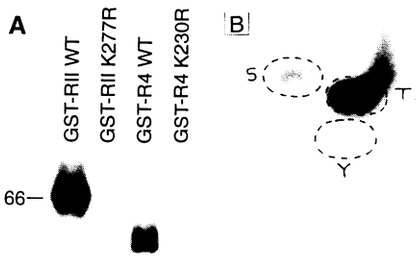

Fig. 5. Autophosphorylation of R4 in vitro. (**A**) In vitro autophosphorylation assay to compare the kinase activity of GST-RII and GST-R4. An equal amount of protein was loaded in each lane and separated by SDS–polyacrylamide gel electrophoresis (8% gel). Autoradiography was done at −80°C for 1.5 hours. (**B**) Phosphoamino acid analysis of in vitro autophosphorylated GST-R4.

binding of TGF-β1 to the 63- to 67-kD protein. These results demonstrate that R4 has the binding properties of the type I TGF-β receptor (*4*).

The type II TGF-β receptor is an active kinase capable of autophosphorylation in an in vitro kinase assay (Fig. 5A) (*5*). Because the cytoplasmic domain of R4 also contains a putative serine-threonine kinase domain, which has sequence similarity to the type II receptor kinase (41% identity), we tested whether R4 could act as a functional serine-threonine kinase in vitro. A fusion protein between glutathione-S-transferase and the type I receptor cytoplasmic domain was generated and used in an in vitro kinase assay (*15*). As a control, we constructed a similar fusion protein using the R4 cytoplasmic domain containing the K230R mutation. The wild-type R4 kinase was active whereas the fusion protein containing the K230R mutation did not autophosphorylate (Fig. 5A). The type II receptor fusion protein showed four times more activity than the R4 protein (Fig. 5A). We also assayed a GST-R1 construct for autophosphorylation activity and found its activity to be 1/100 that of the type II receptor (*12*). This result suggests that in vitro the R4 type I receptor, like the type II receptor, acts as a functional serine-threonine kinase. The GST-RII kinase is mainly a serine kinase with a small amount of activity toward threonine (*5*). Phosphoamino acid analysis of the autophosphorylated GST-R4 kinase revealed mainly phosphothreonine with a small amount of phosphoserine (Fig. 5B).

We conclude that R4 is a functional type I TGF-β receptor because it restored expression of a reporter gene in response to TGF-β in the nonresponsive R1B mutant cells, it required the presence of a functional type II receptor to bind ligand and to signal, its kinase activity was required for signaling, and the kinase domain was capable of serine-threonine autophosphorylation under in vitro conditions. R4 may represent only one of the functional type I receptors for TGF-β because the functional assay involving TGF-β induction of a specific promoter in a particular cell type may have limited our ability to test the functional nature of the other members of this receptor family. The diversity of biological responses elicited by TGF-β could be derived from the existence of multiple receptors that can functionally mediate various effects in different biological systems.

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13. The R4 (K230R) mutant was created by oligonucleotide site-directed mutagenesis with the unique site elimination system (Pharmacia). The template was generated by subcloning the R4 cDNA into the Eco RI site of the pBSK$^+$ vector (Stratagene). The mutation was verified by dideoxy chain termination sequence analysis.
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15. To create the in-frame fusion protein, we subcloned the portion of R4 cDNA encoding the cytoplasmic domain, beginning with Asp^{161}, into the plasmid pGEX-1. In vitro kinase assays were done with the proteins bound to glutathione-Sepharose beads in 50 mM tris (pH 7.4), 150 mM NaCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM Na_2MoO_4, 2 mM NaF, 1 mM DTT, and 10 μCi [γ-^{32}P]ATP at 30°C for 40 min.

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

Claudia Lütticken, Ursula M. Wegenka, Juping Yuan, Jan Buschmann, Chris Schindler, Andrew Ziemiecki, Ailsa G. Harpur, Andrew F. Wilks, Kiyoshi Yasukawa, Tetsuya Taga, Tadamitsu Kishimoto, Giovanna Barbieri, Sandra Pellegrini, Michael Sendtner, Peter C. Heinrich, Friedemann Horn*

Interleukin-6 (IL-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, leukemia inhibitory factor (LIF), oncostatin M (OSM), interleukin-11 (IL-11), 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).

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 antiserum 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

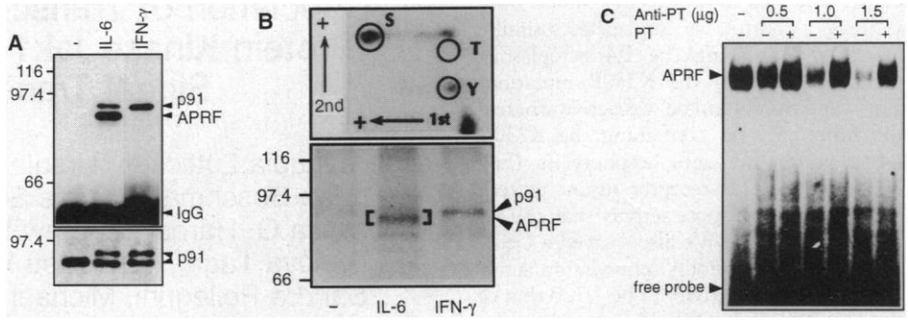

Fig. 1. Tyrosine phosphorylation of APRF and ISGF-3α p91 in response to IL-6. (**A**) HepG2 cells (10^7) were treated for 20 min without or with human IL-6 (100 units/ml) or IFN-γ (25 ng/ml). Cells were rinsed with cold phosphate-buffered saline and lysed in 0.5-ml lysis buffer (24) for 20 min at 0°C. 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 (PVDF) membrane, and probed with monoclonal antibodies to phosphotyrosine (PY20) [International Chemical and Nuclear (ICN), Costa Mesa, CA]. 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 U/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).

C. Lütticken, U. M. Wegenka, J. Yuan, J. Buschmann, P. C. Heinrich, F. Horn, Institute for Biochemistry, RWTH Aachen, Pauwelsstrasse 30, D-52057 Aachen, Germany.
C. Schindler, College of Physicians and Surgeons of Columbia University, Department of Medicine, New York, NY 10032.
A. Ziemiecki, Laboratory for Clinical and Experimental Cancer Research, University of Berne, Tiefenaustrasse 120, CH-3004 Bern, Switzerland.
A. G. Harpur and A. F. Wilks, Ludwig Institute for Cancer Research, Royal Melbourne Hospital, Victoria 3050, Australia.
K. Yasukawa, Biotechnology Research Laboratory, Tosoh Corporation, 2743-1 Hayakawa, Ayase, Kanagawa 252, Japan.
T. Taga, Institute for Molecular and Cellular Biology, Osaka University, 1-3, Yamadaoka, Suita, Osaka 565, Japan.
T. Kishimoto, Department of Medicine III, Osaka University Medical School, 2-2, Yamadaoka, Suita, Osaka 565, Japan.
G. Barbieri and S. Pellegrini, Unité INSERM 276, Institut Pasteur, 75724 Paris Cedex 15, France.
M. Sendtner, Max Planck Institute for Psychiatry, Am Klopferspitz, D-82152 Martinsried, Germany.
*To whom correspondence should be addressed.

p91 and Jak1 for at least 1 hour (*15*), confirming a close correlation between tyrosine phosphorylation of members of the Jak and ISGF-3α 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 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.

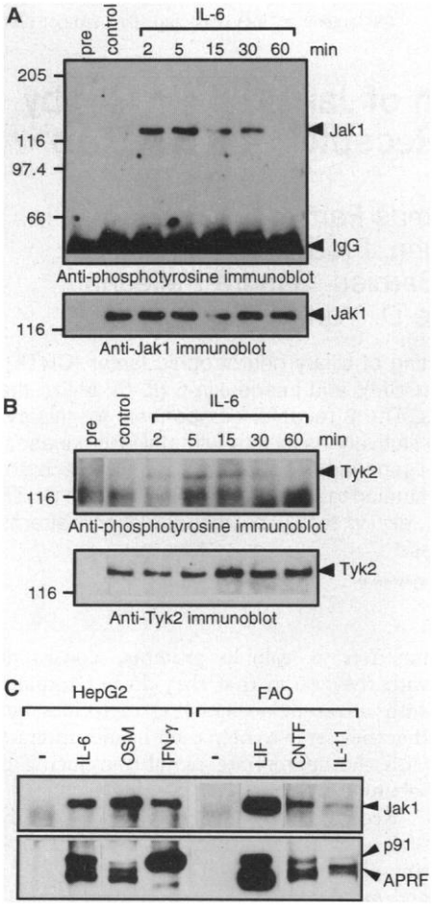

Fig. 2. Tyrosine phosphorylation of Jak family protein kinases in response to IL-6 and other cytokines. (**A**) Time course of Jak1 phosphorylation in response to IL-6. Lysates from HepG2 cells treated with IL-6 (200 U/ml) for various periods (labeled in minutes) were immunoprecipitated with antiserum 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 U/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*).

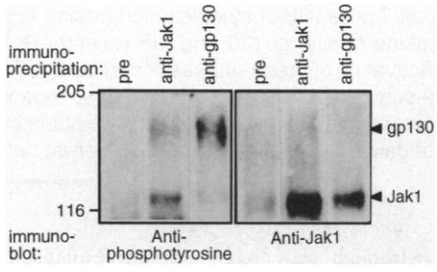

Fig. 3. Association of Jak1 with the IL-6 signal transducer gp130. Untreated HepG2 cells (right panel) or cells treated for 5 min with IL-6 (200 units/ml) (left panel) were washed with ice-cold phosphate-buffered saline and collected. Cells were cross-linked (*32*) and lysed, and proteins were immunoprecipitated 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.

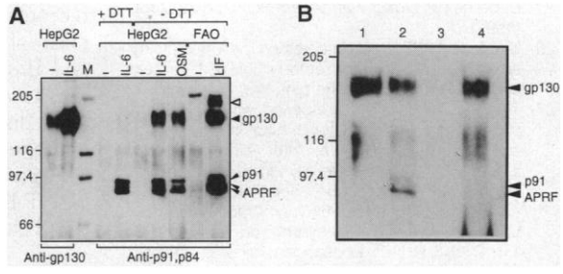

Fig. 4. Coprecipitation of gp130 with APRF from IL-6–stimulated hepatoma cells. (**A**) HepG2 or FAO cells were treated for 10 min with human IL-6 (200 U/ml), OSM (20 ng/ml), or LIF (25 ng/ml). Cell lysates prepared in the presence or absence of dithiothreitol (DTT) were immunoprecipitated with monoclonal 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 U/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 analyzed by immunoblotting with antibodies to phosphotyrosine.

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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 PVDF membrane. 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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Association and Activation of Jak-Tyk Kinases by CNTF-LIF-OSM–IL-6 β Receptor Components

Neil Stahl,* Teri G. Boulton, Thomas Farruggella, Nancy Y. Ip, Sam Davis, Bruce A. Witthuhn, Frederick W. Quelle, Olli Silvennoinen, Giovanna Barbieri, Sandra Pellegrini, James N. Ihle, George D. Yancopoulos*

A recently defined family of cytokines, consisting of ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), and interleukin-6 (IL-6), utilize the Jak-Tyk family of cytoplasmic tyrosine kinases. The β receptor components for this cytokine family, gp130 and LIF receptor β, constitutively associate with Jak-Tyk kinases. Activation of these kinases occurs as a result of ligand-induced dimerization of the receptor β components. Unlike other cytokine receptors studied to date, the receptors for the CNTF cytokine family utilize all known members of the Jak-Tyk family, but induce distinct patterns of Jak-Tyk phosphorylation in different cell lines.

Although they have different biological activities, CNTF, LIF, OSM, and IL-6 make up a cytokine family on the basis of their predicted structural similarities (*1, 2*) and shared β signal-transducing receptor components (*3–8*). Receptor activation by this cytokine family results from either homo- or heterodimerization of these β components (*6, 8, 9*). IL-6 receptor activation involves homodimerization of gp130 (*9*), a protein initially identified as the IL-6 signal transducer (*10*). CNTF, LIF, and OSM receptor activation requires heterodimerization between gp130 and a second gp130-related protein known as the LIF receptor β (LIFRβ) (*6*), initially identified for its ability to bind LIF (*11*). Both CNTF and IL-6 also require specificity-determining α components that are more limited in their distribution than the β components and thus determine the cellular targets of the particular cytokines (*8*). Both the CNTF binding protein CNTFRα (*12*) and the IL-6 receptor IL-6Rα (*10, 13*) can function as soluble proteins, consistent with the notion that they do not interact with intracellular signaling molecules but that they serve to help their ligands interact with the appropriate signal-transducing β subunits (*8*).

Receptor activation by the CNTF family of cytokines results in the immediate tyrosine phosphorylation of the β components and other cellular proteins (*3, 5, 14–17*). Because the β receptor components do not contain intrinsic tyrosine kinase domains, tyrosine phosphorylation was hypothesized to result from the activation of one or more receptor-associated tyrosine kinases, which appear to be essential for downstream events because tyrosine kinase inhibitors were found to block the early tyrosine phosphorylations and subsequent physiological responses (*3, 14*). We report here that gp130 and LIFRβ associate with the Jak-Tyk family of cytoplasmic protein tyrosine kinases (*18–21*) in the absence of ligand and that Jak-Tyk kinase activity is induced after cytokine binding. Unlike other cytokines studied to date (*21–24*), the CNTF family of factors utilizes all known members of the Jak-Tyk family but elicits distinct profiles of Jak-Tyk activation in different cells.

Stimulation of responsive cells with CNTF or LIF induces tyrosine phosphorylation of both gp130 and LIFRβ, which form a complex that can be recovered after cell

N. Stahl, T. G. Boulton, T. Farruggella, N. Y. Ip, S. Davis, G. D. Yancopoulos, Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, NY 10591.
B. A. Witthuhn, F. W. Quelle, O. Silvennoinen, J. N. Ihle, St. Jude Children's Research Hospital, Department of Biochemistry, 332 North Lauderdale, Memphis, TN 38105.
G. Barbieri and S. Pellegrini, Unité INSERM 276, Institut Pasteur, 75724 Paris Cedex 15, France.

*To whom correspondence should be addressed.