Stat2 recombinant molecules required either two Stat1-SH2 groups or one Stat1- and one Stat2-SH2 group; two Stat2-SH2 groups did not permit interaction, even though the two potentially interacting proteins were phosphorylated.

The following model for the operation of the Jak-STAT pathway is supported by these and other data (*1*). Ligand-driven receptor-kinase assembly occurs between receptor chains and associated Jak protein kinases. Phosphorylation (intermolecular) of the Jaks ensues, and these active enzymes phosphorylate one or more of the receptor chains. The substrate STAT molecule is directed by its SH2 group to a tyrosine phosphate on a specific receptor-kinase complex. Binding of the SH2 group brings the closely neighboring tyrosine on the STAT protein into proximity of a kinase. If the SH2 domains and tyrosine sites of the phosphorylated STATs are mutually compatible, the proteins form homo- or heterodimers. After translocation into the nucleus, specific binding to DNA, alone or in conjunction with other proteins, directs specific transcriptional responses. This set of specific protein-protein and protein-DNA interactions allows a distinct response to a particular extracellular signaling protein.

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27. U3A cells (5×10^5) (*14*) or U6A cells (*15*) were transfected with DNA (20 μg) by the calcium phosphate procedure (*23*). Colonies were selected in Dulbecco's modified Eagle's medium supplemented with bovine calf serum containing G418 (500 μg/ml; Gibco, BRL) 48 hours after transfection. Cell lines were maintained in G418. Cells were lysed for 30 min on ice in 50 mM tris (pH 8.0), 280 mM NaCl, 0.5% NP-40, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM Na_3VO_4, leupeptin (1 μg/ml), aprotinin (2 μg/ml), and pepstatin (1 μg/ml). Precipitates were washed twice in lysis buffer and once in phosphate-buffered saline, then boiled for 2 min in sample loading buffer [50 mM tris (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue, 1% β-mercaptoethanol]. Precipitates were subjected to SDS-PAGE (7% gel), transferred to nitrocellulose membranes, and probed with the antisera indicated above.

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Choice of STATs and Other Substrates Specified by Modular Tyrosine-Based Motifs in Cytokine Receptors

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Many members of the cytokine receptor superfamily initiate intracellular signaling by activating members of the Jak family of tyrosine kinases. Activation of the same Jaks by multiple cytokines raises the question of how these cytokines activate distinct intracellular signaling pathways. Selection of particular substrates—the transcriptional activator Stat3 and protein tyrosine phosphatase PTP1D—that characterize responses to the ciliary neurotrophic factor–interleukin-6 cytokine family depended not on which Jak was activated, but was instead determined by specific tyrosine-based motifs in the receptor components—gp130 and LIFR—shared by these cytokines. Further, these tyrosine-based motifs were modular, because addition of a Stat3-specifying motif to another cytokine receptor, that for erythropoietin, caused it to activate Stat3 in a ligand-dependent fashion.

The cytokine receptor superfamily comprises structurally related receptors characterized by conserved motifs in their ectodomains (*1*). Many different cytokines—with biological actions as distinct as those of erythropoietin (EPO), ciliary neurotrophic factor (CNTF), and the interferons (IFNs)—activate members of this receptor superfamily (*2, 3*). The cytoplasmic domains of cytokine receptors lack obvious catalytic function. However, binding of ligand to the cytokine receptors initiates intracellular signaling by activating members of a family of receptor-associated tyrosine kinases, referred to as the Janus kinases (Jaks) (*2, 4*). These kinases are constitutively associated with the membrane-proximal portions of cytokine receptor cytoplasmic domains and become activated upon ligand-induced receptor homo- or heterodimerization (*2, 5, 6–10*), a process reminiscent of epidermal growth factor (EGF)– and platelet-derived growth factor (PDGF)–mediated dimerization and subsequent activation of receptor tyrosine kinases (*11*). EPO causes homodimerization of its receptor, which leads to specific activation of the attached kinase, Jak2 (*7*). The CNTF family of cytokines, including interleukin-6 (IL-6), leukemia inhibitory factor (LIF), and oncostatin M (OSM), use more complicated receptor systems that contain two different Jak-associated receptor components, gp130 and LIFR (*2*). The CNTF and IL-6 receptor complexes also include α receptor components that confer cytokine specificity; these α components do not bind to Jaks and their cytoplasmic domains are apparently not required for signaling (*2*). Ligand-mediated activation results from either homodimerization of gp130 for IL-6 or heterodimerization of gp130 with LIFR for CNTF, LIF, and OSM (*8, 9*). Both gp130 and LIFR can associate with and activate at least three members of the Jak family—Jak1, Jak2, and Tyk2—but activate distinct combinations of these Jaks in different cells (*10*). Although the receptor systems for the IFNs are not fully understood, signaling by IFN-α requires Jak1 and Tyk2, whereas signaling by IFN-γ specifically requires Jak1 and Jak2 (*12–14*).

It is not clear how different cytokines, often activating the same Jaks, can uniquely specify activation of distinct intracellular

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signal-transduction pathways. For example, different cytokines activate particular members of a family of transcription factors known as STATs (signal transducers and activators of transcription), which are activated by tyrosine phosphorylation in the cytoplasm before translocation to the nucleus (*3*). For example, IFN-γ activates Stat1 and IFN-α activates Stat1, Stat2, and Stat3 (*3*, *15*), whereas the CNTF family of cytokines all preferentially activate Stat3 (*15–18*). The Jaks might determine the choice of downstream substrates: Definition of the particular Jaks and STATs differentially activated by the interferons led to proposals that Jak1 specifically activates Stat1, whereas Tyk2 specifically targets Stat2 (*4*, *19*). However, the CNTF-related cytokines consistently induce phosphorylation of Stat3 despite activating different Jaks in different cells (*10*, *15*, *20*). Thus, it was proposed that cytokine receptor cytoplasmic domains not only contain a Jak-binding domain, but also selectively bind distinct substrates, allowing them to be activated by the associated Jak (*2*, *10*, *21*). This proposal is supported by observations that Stat1 activation by IFN-γ requires phosphorylation of a particular tyrosine in one of the IFN-γ receptor components and that phosphopeptides spanning this tyrosine specifically bind to Stat1 (*21*).

We found that tyrosine-based motifs in the cytoplasmic domains of gp130 and LIFR were required for selection of two substrates with Src homology 2 (SH2) domains (*22*) that characterize responses to members of the CNTF cytokine family (*20*)—Stat3 and the protein tyrosine phosphatase PTP1D; the latter may couple PDGF receptor activation to the Ras signaling pathway (*23*, *24*). The motifs we have defined are modular determinants that specify selection of particular signal-transduction pathways because a five–amino acid Stat3-specifying motif from gp130 appended to the cytoplasmic domain of the EPO receptor caused that receptor to activate Stat3.

To facilitate mutational analysis of the cytoplasmic domains of gp130 and LIFR, we made expression constructs encoding epitope-tagged chimeric receptors in which the extracellular domains from the receptor tyrosine kinases TrkC and EGF receptor were fused to the cytoplasmic domains of the cytokine receptors (*25*). Expression of these chimeric receptors in transfected cells allowed specific ligand-mediated homodimerization and activation of only the mutant cytokine receptor cytoplasmic domains, by either neurotrophin-3 (NT-3) (for TrkC) or EGF, even in cells that express endogenous gp130 or LIFR. Chimeric receptors containing the cytoplasmic domain of gp130 (called EG for EGFR:gp130 or TG for TrkC:gp130) underwent factor-induced tyrosine phosphorylation of both the receptor and the associated Jaks (Fig. 1, A and C). Although there is no known cytokine that induces homodimerization of LIFR, a fusion protein (EL for EGFR:LIFR) containing the EGFR extracellular domain and the transmembrane and cytoplasmic domains of LIFR underwent EGF-induced tyrosine phosphorylation of the receptor and the associated Jaks (Fig. 1B). This result provides further evidence for the conclusion that LIFR functions as a full signal-transducing partner to gp130 (*2*, *26*), and raises the possibility that an as yet undiscovered cytokine could induce downstream signaling through homodimerization of LIFR.

To investigate the contribution of the cytoplasmic domains of gp130 and LIFR in the activation of signal-transduction pathways, we constructed a series of chimeric

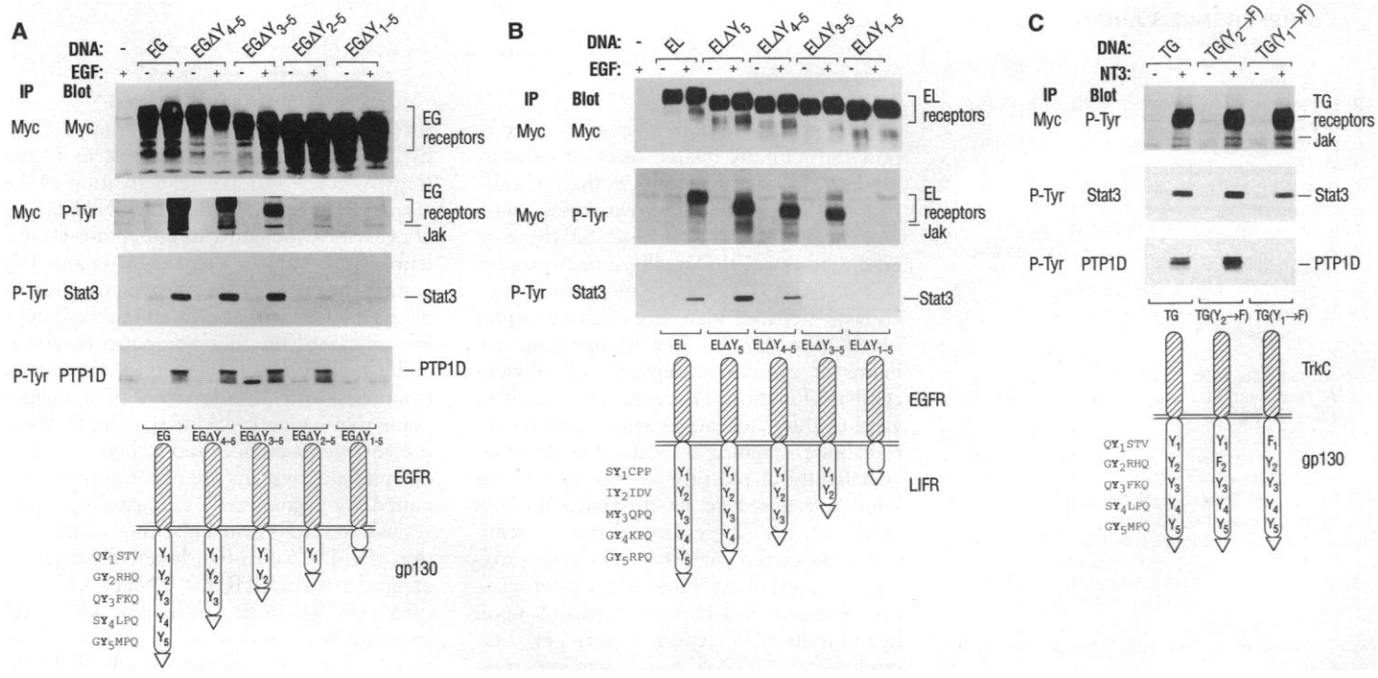

Fig. 1. Phosphorylation of Stat3 and PTP1D upon activation of chimeric receptors containing truncated or mutated forms of the cytoplasmic domains of gp130 and LIFR. Chimeric receptors were constructed (*25*) containing the extracellular portion of the human EGF receptor fused to complete or truncated versions of the cytoplasmic domains of either gp130 (the EG series) (**A**) or LIFR (the EL series) (**B**), or containing the extracellular and transmembrane portions of TrkC fused to complete or mutated versions of the cytoplasmic domain of gp130 (the TG series) (**C**); each receptor also contained a COOH-terminal triple Myc epitope tag (indicated by the triangle) recognized by the monoclonal antibody 9E10 (*10*). Vectors encoding these constructs were transfected into COS7 cells, which were then stimulated with either EGF (100 ng/ml) (A and B) or NT-3 (50 ng/ml) (C) for 10 min and washed and lysed as described (*10*). The lysates were immunoprecipitated (IP) with antibody 9E10 and then immunoblotted (Blot) with either antibody to phosphotyrosine 4G10 or with antibody 9E10. The same lysates were subsequently immunoprecipitated with 4G10 and then immunoblotted with antibody to Stat3 (Transduction Laboratories, Lexington, Kentucky); after stripping (*20*), the blots were probed with antibody to PTP1D (Transduction Laboratories). All immunoblots were visualized with enhanced chemiluminescence (*20*). Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

receptors with progressively larger COOH-terminal deletions of the gp130 or LIFR cytoplasmic domains. These truncations successively removed the five distal tyrosines in gp130 or LIFR (designated Y_1 to Y_5, Fig. 1), but all receptors retained the membrane-proximal Jak-binding domain. Each of these chimeric receptors was then expressed in COS cells and analyzed for its ability to mediate tyrosine phosphorylation of Jak, Stat3, or PTP1D. Analysis of the EG chimeric receptor truncation series revealed that both Stat3 and PTP1D were inducibly tyrosine phosphorylated (*27*) upon stimulation with EGF, regardless of whether the cytoplasmic domain lacked the three distal tyrosines (Fig. 1A). However, further truncation to remove Y_2 created a receptor that did not mediate Stat3 phosphorylation, yet did phosphorylate PTP1D and the associated Jak. Further truncation to remove Y_1 also eliminated the phosphorylation of PTP1D, despite expression of the receptor at equal or greater levels relative to the other truncated receptors, and tyrosine phosphorylation of the associated Jak kinase was still observed; the diminished Jak phosphorylation observed with the truncated receptor in this particular experiment was not reproducible, because the truncated receptor gave maximal Jak phosphorylation in other experiments (for example, Fig. 2A). Results comparable to those obtained with the EG chimeric receptor truncation series were also obtained with the analogous truncation series made with the TG chimeric receptor (*15*). Together, these results implicate the region spanning Y_2 in mediating the tyrosine phosphorylation of Stat3, and the region spanning Y_1 in the tyrosine phosphorylation of PTP1D. Indeed, Y_1 is embedded within a consensus motif proposed for interaction of PTP1D with receptor tyrosine kinases (*28*). Analysis of chimeric receptors (EL) containing the LIFR cytoplasmic domain revealed that dimerization of LIFR also resulted in tyrosine phosphorylation of Stat3 (Fig. 1B), but not PTP1D (*15*). Chimeric EL receptors with truncations of the Y_5 and Y_4 motifs retained the ability to mediate Stat3 activation, whereas further truncation of the Y_3 motif resulted in loss of Stat3 activation, although tyrosine phosphorylation of this chimeric receptor and the associated Jak was still observed (Fig. 1B). These data suggest that the region of LIFR containing Y_3 is critical for activation of Stat3. The results with the TG and EL receptors demonstrate that activation of a Jak kinase is not sufficient to mediate phosphorylation of Stat3 and PTP1D and that sequences in the cytokine receptors themselves are required to specify phosphorylation of these substrates.

We constructed point mutations of the full-length TG chimeric receptor in which either the Y_1 or Y_2 tyrosines were changed to phenylalanine to ascertain whether these residues were essential for induction of tyrosine phosphorylation of PTP1D and Stat3, respectively. Subsequent analysis confirmed that the mutant TG($Y_1 \rightarrow$ F) failed to mediate phosphorylation of PTP1D, but still mediated phosphorylation of the Jaks and Stat3 (Fig. 1C). However, the mutant receptor TG($Y_2 \rightarrow$ F) retained the ability to mediate tyrosine phosphorylation of Stat3 (Fig. 1C). One interpretation of this result is that there are multiple sites in the gp130 cytoplasmic domain that are capable of mediating Stat3 phosphorylation, the last of which is removed in the truncation mutant TGΔY_{2-5}. Indeed, the sequences spanning the four COOH-terminal tyrosines in gp130 (Fig. 1A) and the three COOH-terminal tyrosines in the LIFR (Fig. 1B) all have the sequence YXXQ; five of the seven also have proline at Y + 2, and a small amino acid such as serine or glycine at Y – 1. The second and third positions downstream from a phosphotyrosine may be of particular importance in SH_2-mediated phosphotyrosine binding (*29*).

To further investigate whether other tyrosine motifs in gp130 can mediate phosphorylation of Stat3, we designed a series of epitope-tagged chimeric receptors in which the Y_2, Y_3, Y_4, or Y_5 motifs (all fitting the YXXQ consensus) were individually appended to either of the truncated receptors TGΔY_{1-5} or EGΔY_{1-5} (referred to as TGt or EGt). Although the TGt and EGt receptors did not become tyrosine phosphorylated nor mediate Stat3 activation despite inducing Jak phosphorylation (Fig. 1A and 2A), truncated receptors containing any single tyrosine-based motif from gp130 with the sequence YXXQ became phosphorylated on that tyrosine and mediated tyrosine phosphorylation of Stat3, albeit to different extents (Fig. 2A). These results demonstrate that the consensus sequence YXXQ is sufficient to endow a truncated receptor with the ability to induce tyrosine phosphorylation of Stat3. In addition, the results suggest that these short tyrosine motifs are modular, in that they can be moved from one portion of the gp130 cytoplasmic domain to another, yet still function in mediating Stat3 phosphorylation.

To ascertain whether modular tyrosine-containing motifs are the critical determinants of which STATs are activated by a particular cytokine receptor, we investigated whether addition of a Stat3-specifying motif from gp130 could alter the normal selection of STATs by another cytokine receptor. We appended the gp130 Y_5 motif (GYMPQ) to a chimeric receptor contain-

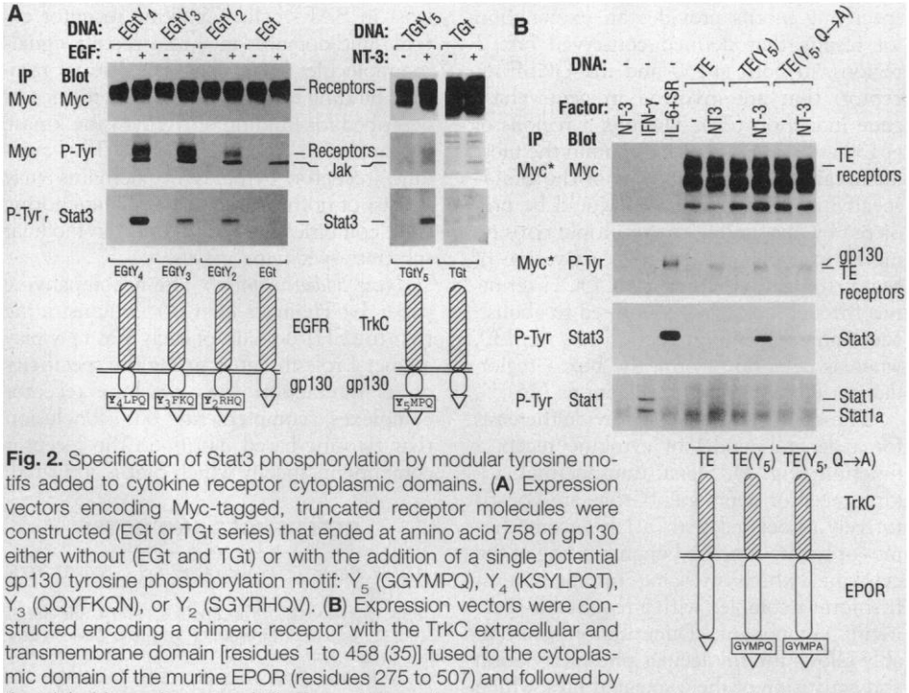

Fig. 2. Specification of Stat3 phosphorylation by modular tyrosine motifs added to cytokine receptor cytoplasmic domains. (**A**) Expression vectors encoding Myc-tagged, truncated receptor molecules were constructed (EGt or TGt series) that ended at amino acid 758 of gp130 either without (EGt and TGt) or with the addition of a single potential gp130 tyrosine phosphorylation motif: Y_5 (GGYMPQ), Y_4 (KSYLPQT), Y_3 (QQYFKQN), or Y_2 (SGYRHQV). (**B**) Expression vectors were constructed encoding a chimeric receptor with the TrkC extracellular and transmembrane domain [residues 1 to 458 (*35*)] fused to the cytoplasmic domain of the murine EPOR (residues 275 to 507) and followed by the triple Myc epitope tag (*10*). Two other versions were also constructed in which the sequences GYMPQ or GYMPA were inserted between the end of the EPOR sequences and the triple Myc tag. Vectors encoding the indicated constructs were transfected into COS7 cells and analyzed as described (Fig. 1), except that antibody to gp130 (Upstate Biotechnology) was used instead of 9E10 on the sample stimulated with IL-6, and the proteins immunoprecipitated with antibody 4G10 were first probed with antibody to Stat1 (Transduction Laboratories) and then probed with antibody to Stat3. Human IFN-γ (1 μg/ml), IL-6 (50 ng/ml), and soluble IL-6 receptor (SR, 1 μg/ml; R & D Systems) were used.

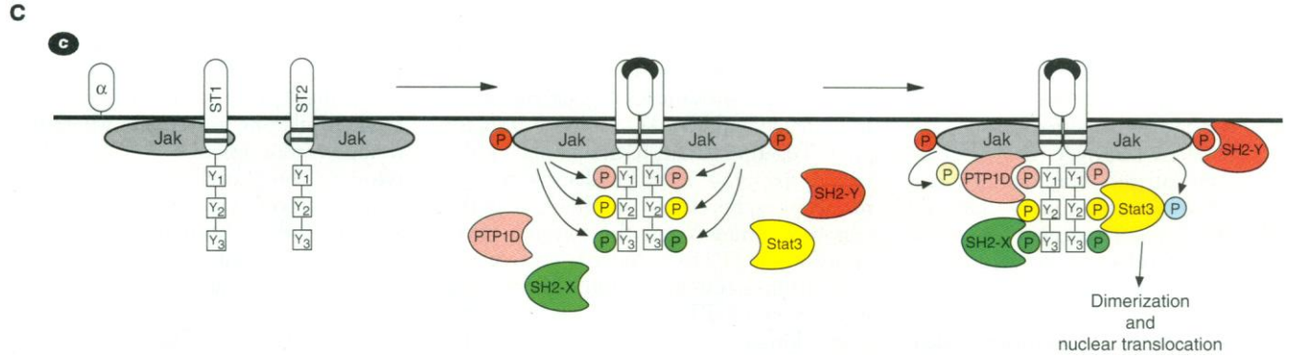

Fig. 3. Signal-transducing cytokine receptor components specify activation of particular pathways. Signal-transducing (ST) receptor components contain modular tyrosine-based motifs (Y_1 to Y_3) and are constitutively preassociated with inactive Jak kinases, which interact with the membrane proximal box1 and box2 sequences of the ST component (stippled regions). Cytokine (C) binding, which for CNTF and IL-6 requires an α receptor component, induces hetero- or homodimerization of the ST components and the associated Jak kinase, resulting in tyrosine phosphorylation and activation of the Jaks, which then tyrosine phosphorylate the ST cytoplasmic domains (tyrosine phosphorylation denoted by curved arrows). The phosphorylated tyrosine-based motifs form binding sites for particular SH2-containing substrates such as PTP1D, Stat3, and perhaps other undetermined substrates (SH2-X) that bind to the receptor and may themselves become tyrosine phosphorylated—presumably by the associated Jak. Stat3 subsequently dissociates, dimerizes with itself or Stat1, and translocates to the nucleus. According to this model, the sequence of the tyrosine-based motifs in the ST receptor component, and not the identity of the associated Jak kinase, specifies choice of many substrates such as the STATs and PTP1D. However, some substrates (SH2-Y) may bind directly to the Jaks and be activated without requiring receptor-based motifs.

ing the EPOR cytoplasmic domain. Whereas IL-6 (utilizing gp130) preferentially induced Stat3 and IFN-γ preferentially induced Stat1, the unmodified cytoplasmic domain of the EPOR did not induce either (Fig. 2B). However, a chimeric EPOR containing five amino acids spanning the gp130 Y5 motif mediated tyrosine phosphorylation of Stat3 but not of Stat1, just as IL-6 normally does on these cells (Fig. 2B). In addition, a tyrosine-based motif with an alanine replacing the glutamine at the Y + 3 position did not mediate phosphorylation of Stat3, consistent with a critical role for this residue (Fig. 2B). These results demonstrate that a short tyrosine-containing motif added to the cytoplasmic domain of a cytokine receptor component can determine the signaling pathways activated by that receptor.

Granulocyte colony-stimulating factor (GCSF) activates Stat3, whereas granulocyte-macrophage colony-stimulating factor (GM-CSF) does not (*15*). Consistent with our proposal that YXXQ is a consensus sequence for designating activation of Stat3, the murine GCSF receptor contains the sequence YVLQ, whereas neither the GM-CSF receptor nor the EPO receptor have sequences matching this motif. These YXXQ motifs differ from the motifs in Stat3 (YLKT) or Stat1α (YIKT) that are implicated in mediating homo- or heterodimerization of the STATs (*30*). It is not known whether the SH2 domain of Stat3 can directly bind to the YXXQ motif in receptor components—perhaps with a lower affinity than to the dimerization motifs in the STATs themselves, thus favoring rapid release from the receptor and subsequent STAT dimerization—or whether association of Stat3 with the receptor requires an intermediary adaptor protein that interacts directly with the phosphorylated YXXQ motif. Analogously, the YDKPH motif in the IFN-γ receptor that is required for Stat1 activation (*21*) also differs from the tyrosine-based motif on Stat1 that mediates dimerization.

Stat3, alternatively known as acute-phase response factor, has been implicated in mediating the acute-phase gene responses characteristic of the IL-6 family of cytokines (*17, 18*). Our identification of Stat3-specifying motifs provides an explanation for results that defined conserved box 3 regions, in both gp130 and the GCSF receptor, that are involved in acute-phase gene induction (*26*). The box 3 regions of gp130 and GCSF receptor contain the most membrane-proximal versions of the Stat3-specifying YXXQ motif. As would be predicted by our finding that multiple tyrosine motifs in gp130 can mediate activation of Stat3, truncations from the COOH-terminus through box 3 were required to abolish acute-phase gene induction by gp130, whereas deletion of only the box 3 region did not eliminate these inductions (*26*).

These and other results provide the basis for a general model of cytokine receptor function (Fig. 3). Signal-transducing cytokine receptor components that are constitutively associated with a Jak become homo- or heterodimerized upon binding of the cytokine, which in some instances must first form a complex with a required α-specificity component. Dimerization presumably allows intermolecular phosphorylation and activation of the associated Jaks, which then phosphorylate tyrosines on the cytokine receptor components. These receptor phosphotyrosines act as docking sites that selectively bind particular STATs and other SH2-containing downstream targets, which in turn can be phosphorylated by the associated Jak. However, the associated Jaks may also directly activate some signaling molecules without recruitment by the receptor component, because a truncated gp130 that retains the ability to activate the associated Jak, but contains no phosphorylatable tyrosine motifs, can promote DNA synthesis in BAF3 cells (*31, 32*); the latter result, coupled with our findings, implies that neither Stat3 nor PTP1D activation is required for IL-6–mediated proliferation, at least in BAF3 cells. Cytokine receptor cytoplasmic domains may also recruit signaling molecules by mechanisms distinct from SH2 binding to phosphorylated tyrosines, as described for binding of the tyrosine kinase lck to the IL-2 receptor (*33*). Thus, cytokine receptor cytoplasmic domains may consist of nothing more than Jak anchoring sites combined with an array of modular substrate-specifying motifs.

Note added in proof: The accompanying paper by Heim *et al.* (*34*) demonstrating that the SH2 domain of each Stat may play a crucial role in determining the specificity of its interaction with cytokine receptor complexes, complements our conclusion that tyrosine-based motifs on the receptor components specify which Stat is activated.

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A p53-Dependent Mouse Spindle Checkpoint

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Cell cycle checkpoints enhance genetic fidelity by causing arrest at specific stages of the cell cycle when previous events have not been completed. The tumor suppressor p53 has been implicated in a G_1 checkpoint. To investigate whether p53 also participates in a mitotic checkpoint, cultured fibroblasts from p53-deficient mouse embryos were exposed to spindle inhibitors. The fibroblasts underwent multiple rounds of DNA synthesis without completing chromosome segregation, thus forming tetraploid and octaploid cells. Deficiency of p53 was also associated with the development of tetraploidy in vivo. These results suggest that murine p53 is a component of a spindle checkpoint that ensures the maintenance of diploidy.

Genetic fidelity is achieved by the coordinated activity of genes and proteins that participate in DNA replication and chromosome segregation, cell cycle checkpoint controls, and repair pathways (*1*, *2*). In general, the onset of each stage of the cell cycle is dependent on successful completion of previous cell cycle events, and these dependencies are maintained by checkpoints (*2*). In the yeast *Saccharomyces cerevisiae*, several genes have been identified as components of a checkpoint that responds to spindle aberrations by causing cells to arrest in mitosis (*3*). Mutations in these genes can relieve the normal dependency of DNA synthesis on the completion of mitosis, permitting premature rounds of DNA synthesis in the presence of inhibitors that normally cause mitotic arrest, thereby leading to polyploidy. A similar checkpoint has been described in mammalian cells, but the genes that control it have not yet been identified.

The tumor suppressor p53 can be inactivated by allelic loss and mutation (*4*) and by interaction with viral oncoproteins, including SV40 T antigen (*5*). A p53-dependent G_1 checkpoint can prevent gene amplification by causing G_1 arrest after exposure to DNA damaging agents (*6*, *7*). However, the results of several studies suggest that p53 may be required for the maintenance of diploidy because loss or inactivation of p53 can be associated with tetraploidy or aneuploidy (*7*–*12*).

To investigate whether p53 participates in a mitotic checkpoint, we assessed the ability of fibroblasts from p53-deficient ($p53^{-/-}$) mouse embryos to arrest after exposure to spindle inhibitors in vitro. Wild-type ($p53^{+/+}$) mouse embryonic fibroblasts (MEFs) that were exposed to nocodazole or colcemid for 22 hours accumulated with a 4N DNA content and only a minority of cells reentered the S phase prematurely (Fig. 1, C and E). In contrast, $p53^{-/-}$ MEFs continued cell cycle progression after treatment with nocodazole or colcemid, forming cycling tetraploid and octaploid cell populations (Fig. 1, D and F).

To determine the fraction of $p53^{-/-}$ MEFs that escaped the spindle checkpoint and became polyploid, we investigated the proportion of cells that developed DNA contents greater than 4N (tetraploid S phase, octaploid) and 8N (octaploid S phase, greater ploidies) after 22 and 44 hours of exposure to each inhibitor. DNA contents greater than 4N were observed in 70.5 ± 2.2% and 66.9 ± 4.5% of $p53^{-/-}$ MEFs after 22 hours of exposure to nocodazole or colcemid, respectively, whereas only 22 ± 1.9% and 22.1 ± 0.3% of $p53^{+/+}$ MEFs developed DNA contents greater than 4N under the same conditions. DNA contents greater than 8N were observed in 49.8% and 48.5% of $p53^{-/-}$ cells after 44 hours of exposure to nocodazole or colcemid, respectively, compared with 0% of $p53^{+/+}$ cells. There was no evidence of aggregation artifacts that could lead to tetraploid and octaploid DNA contents, and the results were reproduced by flow cytometric protocols with the use of three different DNA dyes [4,6-diamidino-2-phenylindole (DAPI), propidium iodide, and Hoechst 33342].

Cycling tetraploid and octaploid cell populations were not observed in $p53^{-/-}$ MEFs after exposure to mimosine, a G_1 inhibitor, or to either aphidicolin or hydroxyurea, which are S phase inhibitors; less than 10% of $p53^{-/-}$ MEFs had DNA contents greater than 4N after a 22-hour exposure and none had DNA contents greater than 8N after 44 hours. The $p53^{-/-}$ MEFs that were exposed to VM26, a G_2 inhibitor, developed a small tetraploid S phase (15.2 ± 1.2% of cells had DNA contents greater than 4N after 22 hours), whereas $p53^{+/+}$ MEFs did not. The $p53^{-/-}$ cells exposed to VM26 did not develop cycling octaploid cell populations. Cytoge-

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