The Carboxyl-terminal Domains of gp130-related Cytokine Receptors Are Necessary for Suppressing Embryonic Stem Cell Differentiation

IN Volvement of Stat3*

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Cell type-specific responses to the leukemia inhibitory factor (LIF)/interleukin 6 cytokine family are mediated by dimerization of the LIF receptor α-chain (LIFRα) with the signal transducer gp130 or of two gp130 molecules followed by activation of the JAK/STAT and Ras/mitogen-activated protein kinase cascades. In order to dissect the contribution of gp130 and LIFRα individually, chimeric molecules consisting of the extracellular domain of the granulocyte colony stimulating factor receptor (GCSF-R) and various mutant forms of the cytoplasmic domains of gp130 or LIFRα were expressed in embryonic stem (ES) cells to test for suppression of differentiation, or in a factor-dependent plasma cytoma cell line to assess for induction of proliferation. Carboxyl-terminal domains downstream of the phosphatase (SHP2)-binding sites were dispensable for mitogen-activated protein kinase activation and the transduction of proliferative signals. Moreover, carboxyl-terminal truncation mutants which lacked intact Box 3 homology domains showed decreased STAT3 activation, failed to induce Hck kinase activity and suppress ES cell differentiation. Moreover, STAT3 antisense oligonucleotides impaired LIF-dependent inhibition of differentiation. Substitution of the tyrosine residue within the Box 3 region of the GSCF-R abolished receptor-mediated suppression of differentiation without affecting the transduction of proliferative signals. Thus, distinct cytoplasmic domains within the LIFRα, gp130, and GCSF-R transduce proliferative and differentiation signals.

Cytokines, such as the interleukins (IL)† and colony stimulating factors (CSF), regulate a wide range of biological activi-

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† The abbreviations used are: IL, interleukin; AS, antisense; ES, embryonic stem; FCS, fetal calf serum; GCSF, granulocyte colony stimulating factor; GCSF-R, granulocyte colony stimulating factor receptor; JAK, Janus kinase; LIF, leukemia inhibitory factor; LIFRα, LIF receptor α-chain; PAGE, polyacrylamide gel electrophoresis; SH2, Src homology 2; STAT, signal transducer and activator of transcription; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PIPES, 1,4-piperazineethanesulfonic acid; MAPK, mitogen-activated protein kinase; HAT, hypoxanthine/aminopterin/thymidine.

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phosphorylate tyrosine residues on gp130 and LIFRα (12, 13). In turn, the phosphorylated tyrosine residues on the cytokine receptors recruit various Src homology (SH2) domain-containing intermediate signal transducing molecules which often result in their subsequent tyrosine phosphorylation and activation. Two such molecules, the tyrosine phosphatase SHP2 and the latent signal transducer and activator of transcription 3 (STAT3), are phosphotyrosine binding partners for gp130 and LIFRα (16, 17), which contain 6 and 5 tyrosine residues in their respective cytoplasmic domains of gp130, LIFRα, respectively. These complexes mediate signal transduction molecules which often result in the transmembrane and cytosolic domain of gp130 and LIFRα have been described previously (20). The full-length G/Cpml and the truncated chimeric receptor were obtained by fusing the extracellular domain of the GCSF-R via an introduced BamHI site (20) with fragments encoding the transmembrane and cytoplasmic domains of gp130 (136 in gp130), and the LIFRα, respectively. These fragments were generated by polymerase chain reaction and contained at their 5′-end a silent BamHI restriction site over the second last amino acid of the extracellular domain and at their 3′-end translational stop codons at amino acid positions 220, 156, and 130 in gp130 (counting from the most membrane-proximal amino acid of the cytoplasmic domain) and position 136 in LIFRα yielding construct Ggp130 trunc®220, Gp130 trunc®156, and Gp130 trunc®130, and LIFRα trunc®130 (Table I). In the Gp130 trunc®130 construct, the tyrosine (Y) residue in the putative STAT3 binding sequence YXXQ in Box 3 was mutated to phenylalanine (F). In order to facilitate subcloning of isolated fragments into the mammalian expression vectors, the 3′-poly(A) tail reaction oligonucleotides also contained a XhoI restriction site. All newly generated chimeric receptor cDNA constructs were sequenced throughout their coding regions on an Applied Biosystem 373A DNA sequencing system and subcloned into the XbaI site of the mammalian expression vector 6–16PGK-IRE5neo designed to give rise to dicstronic mRNAs (20). For analysis of the full-length GCSF-R, Tyr→Asp substitutions were introduced by site-directed mutagenesis at Tyr114 and Tyr144 (29) and at both tyrosine residues (Tyr114, Tyr144) as described previously (29). The GCSF-R constructs were cloned into the XbaI site of the mammalian expression vector pEF-BOS (29) and co-transfected with the resistance marker plasmid PigKneo (14). High performance liquid chromatography-purified phosphothioate AS oligonucleotides directed against the amino-terminal region of mouse STAT1 (5′-GCCGACACTCGCGGACGCGC-3′) or a randomized sequence of a similar nucleotide composition (5′-AATGCAGGCCCATGGTCCG-3′) were purchased from Bresneca (Australia).

The LIF/IL6 cytokine family has been shown to induce tyrosine phosphorylation and DNA binding of a subset of STAT proteins, namely STAT1, STAT3 via a receptor binding dependent mechanism, and possibly STAT5a via a direct interaction with JAK kinases (19–21). In gp130 and LIFRα, activation of STAT3 depends on phosphorylation of the most carboxyl-terminal tyrosine residues while a different, more carboxyl-terminal truncations or mutation of specific tyrosine residues impairs the differentiation-inhibiting activity of full-length gp130 and LIFRα chains on ES cells in vitro.

**Experimental Procedures**

**Biological Reagents and Cell Cultures**—Recombinant human LIF (ES-GRO) was purchased from Amrad (Melbourne, Australia) and recombinant human GCSF and murine IL6 were kindly provided by Amgen (Thousand Oaks, CA) and R. Simpson (Ludwig Institute, Melbourne, Australia), respectively. The monoclonal antibodies used for Western blotting of STAT3 and anti-phosphotyrosine (4G10) were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Immunoprecipitation assays and immunoblotting were carried out by incubating 24-h cultures (inoculated at 5000 cells/cm2) for 10 min with streptolysin-O-containing (5 units/ml, Sigma) permeabilization buffer (150 mm RCl, 37.5 mm NaCl, 6.25 mm MgCl2, 0.8 mm EGTA, 1 mm CaCl2, 1.24 mm ATP, 12.5 mm PIPES, pH 7.5) supplemented with 3 mm HEPES. STAT3-dependent Signal Transduction in ES Cells
AS oligonucleotide. Cultures were then rinsed carefully several times with ES cell medium and incubated for 2 days in the presence of 3 μM AS oligonucleotide in ES cell medium supplemented with the indicated concentration of LIF. At this time, the medium was replaced with fresh medium containing 3 μM AS oligonucleotide and the cultures were incubated for an additional 3 days.

The GCSF-dependent survival/proliferation of G418-resistant, chimeric receptor expressing 7-TDI cells was assessed by determining cell numbers following an 8-day exposure of cultures (inoculated in 6-well multiculure dishes at a concentration 2 × 10^4 cells/well) to either GCSF (50 ng/ml) or 0.5 μM IL6 as a control. The mitogenic activity of GCSF-R-expressing Ba/F03 cells was determined by thymidine incorporation. For this purpose, the cells were inoculated in 96-well multi-culture dishes at a density of 10^4 cells/well, stimulated for 48 h with GCSF (10 ng/ml) in RPMI 1640 medium supplemented with 10% FCS. The cultures were pulsed for 4 h with 0.5 μCi/well [methyl-3H]thymidine (NEN Life Science Products Inc.) prior to harvesting and incorporated radioactivity was measured using a Betaplate liquid scintillation counter (Pharmacia Biotech Inc.).

Immunoblotting, Electrophoretic Mobility Shift Assay, and in Vitro Kinase Assays—For all assays, confluent cultures of undifferentiated ES cells were starved of LIF in ES cell medium containing reduced FCS (1%) levels. Immunoprecipitation experiments were carried out with anti-STAT3 or SHP2 antiserum on 500 μg of cell lysates in lysis buffer (1% Triton X-100, 50 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Trasylol, 0.1 mM Na3VO4) prepared from cultures stimulated for 10 min with LIF (500 units/ml), GCSF (10 ng/ml), or saline as described previously (51). The resulting immunoprecipitates were separated by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose (Schleicher & Schuell). The membranes were incubated with anti-phosphotyrosine antibodies (1:1500 dilution) and the proteins visualized with peroxidase-conjugated rabbit anti-mouse immunoglobulin (1:20000; Bio-Rad) using the ECL system (Amersham Corp.). Before reprobing the membranes with anti-STAT3 (1:500 dilution) or anti-SHP2 (1:2500) antibodies, they were stripped of antibodies in 62.5 mM Tris-HCl, pH 7.5, 2% SDS, 100 mM 2-mercaptoethanol. Sixty μg of total cell lysates were also used for MAPK mobility shift assays as described previously (14).

Electrophoretic mobility shift assays using the high affinity e-sis, factor inducible-binding site m67 were carried out as described previously (20, 30) with 16 μg of extracts prepared from ES cultures stimulated for 15 min with LIF (500 units/ml), GCSF (10 ng/ml), or saline and, where indicated, with STAT1 or STAT3 antisera. The DNA-binding complexes were then subjected to electrophoresis and visualized using PhosphorImager (Molecular Dynamics).

For Hck in vitro kinase experiments, 500 μg of cell lysate were immunoprecipitated with an Hck-specific antisera 1077 (1:5000 dilution, gift of C. Lowell, University of California, San Francisco, CA) and the tyrosine autophosphorylation reaction was carried out in the presence of 10 μCi of [γ-32P]ATP (3000 Ci/mmol, Bresatec, Australia) as described previously (14, 51).

RESULTS

The Membrane-distal Cytoplasmic Receptor Domains Are Required for the Regulation of ES Cell Differentiation—In order to test for the independent signaling capacity of the two components of the native heterodimeric LIF receptor complex, we exploited the fact that undifferentiated ES cells do not express GCSF-R. Cell lines expressing chimeric receptor constructs, in which the intracellular domain of either gp130 or LIFRα was fused to the extracellular domain of the GCSF-R, were established (Table I). This strategy permits GCSF-dependent dimerization and activation of chimeric receptor chains without the interference of the endogenous LIFRα and gp130 receptor chains. Clonally derived ES cell lines expressing comparable numbers of chimeric and endogenous receptor chains (800–1400 per cell) as assessed by binding of radiolabeled GCSF and LIF (Ref. 20, and data not shown) were chosen for subsequent analysis. The ability of the introduced receptor chains to suppress ES cell differentiation was determined in two independent assay systems. The first takes advantage of the striking morphological differences between the densely packed colonies of undifferentiated ES cells and the flattened morphology of differentiated cells (14, 51). Homodimerization of the full-length cytoplasmic domain of gp130 (G/gp130) or LIFRα (G/LIFRα), respectively, prevented ES cell differentiation in a GCSF-dependent manner (Fig. 1a). Moreover, GCSF also prevented differentiation of ES cells transfected with the full-length GCSF-R (Fig. 1c), but had no effect on mock-transfected wild-type cells (20). Signaling through the endogenous LIFRα/gp130 receptor heterodimer, formed in response to stimulation with LIF, prevented differentiation of approximately 95% of colonies in all ES cell lines investigated, indicating that the presence of chimeric receptors did not interfere with activation of the endogenous receptor chains. Since the scoring of cell morphology is potentially subject to investigator bias, we also carried out a colony assay based on selection against differentiated cells. This sensitive “killing assay” (Fig. 1, b and d) facilitated direct measurement of the proportion of undifferentiated ES cells after pulsing the cultures with the MTT dye. For this purpose, we exploited the hck-hprt ES cell line which harbors a hprt minigene under control of the murine hck promoter that is transcriptionally inactivated in differentiating ES cells (20, 51). Thus, differentiating cells are killed when incubated continuously in HAT-containing medium, while undifferentiated, HAT-resistant cells proliferate and metabolize the MTT dye to a product which can be measured photometrically.

Since the YXXQ motifs in the COOH-terminal region of gp130 are required for the induction of differentiation in myeloid M1 cells in response to IL6 (18, 26), we next tested the biological effect of carboxyl-terminal truncations of the cytoplasmic tail of gp130 and LIFRα. Ligand-dependent suppression of differentiation was fully maintained by the G/gp130Δ220 receptor retaining two out of four YXXQ motifs, but was significantly reduced with the G/gp130Δ315 chimera in which only the single YXXQ motif within Box 3 is retained (Figs. 1, a and c). Extending the truncation further to the membrane-distal border of Box 3 in conjunction with a phenylalanine substitution of the fourth YXXQ motif (F268XXQ) within Box 3 abolished the capacity of the G/gp130Δ310 receptor to suppress differentiation. Similarly, a truncated LIFRα with a deletion of the Box 3 region and lacking two out of three YXXQ motifs (G/LIFRαΔ130), was no longer capable of preventing ES cell differentiation (Fig. 1, b and d). Furthermore, GCSF-dependent activation of a chimeric receptor containing the full-length cytoplasmic domain of the thrombopoietin receptor c-Mpl, which lacks the Box 3 homology region but contains one YXXQ motif, was also unable to maintain the undifferentiated ES cell phenotype.

We next investigated the potential of wild-type and truncated chimeric receptor homodimers to mediate a proliferative response in the IL6-dependent cell line 7-TDI. Unlike the traditionally employed, factor-dependent pro-B cell line Ba/F03, the plasmaocyte cell line 7-TDI shows marked induction of proliferation upon activation of gp130 mediated signaling. As revealed in Fig. 2, both full-length, as well as the most truncated receptors, mediated a GCSF-dependent increase in cell number which was independent of STAT3 phosphorylation as assessed by anti-phosphotyrosine blotting of cell lysates. These data, therefore, suggest that the carboxyl-terminal membrane-distal regions of gp130 and LIFRα which contain the Box 3 homology region as well as several YXXQ motifs play a critical role in the signal transduction pathways required to suppress differentiation of ES cells in vitro. By contrast, the carboxyl-terminal regions of gp130 and the LIFRα are dispensable for the transduction of proliferative signals in 7-TDI cells which appears to be independent of STAT3 phosphorylation.

Activation of Intermediate Signaling Molecules—The heterodimeric LIFRα-gp130 receptor complex is physically and functionally associated with the Src-related tyrosine kinase
Hck and the JAK/STAT signaling cascade in ES cells (14, 51). We therefore investigated the capacity of the chimeric receptors to activate p56/59Hck and to phosphorylate STAT3, the predominant STAT protein activated in response to the LIF/IL6 family of cytokines. GCSF stimulation of cells expressing the full-length G/gp130, G/LIFRa, or the truncated G/gp130Δ220 receptors led to increased in vitro autophosphorylation of the two Hck isoforms (p56/59Hck) in cell lysates immunoprecipitated with an anti-Hck antiserum comparable to the increase observed after LIF-dependent stimulation of the endogenous gp130LIFRa complex (Fig. 3). By contrast, the truncated G/gp130Δ156, G/gp130Δ130F, nor G/LIFRaΔ136 receptors were capable of mediating significant activation of Hck. Furthermore, Hck kinase activity remained unaffected after stimulation of the G/mpl receptor, indicating that the most carboxyl-terminal sequences of the cytoplasmic tails of gp130 and the LIFRa-a are required for ligand-dependent Hck activation. Immunoprecipitation of STAT3 proteins followed by Western blotting with anti-phosphotyrosine antibody showed a prominent increase in tyrosine phosphorylation of STAT3 in response to GCSF in ES cells expressing chimeric receptors following LIF or GCSF stimulation (Fig. 4a, bottom), confirming that gp130 and LIFRa-dependent activation of MAPK occurs independently of YXXQ motifs (31). Furthermore, ligand-dependent activation of MAPK in ES cells correlated with an increase in tyrosine phosphorylation of the phosphatase SHP2 (Fig. 4b) which has been suggested as a molecular mechanism by which gp130 connects to the Ras/MAPK pathway (27). Thus, ligand-dependent activation of SHP2 and MAPK correlates with the receptors' capacity to stimulate proliferation, however, activation of this pathway in isolation does not suppress ES cell differentiation.

**STAT3 Is Required for the Suppression of ES Cell Differentiation**—The heterodimeric LIFRa-gp130 receptor complex in
ES cells is known to induce binding of STAT1 and STAT3 containing protein complexes to the high affinity sis-inducible SIE element (17, 20). Based on the retardation of these complexes by STAT antibodies, the most prominent SIF-A complex consists of STAT3 homodimers whereas the less abundant SIF-B and SIF-C complexes represent STAT3/STAT1 heterodimers and STAT1 homodimers, respectively (19, 20). Consistent with decreased or absent STAT3 phosphorylation following GCSF stimulation of the G/LIFRa D136, G/mpl, or G/gp130 D130F receptors, we observed reduced STAT-DNA binding in cell lines expressing G/LIFRa D136 or G/mpl, when compared with the pattern obtained after activation of the full-length G/gp130 and G/LIFRa, respectively (Fig. 5). Activation of the G/gp130 D130F receptor failed to induce STAT binding activity all together.

Receptor-dependent engagement of particular STAT family members is one of the mechanisms by which different biological outcomes may be achieved through JAK-STAT signal transduction pathways (4). Consistent with this finding, stimulation of the interferon-α receptor, which activates STAT1 and STAT2, fails to suppress ES cell differentiation (14). We therefore focused on possible functions of STAT3 by attempting to reduce the intracellular protein levels of STAT3 by exploiting phosphothioate AS oligonucleotides directed against the aminoterminal sequences of murine STAT3. A 5-day treatment of undifferentiated ES cells with 3 μM STAT3-AS oligonucleotides specifically decreased the STAT3 protein level by more than 90% when compared with cells exposed to a control AS oligonucleotide consisting of a randomized sequence with a similar nucleotide composition (Fig. 6a). Furthermore, the effect of the STAT3 AS oligonucleotide was specific, since in the same cells the protein level of STAT5a was not affected. The addition of STAT3 AS oligonucleotides also decreased the proportion of undifferentiated colonies in GCSF-treated cultures of cells expressing either G/gp130 or G/LIFRa (Fig. 6b and data not shown). We also tested the effect of the STAT3 AS oligonucleotide on the mitogenic response of 7-TD1 cells stimulated with IL6. Fig. 6c shows that inhibition of STAT3 protein production by STAT3 AS oligonucleotides had no effect on IL6-induced DNA synthesis. By contrast, the IL6 effect was abolished in cultures treated with the specific MAPK kinase (MEK) inhibitor PD098059, consistent with activation of the Ras/MAPK pathway via the membrane-most proximal SHP2 phosphotyrosine-binding sites in gp130 and LIFRa.

Taken together, our data obtained in ES and 7-TD1 cells suggest a selective functional involvement of STAT3 in differentiation suppressing signal(s) generated by the heterodimeric LIFRa gp130 complex or their respective homodimeric receptor counterparts.
Tyrosine Substitution in GCSF-R Impairs the Regulation of ES Cell Differentiation—Our limited survey of cytokine receptors suggests that activation of the Box 3-containing cytoplasmic GCSF-R domain can suppress ES cell differentiation (Fig. 1). Since the tyrosine residues within the cytoplasmic tail of the GCSF-R contribute to specific signaling pathways (31), we focused on the two tyrosine residues which showed the most prominent impairment of GCSF-mediated differentiation in the myeloid M1 cell line (29, 30). Stimulation of a GCSF-R mutant with a phenylalanine substitution of the membrane most proximal tyrosine residue (Y74F) suppressed ES cell differentiation to a similar extent as stimulation of the wild-type GCSF-R (Fig. 7). By contrast, substitution of the tyrosine residue within Box 3 (Y144F) decreased the capacity to inhibit differentiation in a GCSF-dependent manner by approximately 80%. The capacity to suppress differentiation was entirely lost in ES cells expressing GCSF-R containing the double substitution (Y74F, Y144F). In all cases, stimulation through the endogenous LIFRα gp130 receptor complex suppressed differentiation of more than 90% of ES cell colonies expressing the various versions of the GCSF-R constructs.

We also tested whether the tyrosine residues in GCSF-R required for signaling in ES cells contribute to propagation of mitogenic signals. For this purpose, we introduced the various GCSF-R expression constructs into the factor-dependent Ba/F03 cell line and screened for surface expression of GCSF-R protein by flow cytometry (data not shown) and representative cell lines were chosen which showed comparable levels of receptor expression. With all GCSF-R constructs tested, we were able to derive GCSF-dependent Ba/F03 cell lines. Moreover, GCSF-dependent [3H]thymidine incorporation was recorded in Ba/F03 cells expressing wild-type, Y74F, and Y144F GCSF-R, respectively, while some of the cell lines containing the Y74F,Y144F double substitution responded slightly less than wild-type receptor expressing cells (Fig. 8). These results suggest that Tyr144 within the GCSF-R Box 3 region is absolutely required for the regulation of ES cell differentiation, but is dispensable for the transmission of a mitogenic signal.
Analysis of STAT3 phosphorylation indicated reduced tyrosine phosphorylation following activation of the GCSF-R mutants Y144F when compared with the pattern observed with the wild-type and the Y74F receptor, respectively (Fig. 9). Furthermore, we could not detect significant STAT3 phosphorylation following stimulation of ES cell lines expressing GCSF-R (Y74F,Y114F). Similar results were obtained in electrophoretic mobility shift assays using the SIE probe (data not shown). Thus, the decreased STAT3 phosphorylation and DNA-binding observed with the GCSF-R(74F,Y114F) correlates with an inability to prevent differentiation of ES cells.

**DISCUSSION**

In this study we demonstrate that the membrane-proximal region of the cytoplasmic region of gp130 and LIFRα are required for regulation of mitogenic activity while the membrane-distal region with an intact Box 3 homology motif is critical for suppressing differentiation of ES cells *in vitro*. This observation correlates with ligand-dependent activation of Hck kinase and STAT3 phosphorylation and, unlike the transduction of mitogenic signal, is selectively impaired in the presence of STAT3 AS oligonucleotides. Therefore, our data support findings by Niwa et al. (32) who suggested a critical role for STAT3 in maintaining the pluripotent phenotype of ES cells *in vitro*.

Many cytokine receptors, including those for the ILF/LIF6 family, initiate divergent intracellular signaling pathways through domains that are specifically required for regulation of proliferation, differentiation, or apoptosis (33–35). Signaling by most type I cytokine receptors depends minimally on the Box 1 domain that binds the cytosolic kinase Lck and the T-cell co-receptors CD4/CD8 (39). The presence of conspicuous cysteine residues in gp130 and LIFRα as sites required for the induction of ligand-dependent Hck activation. While the molecular nature of this interaction remains to be established, the absence of conspicuous cysteine residues in gp130 and LIFRα suggests a different mechanism to that identified between the Src family kinase Lck and the T-cell co-receptors CD4/CD8 (39). The present study suggests a correlation between gp130/LIFRα-mediated...
...transfected with the indicated receptor construct are shown, mean ± S.D. The proportion of morphologically undifferentiated ES cell colonies was assessed as described in the legend to Fig. 1 in triplicate culture dishes, mean ± S.D. b, assessment by chemical selection. Transfected cells were plated in 24-well plates and grown for 6 days in ES cell medium supplemented with HAT and the indicated concentration of GCSF and an MTT assay was carried out in quadruplicate cultures as described in the legend to Fig. 1. The results for one representative cell line transfected with the indicated receptor construct are shown, mean ± S.D.

![Graph](image)

**Fig. 7.** Differentiation suppressing activity of GCSF-R mutants in ES cells. a, assessment by cell morphology. Transfected ES cells were cultured for 5 days with the indicated concentration of GCSF. The proportion of morphologically undifferentiated ES cell colonies was assessed as described in the legend to Fig. 1 in triplicate culture dishes, mean ± S.D. b, assessment by chemical selection. Transfected cells were plated in 24-well plates and grown for 6 days in ES cell medium supplemented with HAT and the indicated concentration of GCSF and an MTT assay was carried out in quadruplicate cultures as described in the legend to Fig. 1. The results for one representative cell line transfected with the indicated receptor construct are shown, mean ± S.D.

![Graph](image)

**Fig. 8.**[^1] Thymidine incorporation into Ba/F03 cells expressing mutant GCSF-R. Clonally derived Ba/F03 cell lines expressing the indicated GCSF-R mutants were plated in 96-well plates and stimulated for 48 h with 10 ng/ml GSF. Triplicate cultures were labeled with [3H]thymidine for the last 4 h and incorporated acid-precipitable radioactivity was determined in a β-scintillation counter. Similar results were obtained with at least one more independently derived cell line for each receptor construct, mean ± S.D.

![Graph](image)

**Fig. 9.** GCSF-dependent STAT3 phosphorylation and in ES cells expressing mutant GCSF-R. Confluent cultures of ES cells, expressing the indicated GCSF-R construct, were starved for 12 h of LIF in medium containing 1% FCS before stimulating for 10 min with 20 ng/ml GCSF (G), 500 units/ml LIF (L), or saline (c). Cell lysates were immunoprecipitated with anti-STAT3 antibodies, separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-phosphotyrosine antibodies (Tyr(P)). Blots were stripped and reprobed to assess for equal amounts of STAT3. The results for one representative cell line transfected with the indicated receptor construct are shown.

Expression of the JAK-STAT pathway is controlled at the level of JAK kinase engagement (14, 41), SH2 domain-dependent and independent interaction of STAT proteins with specific cytokine receptors, STAT dimerization, and the resulting specificity in DNA binding (42-44). Receptor phosphotyrosine-independent STAT5 activation and cell type-specific differences in STAT expression patterns may therefore account for the fact that the truncated G/gp130^{1130F} receptor induced growth of the plasmacytoma cell line 7-TD1 but only induced a transient mitogenic signal in the pro-B cell line Ba/F03 (27). In fact, STAT5 activation is required for maximal proliferation in response to IL2 receptor activation (45) and we found in 7-TD1 cells weak tyrosine phosphorylation of STAT5a with all truncated chimeric gp130 and LIFRa constructs (data not shown).

Based on the severity of the phenotype observed in mice with deletions for either STAT3 or gp130, STAT3 appears to be a crucial signaling intermediate for a number of growth factors and cytokine receptors in addition to those for the LIF/IL6 cytokine family. STAT3 plays an important role in self-renewal of lymphocytes (46) and is critically involved in transducing some of the biological responses of the LIF/IL6 cytokine family, including IL6-induced terminal differentiation, growth arrest, and macrophage differentiation of myeloid leukemia M1 cells (18, 26) and prevention of apoptosis in factor-dependent Ba/F03 cells (27). Similarly, phenylalanine substitutions or deletions of all YXXQ motifs in gp130 or LIFRa, respectively, abolish the ligand-dependent transduction of differentiation promoting signals in M1 cells and the induction of acute-phase proteins in hepatoma cells (5). However, the presence of a single YXXQ motif retained ligand-dependent differentiation in M1 cells, but not β-fibrinogen expression in HepG2 cells (5). In response to IL6, STAT3 activation occurs rapidly and transiently in ES and HepG2 cells but persists for more than 24 h in M1 cells (26). The sustained nature of STAT3 activity may be important in causing growth arrest and terminal differentiation, as in the case of sustained MAPK activation in nerve growth factor-induced PC12 cell differentiation (47) and gp130-mediated prevention of ES cell differentiation (14). Gp130 and LIFRa-dependent STAT3 activation requires at least one YXXQ consensus motif (51). Two of these motifs in gp130 (pY^{126RHQ} and pY^{177QFQ}) mediate activation of STAT3 while the other two motifs (pY^{265LPQ} and pY^{275MQ}) are capable of mediating the activation of STAT3 and STAT1 (48). Thus, substitution of the tyrosine residue in the truncated G/gp130^{1130F} receptor blunts the ligand-dependent formation of the SIF-A, SIF-B, and SIF-C DNA-binding complexes (Fig. 5 and Ref. 19). STAT1 appears to be a minor component of the total STAT proteins activated by gp130. It is therefore difficult to assess the role of STAT1 in ES cell directly, because (as observed in M1 cells)

[^1]: M. Ernst, unpublished observation.
STAT1 activity is weak and only detected when STAT3 is highly activated. STAT3-dependent induction of M1 cell differentiation and suppression of BA/F30 cell apoptosis appears to be independent of the number and position of these motifs in gp130 (18, 26, 27). In ES cells, however, a single XXXQ motif outside of the Box 3 motif (G/LIFR) failed to suppress differentiation, while the single XXXQ motif within the Box 3 homology domain (G/gp130) partially retained the activity in ES cells. These findings are reminiscent of observations that only Box 3 containing receptors, but not c-Mpl, induced transcriptional activation of acute-phase protein genes (5).

The presence of the common Box 3 motif in the cytoplasmic domain of GCSF-R, LIFR, and gp130 is likely to contribute to the remarkably similar pattern of biological activities transduced in a number of cell systems (4, 5, 20, 29). However, GCSF-R-mediated STAT3 phosphorylation and M1 cell differentiation is less tightly correlated with individual tyrosine residues when compared with gp130 and LIFR (29) and the Y74F substitution of the only XXXQ motif in the human GCSF-R did not have a significant effect on STAT3 phosphorylation and GCSF-mediated inhibition of ES cell differentiation. By contrast, the Y114L → FLRC substitution within the Box 3 sequence decreased STAT3 phosphorylation and GCSF-mediated suppression of differentiation. Thus, the regulation of ES cell differentiation by the cytoplasmic domain of the Box 3-containing gp130, LIFR, and GCSF-R appears to depend largely on STAT3, however, the mechanisms by which GCSF-R mediates STAT3 activation may be cell type-specific and more complex than SH2 domain-dependent binding to the XXXQ motif. In fact, GCSF-R-mediated STAT3 activation is not observed in neutrophils (40), while STAT3 and R. Simpson for recombinant human IL6.

Amgen for recombinant human GCSF, C. Lowell for the Hck antiserum, and R. Simpson for recombinant human IL6.

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