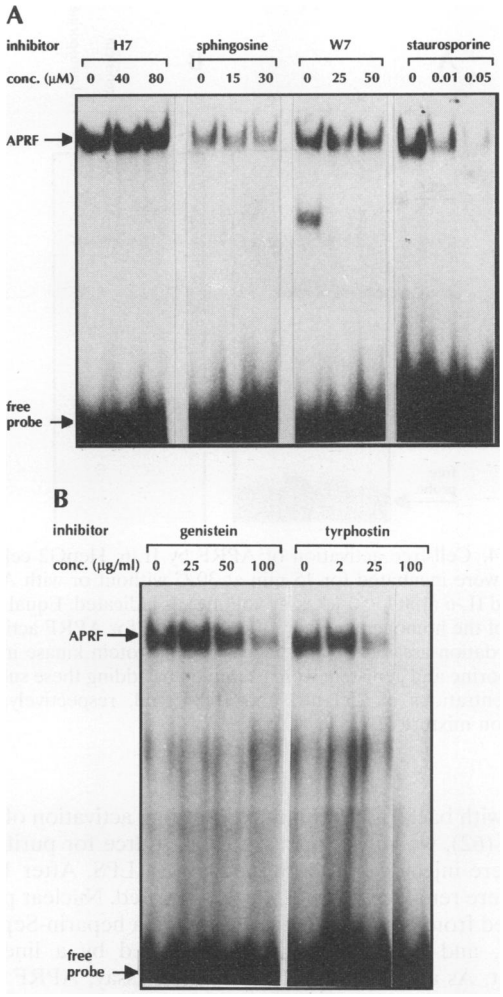

FIG. 3. Effects of protein kinase inhibitors on the APRF activation by IL-6. HepG2 cells were incubated for 1 h at 37°C with H7, W7, sphingosin, or staurosporin (A) or with genistein or tyrphostin (B) at the concentrations indicated. Then IL-6 (10 U/ml) was added to the medium, the cells were harvested, and nuclear extracts were prepared after additional 15 min. APRF activity was determined by gel retardation assays as described for Fig. 1.

Purification of APRF from rat liver. Further elucidation of the functional role of APRF and the mechanism of its activation requires knowledge of the molecular composition of APRF. We therefore decided to purify APRF. Since treatment

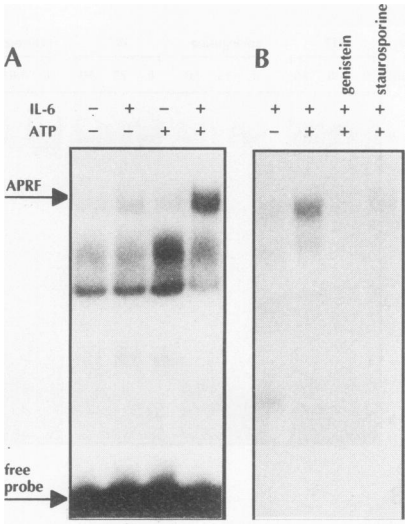

FIG. 4. Cell-free activation of APRF by IL-6. HepG2 cell homogenates were incubated for 15 min at 30°C without or with ATP (0.2 mM) and IL-6 (100 U/50-μl assay volume) as indicated. Equal aliquots (10 μl) of the homogenates were then analyzed for APRF activity by a gel retardation assay (A). The effects of the protein kinase inhibitors staurosporine and genistein were examined by adding these substances at concentrations of 250 nM and 100 μg/ml, respectively, to the incubation mixture (B).

of rats with bacterial LPS induces a strong activation of APRF in liver (62), we chose this tissue as a source for purification. Rats were injected intraperitoneally with LPS. After 1 h, the livers were removed and nuclei were isolated. Nuclear proteins extracted from the nuclei were applied to a heparin-Sepharose column, and bound proteins were eluted by a linear salt gradient. As assessed by gel retardation assay, APRF activity was eluted between 140 and 350 mM KCl (Fig. 5A) with a yield of 90% of the APRF activity loaded onto the column (Table 1).

For affinity purification of APRF, a DNA affinity resin was prepared by coupling highly ligated and biotinylated synthetic oligonucleotides containing a tandem of two strong APRF binding sites to streptavidin-agarose. By using this resin, the pooled active fractions eluted from the heparin-Sepharose column were further purified by two subsequent rounds of DNA affinity chromatography (Fig. 5B). The total amount of purified protein was estimated as approximately 10 μg (Table I). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of the affinity-purified APRF preparation revealed a single, apparently homogeneous protein band with an apparent molecular size of approximately 87 kDa (Fig. 5C). Purified APRF gave rise to the same retarded DNA-protein complex upon gel retardation analysis as did partially purified preparations (Fig. 5B) or crude nuclear extracts (data not shown).

To obtain partial amino acid sequence data, purified APRF was subjected to cyanogen bromide cleavage and the peptides generated were microsequenced. Two peptide sequences were recovered: peptide 1 (MLEQHLQNVRKRVQDLE) and peptide 2 (MKVVENLQD). Although APRF shares an almost identical DNA-binding sequence specificity with Stat91 (70), these sequences are not found in the Stat91 sequence, nor do they show significant homology to any Stat91 portion, proving that APRF and Stat91 represent distinct factors. Furthermore, a search of protein sequence data bases revealed that the sequences are not part of any previously known protein. It is of

TABLE 1. Purification of APRF

Fraction	Protein	Activity (U)[a]	Sp act (U/mg)	Total yield (%)	Purification (fold)
Liver	400.0 g				
Nuclear extract	100.0 mg	50	0.5	100	1
Heparin-Sepharose	30.0 mg	45	1.5	90	3
DNA affinity	0.08 mg	32	400	64	800
	0.01 mg	30	3,000	60	6,000

[a] One unit was defined as the amount of APRF that retarded 1 pmol of labeled probe under the conditions used in a gel retardation assay.

note, however, that both sequences are highly homologous to a recently cloned novel murine Stat family member, with 16 of 17 and 8 of 8 amino acids of peptides 1 and 2, respectively, matching portions of that protein (12). Therefore, APRF very likely represents a novel STAT family member.

An antiserum raised against a synthetic peptide corresponding to the sequence of peptide 1 specifically recognized purified APRF in an immunoblot experiment (Fig. 5D). This finding demonstrates that peptide 1 is in fact contained in the APRF protein. However, the antiserum did not bind APRF with an affinity high enough to allow detection of the factor in crude extracts.

APRF is recognized by antibodies directed against an NH_2-terminal portion of Stat91. Intrigued by the observation that APRF is likely to belong to the Stat family, we next investigated whether it is recognized by antisera raised to known Stat family members. For this purpose, we used polyclonal antisera specifically recognizing Stat91 and Stat113 (anti-Stat91 and anti-Stat113, respectively) as well as antisera raised against different protein portions shared by Stat91 and its 84-kDa splice variant (anti-Stat91/p84 and anti-Stat91/84-NH_2; see Materials and Methods). Nuclear extracts prepared from IL-6-treated HepG2 cells were incubated with these antisera and then analyzed in gel retardation assays. Neither anti-Stat113 nor anti-Stat91 and anti-Stat91/84 affected the mobility of the APRF-DNA complexes (Fig. 6A). Under the same conditions, the latter two antisera supershift GAF complexes upon incubation with extracts from IFN-γ-treated HepG2 cells (70). However, anti-Stat91/84-NH_2 antiserum gave rise to a supershifted APRF complex (Fig. 6A). The potentials of anti-Stat91/84-NH_2 to shift APRF and GAF complexes were comparable (Fig. 6B). Another antiserum directed against the SH2 domain of Stat91 was also found to recognize APRF in gel retardation assays but did so to a smaller extent (data not shown). When liver nuclear extracts from LPS-treated rats were analyzed, again only anti-Stat91/84-NH_2 was found to interact with APRF (Fig. 6C). We conclude from these data that either APRF itself is recognized by an antiserum raised against the NH_2 terminus of Stat91, indicating the presence of related epitopes in that portion of both proteins, or APRF is coprecipitated with Stat91 or a third protein bound by this antiserum.

Affinity purification and immunoprecipitation of APRF from HepG2 cells. The question of whether APRF is itself recognized by anti-Stat91/84-NH_2 or is coprecipitated with another protein cannot be answered by analyzing crude cell lysates. Therefore, we next investigated whether this antiserum is able to immunoprecipitate affinity-purified APRF as well. To be able to detect the small protein amounts after immunoprecipitation, we took advantage of the fact that active APRF is a phosphoprotein (39, 62). APRF was purified from ^{32}P-labeled HepG2 cells by a procedure similar to that described above for

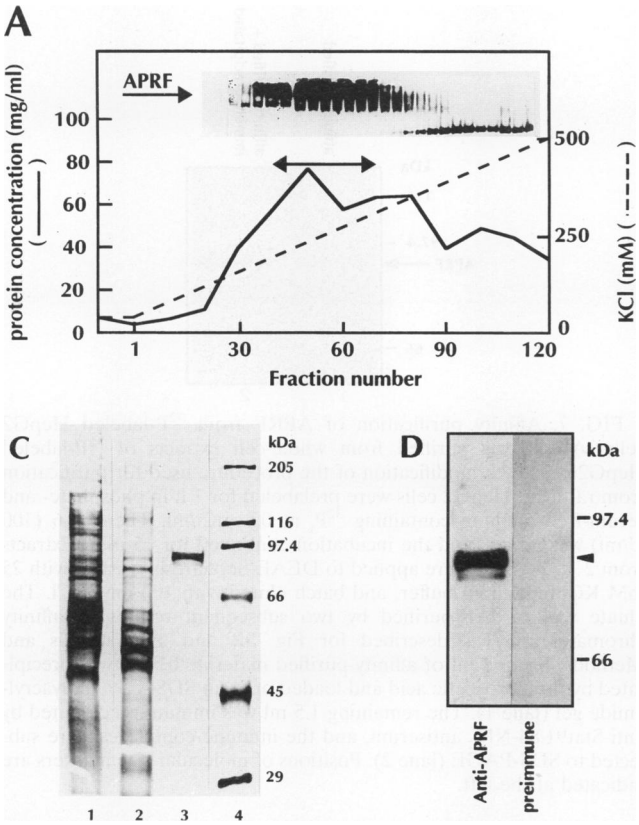

FIG. 5. Purification of APRF from rat liver. (A) Heparin-Sepharose chromatography. Sixty Sprague-Dawley rats were injected intraperitoneally with LPS (10 mg/kg of body weight). After 1 h, the animals were killed and nuclear extracts were prepared from the livers. Then 100 mg of nuclear protein was loaded onto a heparin-Sepharose column equilibrated with 25 mM KCl in column buffer, and bound proteins were eluted by a linear gradient of KCl (25 to 400 mM). APRF activity in the collected fractions (1 ml) was determined by gel retardation analysis as described in the legend to Fig. 1. (B) DNA affinity chromatography. A DNA affinity resin which consisted of immobilized, ligated double-stranded oligonucleotides containing APRF binding sites was used for affinity purification of APRF from the pooled active fractions of the heparin-Sepharose column. After binding to the affinity column, APRF was eluted by stepwise rising the KCl concentration from 250 mM to 1 M. APRF activity in the collected fractions (1 ml) was determined by a gel retardation assay. The fractions indicated by brackets were pooled. Subsequently, a second round of affinity chromatography was carried out and yielded essentially the same elution profile as the first round (not shown). (C) SDS-PAGE. Samples of the nuclear extract, the material purified by heparin-Sepharose chromatography, and the APRF preparation after two affinity purification steps were analyzed by SDS-PAGE (7.5% gel) and silver staining. The positions of molecular size markers are indicated at the right. (D) Immunoblot. Purified APRF (50 ng) was subjected to SDS-PAGE, electroblotted to a PVDF membrane, and analyzed by an antiserum to APRF peptide 1 (anti-APRF) or preimmune serum. Bound antibody was detected by enhanced chemiluminiscence (ECL kit; Amersham).

APRF purification from rat liver. After prelabeling of the cells with $^{32}P_i$ and stimulation with IL-6 for 15 min, whole-cell lysates were subjected to ion-exchange chromatography and subsequently two rounds of affinity chromatography. The purified APRF again produced the same retarded band with an APRF binding site in gel retardation assays as did HepG2 cell lysates or nuclear extracts (data not shown). When the affinity-purified material was analyzed by SDS-PAGE, APRF was consistently detected as an 87/89-kDa doublet of phosphoprotein bands (Fig. 7, lane 1). A contaminating band of 80 kDa was sometimes observed. When the preparation was then subjected to immunoprecipitation with anti-Stat91/84-NH_2 antiserum, the 87/89-kDa bands were precipitated (Fig. 7, lane 2). The proteins forming the two bands are hereafter referred to as APRF-87 and APRF-89. One conclusion which can be drawn from these findings is that APRF can be immunoprecipitated by anti-Stat91/84-NH_2 from an affinity-purified preparation and thus very likely is directly recognized by the antiserum rather than being coprecipitated. The observed heterogeneity of APRF purified from HepG2 cells may be due to partial degradation during the purification. Alternatively, APRF may occur in different posttranslationally modified forms or may be composed of two different proteins.

We have previously demonstrated that APRF as well as Stat91 are tyrosine phosphorylated in response to IL-6 (39). We therefore next studied whether both APRF forms observed in the purified preparation are tyrosine phosphorylated and can be immunoprecipitated from IL-6-treated HepG2 cells. HepG2 cells treated with IL-6 for 10 min were lysed and immunoprecipitated with anti-Stat91/84-NH_2. After the immune complexes were separated by SDS-PAGE and the proteins were blotted to a polyvinylidene difluoride (PVDF) membrane, the membrane was probed by monoclonal antibodies to phosphotyrosine. Three tyrosine-phosphorylated protein bands of 91, 89, and 87 kDa were detected (Fig. 8). The 91-kDa band has recently been identified as Stat91, which is tyrosine phosphorylated in response to IL-6 in HepG2 cells (39). In fact, reprobing the same blot with anti-Stat91/84-NH_2 demonstrated the presence of both phosphorylated and unphosphorylated Stat91 as well as of small amounts of the 84-kDa splice variant in HepG2 lysates (Fig. 8). The two other bands corresponded in size to the APRF-87 and APRF-89 bands observed in purified APRF preparations. Therefore, the appearance of two APRF bands after affinity purification is not due to partial degradation. In fact, when APRF purified from HepG2 cells was analyzed by immunoblotting with antibodies to phosphotyrosine, again the same bands were observed (Fig. 8B). Reprobing the same blot with anti-Stat91/84-NH_2 showed that Stat91 and Stat84 were not present in that preparation. This finding proves that both APRF forms are distinct from Stat91/84. The same result was obtained when APRF purified from rat liver was analyzed by immunoblotting except that again only the 87-kDa band was detected in this case. From Fig. 8, it is also evident that the antiserum raised against the NH_2 terminus of Stat91/84, although capable of immunoprecipitating APRF, did not recognize the factor in immunoblots at the protein amounts used in these experiments.

IL-6 induces Stat91, APRF-89, and APRF-87 tyrosine phosphorylation with different kinetics. When the time course of IL-6-induced tyrosine phosphorylation in HepG2 cells was

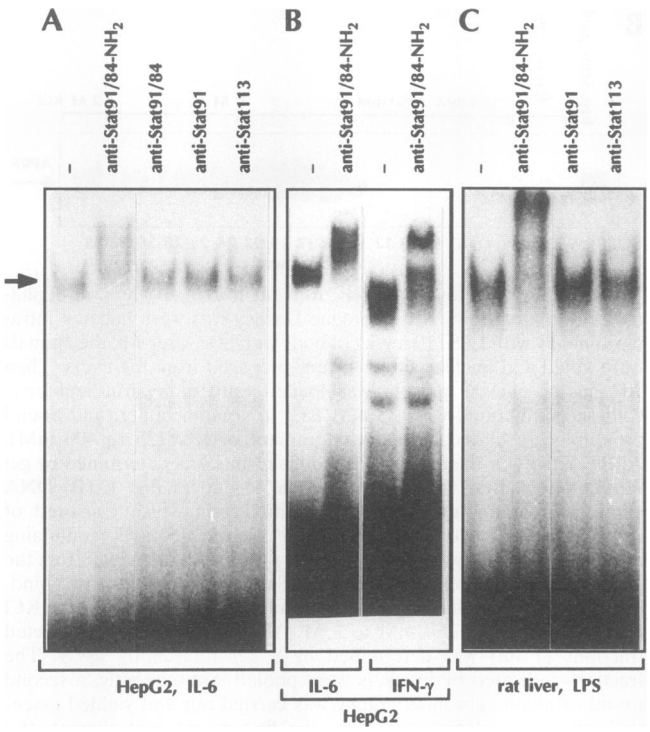

FIG. 6. APRF-DNA complexes are supershifted by anti-Stat91/84-NH_2 antiserum. (A) Incubation of nuclear extract from IL-6-treated HepG2 cells with different anti-Stat antisera. HepG2 cells were treated with IL-6 (100 U/ml) for 20 min. Nuclear extracts (2 μg of protein) prepared from these cells were incubated with the indicated rabbit antisera at 1:20 dilution for 30 min. Then the samples were analyzed by gel retardation assay as described for Fig. 1. (B) Comparison of APRF and GAF reactivities toward the anti-Stat91/84-NH_2 antiserum. Nuclear extracts from HepG2 cells treated with either IL-6 (100 U/ml) or IFN-γ (1,000 U/ml) were incubated without or with a 1:20 dilution of anti-Stat91/84-NH_2 antiserum and analyzed by gel retardation assay. (C) Incubation of rat liver nuclear extract with anti-Stat antisera. Two micrograms of nuclear protein prepared from livers of rats treated with LPS for 1 h was incubated with 1:20 dilutions of the indicated antisera for 30 min and analyzed by gel retardation assay.

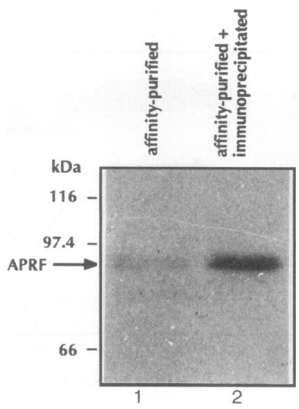

FIG. 7. Affinity purification of APRF from ^{32}P-labeled HepG2 cells. APRF was purified from whole-cell extracts of ^{32}P-labeled HepG2 cells by a modification of the procedure used for purification from rat liver. HepG2 cells were prelabeled for 1 h in phosphate- and serum-free medium containing $^{32}P_i$ at 0.5 mCi/ml. Then IL-6 (100 U/ml) was added, and the incubation continued for 15 min. Extracts from 2×10^7 cells were applied to DEAE-Sepharose, washed with 25 mM KCl in column buffer, and batch eluted with 100 mM KCl. The eluate was further purified by two subsequent rounds of affinity chromatography as described for Fig. 5B and in Materials and Methods. From 2 ml of affinity-purified material, 0.5 ml was precipitated by trichloroacetic acid and loaded onto an SDS–7.5% polyacrylamide gel (lane 1). The remaining 1.5 ml was immunoprecipitated by anti-Stat91/84-NH_2 antiserum, and the immune complexes were subjected to SDS-PAGE (lane 2). Positions of molecular size markers are indicated at the left.

analyzed, it became obvious that Stat91 and the two APRF forms behave differently. Tyrosine phosphorylation of Stat91 was detected after 5 min, was highest after 15 min, and rapidly decreased thereafter (Fig. 9A). Phosphorylation of the two APRF forms, however, followed other kinetics. APRF-87 tyrosine phosphorylation was observed as early as 2 min after IL-6 treatment, was maximal after 5 min, and then declined rapidly, while APRF-89 phosphorylation was delayed with a maximum between 15 and 30 min (Fig. 9A). Interestingly, DNA binding of APRF was detected in HepG2 lysates between 2 and 30 min after IL-6 stimulation (Fig. 9B), indicating that the two APRF forms gave rise to DNA-protein complexes of the same mobility.

Therefore, APRF-89 and APRF-87 may be different DNA-binding proteins which are tyrosine phosphorylated with different kinetics after IL-6 stimulation of HepG2 cells. However, the data also allow the alternative explanation that the two APRF forms represent one protein which between 5 and 15 min after IL-6 treatment underlies an additional posttranslational modification causing a slightly decreased mobility in SDS-polyacrylamide gels.

When HepG2 cells were pretreated with the protein kinase inhibitor genistein or staurosporine, shown above to block the activation of APRF DNA binding, the IL-6-induced tyrosine phosphorylation of both Stat91 and APRF was inhibited (Fig. 10). These data again demonstrate the close correlation between tyrosine phosphorylation and DNA-binding activity of APRF.

DISCUSSION

APRF activation is a general and ubiquitous response to cytokines acting through the IL-6 signal transducer gp130. The mechanism of the IL-6-triggered induction of acute-phase protein genes in liver has been subject to extensive studies, and the IL-6REs of many such genes were localized and characterized (22, 40, 44, 46, 49, 66). In contrast, although IL-6 acts on a great variety of cell types, only little is known about the regulation of IL-6 target genes in nonhepatic cells. Several immediate-early genes are induced by IL-6 and other cytokines acting through gp130 in various cell types, including hepatocytes (2, 6, 37, 43). Therefore, for the transcriptional induction of these genes, one would anticipate the existence of a ubiquitous pathway for the IL-6 signal transduction from plasma membrane to nucleus.

Since the activation of transcription factor APRF by IL-6 in liver and hepatoma cells is a very rapid and transient process and fits well with the time course of immediate-early gene induction (62), we have recently investigated the possibility of an involvement of APRF in the regulation of these genes. We could in fact demonstrate binding of APRF to IL-6REs of the ICAM-1, IFN regulatory factor 1, and *junB* immediate-early genes (11, 38, 70), suggesting that the factor may play a more general role in transcriptional control by IL-6. In the present study, we have demonstrated that APRF is present in various nonhepatic cell types as well. In fact, in all cell types so far tested, APRF activation by IL-6 was observed. Furthermore,

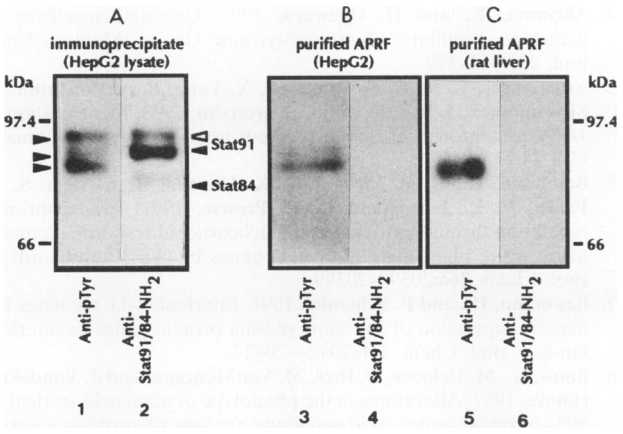

FIG. 8. Immunoblot analysis of HepG2 and rat liver APRF. Immunoblotting was performed with either anti-Stat91/84-NH_2 immunoprecipitates from HepG2 cells treated with IL-6 (200 U/ml) for 10 min (A) or with affinity-purified APRF from HepG2 cells (B) or rat liver (C). All proteins were separated by SDS-PAGE on 7.5% gels, transferred to a PVDF membrane, and analyzed by monoclonal antibodies to phosphotyrosine (lanes 1, 3, and 5). Then the membranes were stripped and reprobed with anti-Stat91/84-NH_2 (lanes 2, 4, and 6). The positions of the 91-, 89-, and 87-kDa phosphoprotein bands immunoprecipitated from HepG2 lysate are indicated by arrowheads at the left; positions of phosphorylated and unphosphorylated Stat91 are shown by open and closed arrowheads, respectively. Molecular size markers are indicated.

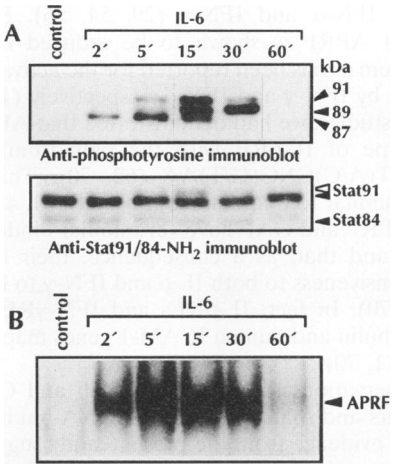

FIG. 9. Time course of APRF tyrosine phosphorylation and activation. HepG2 cells were treated with IL-6 (200 U/ml) for various periods and lysed. (A) The lysates were immunoprecipitated with anti-Stat91/84-NH_2; the immune complexes were separated by SDS-PAGE on a 6% gel, electroblotted to a PVDF membrane, and analyzed first with antibodies to phosphotyrosine (upper panel) and then, after stripping of the membrane, with anti-Stat91/84-NH_2 to control equal protein loading (lower panel). (B) Aliquots of the same lysates were subjected to gel retardation analysis as described for Fig. 1.

APRF is activated by all cytokines binding to receptors containing gp130, i.e., IL-6, LIF, OSM, IL-11, and CNTF. These findings strongly suggest that APRF is ubiquitously implicated in the regulation of immediate-early genes by IL-6 and related cytokines and that its activation represents a pivotal step in gp130-initiated signaling. In view of the pleiotropic actions of the cytokines acting through gp130 which control events as diverse as acute-phase protein synthesis, proliferation, differentiation, and neuron regeneration, it will be tempting to study a potential involvement of APRF in the onset of such processes.

The molecular composition of APRF. In a highly purified APRF preparation from rat liver, a homogeneous protein band with an apparent molecular size of 87 kDa was detected by SDS-PAGE and silver staining. Therefore, rat liver APRF apparently consists of one type of polypeptide. In contrast, two bands of 87 and 89 kDa (APRF-87 and APRF-89, respectively) were obtained when APRF was purified from HepG2 cells. Both forms were found to be tyrosine phosphorylated in response to IL-6. From the data presented, the question as to whether APRF-87 and APRF-89 correspond to two different proteins or represent differently modified forms of the same protein cannot yet be answered. However, recent results from our laboratory strongly indicate that the two APRF forms differ only by an IL-6-induced serine phosphorylation of APRF (38). Interestingly, tyrosine phosphorylation of two Stat91-related proteins in response to CNTF has recently been also observed by Bonni et al. (10). In view of the data presented here, it seems very likely that the 89- and 88-kDa bands observed by that group correspond to APRF-89 and APRF-87.

We had reported earlier that in Southwestern (DNA-protein) blot experiments, the APRF binding site of the α_2-macroglobulin promoter is bound by a protein of approximately 110 kDa and predicted the molecular size of APRF accordingly (62). However, the 110-kDa protein very likely is different from APRF because purified APRF preparations did not reproduce the 110-kDa signal in Southwestern blot experiments (63). This indicates that an additional factor in nuclear extracts distinct from APRF may bind to the α_2-macroglobulin element. In fact, a protein, IL-6RE-BP, has been described to bind the same element and to be activated after IL-6 stimulation by a process requiring ongoing protein synthesis (26, 27). The molecular size of IL-6RE-BP as determined by UV cross-linking was reported as approximately 102 kDa (27).

Evidence that APRF represents a novel member of the Stat family. The observation that APRF is tyrosine phosphorylated in response to IL-6 suggests that APRF is activated by a mechanism analogous to how GAF is activated by IFN-γ. In fact, both phosphorylation and DNA binding of APRF are blocked by inhibitors of protein tyrosine kinases and staurosporine, the same inhibitors which are known to interfere with the tyrosine phosphorylation and activation of Stat factors in

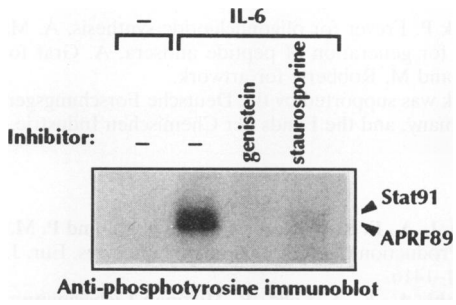

FIG. 10. Genistein and staurosporine inhibit the IL-6-induced tyrosine phosphorylation of APRF and Stat91. HepG2 cells preincubated for 30 min without or with genistein (100 μg/ml) or staurosporine (250 nM) were treated with IL-6 (100 U/ml) for 20 min and lysed. The lysates were analyzed for APRF and Stat91 tyrosine phosphorylation by immunoprecipitation with anti-Stat91/84-NH_2 and immunoblotting with antiphosphotyrosine antibodies as described for Fig. 8 and 9.

response to IFN-α and IFN-γ (29, 54, 56). Furthermore, activation of APRF is shown to be induced by IL-6 in a cell-free system as has been reported for the activation of GAF and ISGF3α by IFN-γ and IFN-α, respectively (13, 29).

In earlier studies, we had demonstrated that APRF binds to a certain type of IL-6RE with the palindromic consensus sequence TT(A/C)CNG(G/T)AA (62, 70). This consensus sequence is similar to that defined for GAF (31, 45). We could show that APRF and GAF have very similar binding sequence specificities and that, as a consequence, their binding sites confer responsiveness to both IL-6 and IFN-γ to heterologous promoters (70). In fact, IL-6REs and IFN-γREs of the rat α_2-macroglobulin and human ICAM-1 genes map to the same sequences (11, 70).

These observations suggest that APRF and GAF may be related factors and share homologous DNA-binding domains. Two lines of evidence from the present study suggest that this is in fact the case. Of four antisera raised against different portions of Stat91, two cross-reacted with APRF, as shown by supershift formation in gel retardation assays and by immunoprecipitation. Most efficient cross-reactivity was observed with an antiserum directed against the Stat91 NH_2 terminus, indicating the presence of shared epitopes in this protein portion. Furthermore, partial amino acid sequence obtained by microsequencing of APRF revealed a high degree of homology to a recently cloned Stat91-related factor (12). Therefore, APRF most likely represents a novel member of the STAT family.

Common features of IL-6 and IFN signal transduction. The two IL-6 receptor subunits belong to a superfamily of cytokine receptors which share both structural and functional features (9). Recent data have shown that cytokine receptors have a novel signaling pathway in common which is characterized by the activation of protein tyrosine kinases of the JAK family and the subsequent recruition of STAT family members. So far, three kinases of the JAK family, Tyk2, Jak1, and Jak2, have been identified (34, 64) and were shown to be components of the signaling pathways of IFN-α, IFN-γ, erythropoietin, IL-3, growth hormone, GM-CSF, and G-CSF (5, 41, 58, 61, 65). The recent findings that IL-6 induces tyrosine phosphorylation and activation of all three JAK family kinases as well as of both APRF and Stat91 (39, 59) demonstrate that signaling through gp130 uses an analogous pathway. The relatedness of APRF to Stat family factors as indicated by the data presented here further underscores the similarities between IL-6 and IFN signaling.

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