gp130-mediated Signal Transduction in Embryonic Stem Cells Involves Activation of Jak and Ras/Mitogen-activated Protein Kinase Pathways*

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The leukemia inhibitory factor/interleukin 6 (LIF/IL6) family of cytokines promotes cell type-specific pleiotropic effects by engaging multimeric receptor complexes that share the common affinity converter/signal transducing subunit gp130. While the maintenance of embryonic stem (ES) cell self-renewal is an activity unique to this family of cytokines, the intracellular signaling events mediated by gp130 remain largely unknown. Here we show a rapid and transient increase in the specific activity of the Src-related kinase Hck as well as of the Janus kinases Jak1, Jak2, and Tyk2 following treatment of ES cells with LIF or a combination of IL6 plus a soluble form of the IL6 receptor. Within 2 min of stimulation, we also observed increased tyrosine phosphorylation of SHC, activation of the guanidine nucleotide exchange activity on p21WRC, and an electrophoretic mobility shift of MAP kinase. Functional involvement of Hck and p21WRED activation in gp130-mediated signaling is supported by the finding that the introduction of constitutively activated Hck or v-Ha-ras partially alleviates the requirement of ES cells for LIF to remain undifferentiated. In contrast, suppression of Jak1 in ES cells by antisense technology increased the amount of LIF required to retain their pluripotentiality. These results are consistent with the notion that gp130-mediated suppression of ES cell differentiation depends on signaling through at least two cascades, namely a p21WRED-dependent pathway that possibly involves Hck, as well as a Jak kinase-dependent pathway.

The leukemia inhibitory factor (LIF)/interleukin-6 (IL6) family of cytokines elicits a wide variety of cellular responses including survival, proliferation, differentiation, and the regulation of end-cell function. Mice that contain targeted disruptions of genes encoding members of the LIF/IL6 family of cytokines, or the corresponding ligand binding chains, have confirmed the importance of these cytokines during fetal development and in the adult (1–4). In particular, LIF has been implicated in early embryogenesis (5, 6) by promoting the survival and proliferation of primordial germ cells in vitro (7) and maintaining the pluripotency of embryonic stem (ES) cells.

Cytokines like LIF and IL6 exert their biological effects through the formation and activation of multimeric hemopoietin receptors. These complexes frequently comprise a ligand-specific binding subunit and an affinity converting/signal transducing subunit common to a particular family of cytokine receptors. The competition of cytokines of the LIF/IL6 family for the shared and ubiquitously expressed signal transducing subunit gp130 defines a group of cytokines with overlapping functions. These include LIF, IL6, IL11, oncostatin M (OncM), ciliary neurotrophic factor (CNTF) (8), and cardiotrophin CT-1 (9). While the cytoplasmic portion of the CNTF- and IL6-ligand binding chains (IL6R) is not required for the generation of intracellular signals (10), the intracellular portion of the IL6-ligand binding chain (LIFR) has been implicated in signal transduction in some cell types (11). By analogy to tyrosine kinase receptors, the current model proposes that each receptor complex must contain two membrane-spanning subunits. This may be fulfilled either by a LIFR/gp130 heterodimer (in the case of LIF, OncM, and CNTF) or a gp130 homodimer (in the case of IL6) (12).

Despite the fact that hemopoietic receptors lack conspicuous catalytic subdomains, the appearance of tyrosine-phosphorylated proteins is among the earliest events following ligand-induced receptor oligomerization. For the LIF/IL6 family of cytokines, it has recently been shown that these effects can be accounted for by the association of the gp130 receptor subunit with members of the cytoplasmic tyrosine kinases of the Src (10) and Janus kinase (Jak) families (13). Indeed, the introduction of a constitutively active version of the Src-related kinase Hck (hckY499F) (10) partially abrogates the requirement of LIF to suppress differentiation of ES cells in vitro. Since tyrosine phosphorylation appears to be an obligatory early event in gp130-mediated signaling, additional cytoplasmic tyrosine kinases must be activated in ES cells in response to LIF either upstream of Hck or as part of Hck-independent signaling cascades. The Jak family of kinases represent likely candidates for the complementation of gp130-dependent signaling in ES cells, since at least 3 family members can be activated in response to LIF and IL6 in a variety of cellular systems (13). However, it appears that activation of individual Jak kinases may well be cell type-specific (13) with an apparent restriction to Jak1 and Jak2 activation in response to LIF in ES cells (14). Whether the differential utilization of Jak kinases (or indeed cytoplasmic kinases in general) may account for the different cell-type specific biological effects exerted by the LIF/IL6 family of cytokines has not yet been established.

For other hemopoietin receptors, such as those for IL2 and G-CSF, it has been shown that different regions of the cytoplasmic domain are required for the transduction of proliferative signals and those related to end-cell function (15, 16). Thus, the
activation of more than one intracellular signaling cascade may also explain the pleiotropic responses observed for the LIF/IL6 family of cytokines. Most studies addressing gp130-mediated intracellular signaling events have been carried out by overexpressing individual receptor chains in heterologous cell systems, including COS and hepatoma cells (11, 17–19). While such transfection experiments aim at reconstituting the normal regulatory systems in operation, they may often yield artificially exaggerated responses to extracellular signals as a direct consequence of overexpressing experimentally introduced molecules. Indeed, for many hematopoietin receptor signaling pathways it has been shown that the relative concentrations of intermediary signaling molecules are critical in specifying transcriptional regulation of target genes (20, 21). To overcome these potential difficulties, we have investigated the impact of LIF/IL6-dependent activation of membrane-proximal signaling molecules on preventing cellular differentiation in ES cells where the signal transducing molecules are at normal physiological concentrations. We provide insights into the signaling events which permit unrestricted self-renewal of ES cells in vitro in response to the LIF/IL6 family of cytokines. Based on “guilt by association and/or activation,” we identify Hck, Jak1, and p21ras as key intracellular signaling molecules. We also present functional evidence for the involvement of these molecules in promoting ES cell self-renewal by enforcing their activation in a LIF/IL6-independent manner. Finally, our data suggest that Hck and Jak kinases activate at least two intracellular signaling cascades which are required for the effective regulation of target genes that suppress differentiation of ES cells.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The expression cassette containing an activated version of v-Ha-ras (G12V,A59T) was constructed by polymerase chain reaction-mediated introduction of XbaI linkers into a full-length cDNA (22) and subsequent ligation into the XhoI site of the mammalian expression vector pEF-BOS (23). The inducible Jak1 antisense construct was obtained by cloning a 2.7-kilobase BglII fragment, derived from pCDHJ5 and encompassing the site of translational initiation of a human Jak1 cDNA (24) in the antisense orientation into the BglII site immediately downstream of the human 6–16 promoter in the expression plasmid pIOX (25). Expression construct were co-transfected with the resistance markers plasmids PGKneo (26) or PGKhprt (10).

**Cell Lines**—All experiments were carried out in the ES cell line E14TG2a which is defective in the hypoxanthine phosphoribosyltransferase (hprt) gene (27). ES cells were routinely subcultured in ES cell medium (Dulbecco’s modified Eagle’s medium containing 15% fetal calf serum, 0.1 mM 2-mercaptoethanol, and supplemented with nonessential amino acids and nucleotides) plus 500 μM (1000 units/mL) LIF (Esgro from AMRAD Operations Pty Ltd.) in the absence of feeder cell lines. Stable transfected ES cell lines were obtained by co-electroporating 15 μg of the linearized expression plasmids together with 1.5 μg of the selectable marker plasmid PGKneo or PGKhprt (500 microfardads; 270 V, Bio-Rad Gene Pulser) into 1.5 × 106 ES cells. The cells were plated into five 100-mm diameter culture dishes (Nunc) and selected for 7 days in ES cell medium containing G418 (Genetec, Life Technologies, Inc.; 175 μg/mL) or HAT (hypoxanthine, 120 μM; aminopterine, 0.4 μM; thymidine, 20 μM) beginning 24 h after the electroporation. Individual resistant colonies were expanded, and the integration of the expression construct was verified by Southern blotting.

**Cell Culture Assays**—The extent to which gp130-mediated signaling prevents ES cell differentiation was monitored by plating cells at a density of 500 cells/cm² in gelatin-coated 6-well multilwell plates (Nunc) in ES cell medium containing the indicated concentration of LIF. Five days after inoculation, the proportion of undifferentiated cells was determined by scoring ES cell colonies that consisted entirely of densely packed cells (“undifferentiated”) and colonies consisting of a mixture of densely packed cells and cells of flattened morphology or entirely of flattened morphology (“differentiated”) (10). The proportion of undifferentiated colonies was calculated after scoring the morphology of 300 randomly chosen colonies in triplicate culture dishes.

**In Vitro Kinase Assay and Immunoblotting**—For each time point, approximately 4 × 107 cells were starved in serum-free ES cell medium in the absence of LIF for 12 h. The cultures were then stimulated with 500 pM LIF or with 64 μM human sIL6R (encompassing amino acids 1–16) at the extracellular domain (28) plus 36 μM LIF at the indicated time period. After harvesting in ice-cold PBS, cells were lysed in 500 μL of KALB buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride, 1% Trasylol, 0.1 mM Na3VO4) for 30 min on ice before removing the nuclei by centrifugation. The protein concentration of the supernatant was determined using a BCA kit (Pierce), and aliquots of 150 μg of protein were precleared with nonimmune serum and immunoprecipitated with either the Hck-specific antis serum 1077 (1:500 dilution, gift of C. Lowell, University of California, San Francisco), the Jak1-specific antis serum M7 (1:100 dilution (30)), a Jak2-specific antis serum (1:200 dilution, UBI), or a Tyk2-specific antis serum (1:200 dilution, Transduction Laboratories) for 2 h at 4 °C with Protein A-Sepharose as a carrier. p21ras was immunoprecipitated with a monoclonal antibody raised against p21ras (31). The efficiency of p21ras protein was monitored by immunoblotting with an antibody directed against the carboxy terminus of p21ras (32). The proteins were subjected to partial tryptic digestion with Hck and Jak kinases. After reprecipitation with Protein A-Sepharose as a carrier (33), the immunoprecipitated pellets were washed four times in digitonin lysis buffer before adding 100 μL of kinase buffer containing 10 μCi of [γ-32P]ATP (3000 Ci/mmol, Amersham, Australia) and 500 μM LIF for 10 min. After preclarification of the samples with Pansorbin (Calbiochem) for 30 min at 4 °C, the lysates were immunoprecipitated with the anti-Hck antisera 1077 or the anti-Jak1 antisera M7 and protein A-Sepharose as a carrier. The immunoprecipitated pellets were suspended in a lysis buffer containing 10 μCi of [γ-32P]ATP and 10 μCi of [γ-32P]ATP and incubating the reaction for 30 min at 25 °C. Following separation of the denatured samples on SDS-PAGE gels, the radiolabeled bands of approximately 56/59-kDa molecular mass were excised from the dried gel and subjected to partial digestion with Staphylococcal V8 protease (Sigma) during the course of electrotheretic separation through a 15% SDS-PAGE gel (16). For immunoblotting of Hck, 150 μg of the total cell lysate used for the immunoprecipitation/autophosphorylation experiments was separated on 10% SDS-PAGE gels, while for the detection of Jak1, Jak2, or Tyk2, aliquots of the respective immunoprecipitates were separated on 7.5% SDS-PAGE gels. The proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) which was subsequently preincubated in blocking buffer (phosphate-buffered saline containing 5% sheep serum, 0.5% bovine serum albumin, 0.2% Tween 20) before adding the anti-Hck antisera 1077 (1:2000 dilution), the anti-Jak1 antisera M7 (1:500 dilution), the anti-Jak2 antisera (1:1000 dilution), or the anti-Tyk2 antisera (1:500 dilution). The bands were visualized following incubation with a peroxidase-coupled goat anti-rabbit IgG antiserum (Bio-Rad) and ECL reagents (Amersham).

For the detection of tyrosine-phosphorylated SHC, cell lysates were prepared from either LIF or IL6 plus sIL6R-stimulated confluent cultures that had been LIF-starved and serum-starved for 40 h prior to the experiment. Four hundred μg of lysates were immunoprecipitated with the anti-phosphotyrosine antibody 4G10 (UBI) at a concentration of 1 μg/mL for 2 h at 4 °C with rabbit anti-mouse immunoglobulins and protein A-Sepharose as carriers. The immunoprecipitates were separated on a 10% SDS-PAGE gel and blotted with an antiserum directed against SHC (1:500 dilution; UBI). The same cell lysates were also used for the MAP kinase mobility shift assay, by separating 60 μg of total cell lysates on a 10% SDS-PAGE gel and subsequent blotting with an anti-MAP kinase antibody (1:3000 dilution; UBI).

**Nucleotide Exchange Activity on p21ras**— Cultures of undifferentiated cells grown in 35-mm diameter culture dishes were starved of LIF and FCS for 12 h prior to the experiment. Cultures were then washed with warm phosphate-buffered saline and 0.8 ml of permeabilization buffer (150 mM KCl, 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) was added (32). Following the addition of 0.2-ml volume of streptolysin O (2 units/ml in water; Wellcome), the cells were permeabilized for 5 min. LIF (500 pM), IL6R (36 nm) plus sIL6R (64 nm) or FCS (10%) were added together with 5 μCi of [α-32P]GTP (3000 Ci/mmole, Bresapect), and the reaction was stopped at the indicated time by removing the supernatant and lysing...
the cells on ice in 1 ml of lysis buffer (100 mM NaCl, 5 mM MgCl2, 1 mg/ml bovine serum albumin, 1% Triton X-100, 30 mM Hepes (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 1% Trasylol, 0.1 mM Na3VO4) containing 0.1 mM unlabelled GTP and the anti-p21ras antibody Y13259 (1:20 dilution of hybridoma supernatant). The lysis buffer was collected and the immunoprecipitation was continued for 1 h at 4°C before adding protein G-Sepharose. After washing the pellets 3 times in lysis buffer, nucleotides were eluted in 1 M KH2PO4 (pH 3.4) for 3 min at 95°C and separated on thin layer chromatography plates using unlabeled GDP and GTP as standards.

RESULTS

The pluripotentiality of ES cells can be maintained in vitro by culturing in the presence of LIF which triggers the formation of a receptor complex containing a LIFR/gp130 heterodimer. While ES cells do not express IL6R, the same biological effect can be produced by IL6 if cells are exposed to a truncated, soluble form of the IL6R (sIL6R) that lacks intracellular and transmembrane sequences (10, 33). In this case, transmembrane signaling is mediated by a gp130 homodimer. To test for a possible contribution of the LIFR subunit to signaling, we compared the potency of LIF and IL6 to maintain self-renewal of ES cells. The concentration-dependent effects of LIF showed half-maximally effective concentrations (EC50) in the range of 4–10 pM (Fig. 1). IL6, in the presence of saturating concentrations of sIL6R, was about 100–300-fold less effective than LIF, while IL6 alone had no biological activity. Furthermore, the dose-response curves for LIF and OncM were comparable and consistent with suggestions by others (33) that both cytokines transduce the differentiation-retarding signal through the same LIFR/gp130 heterodimer. Thus, our results suggest that a LIFR/gp130 heterodimer may be a more effective signaling complex than a gp130 homodimer, although both types of receptor complex transmit signals that ultimately produce the same biological effect.

Several Tyrosine Kinases Are Activated by and Associated with gp130—Receptor complexes containing gp130 have been shown to activate members of the Jak family of tyrosine kinases in a cell type-specific manner (13). We therefore compared the time course of activation of Jak1, Jak2, Tyk2, and Hck in ES cells in response to LIF or IL6 in the presence of its soluble receptor (sIL6R). Since autophosphorylation of cytoplasmic kinases appears to be an important prerequisite for their catalytic activation in vitro, autophosphorylation experiments were carried out on lysates of cells stimulated with either LIF or IL6 plus sIL6R and immunoprecipitated with antibodies directed against Hck, Jak1, Jak2, or Tyk2 (Fig. 2).

As shown previously (10), we observed transient activation of the two isoforms of Hck, p56hck and p59hck (34) in response to LIF. In addition, Jak1 and Jak2 were also activated within 2–5 min of stimulation; the autophosphorylation activity of these kinases returned to basal levels within 120 min. In contrast, Tyk2 activity was only moderately stimulated 10 min after exposure of ES cells to ligands; however, this increase was maintained throughout the 120-min duration of the experiment. We confirmed that the ligand-dependent increase in kinase autophosphorylation reflected an increase in specific kinase activity, since Western blotting showed that the protein levels for all kinases investigated were unaffected over the time course of the experiment. Furthermore, the similar kinetics of p56hck and Jak1 autophosphorylation is consistent with the activation of these kinases independently of each other. This notion is supported by our finding that the kinetics and extent of Jak1 autophosphorylation in ES cells expressing a constitutively activated form of Hck (hckY499F) was unaltered (data not shown). The observed overall pattern of kinase activation was similar in cells treated with either LIF or IL6 plus sIL6R, but stimulation with IL6 plus sIL6R tended to produce delayed and less pronounced autophosphorylation of these cytoplasmic kinases. Thus, while LIFR/gp130 receptor heterodimers (in the case of LIF stimulation) cause activation of Jak1, Jak2, Tyk2, and p56/59hck, our data suggest a functional advantage conferred by the heterodimeric receptor complex.

p56/59hck are physical components of the functional LIFR-gp130 complex in ES cells (10), and various Jak kinases can be immunoprecipitated with gp130 receptor complexes in many transfected cell systems (13, 14). Therefore, we investigated whether Jak1 exists in the same physical complex as p56/59hck in cell lysates (containing 1% digitonin) prepared from LIF-stimulated ES cells. Jak1 immunoprecipitates subjected to an in vitro kinase assay showed a tyrosine-phosphorylated species of approximately 116 kDa (corresponding to Jak1) and two additional products with identical electrophoretic mobilities as p56/59hck immunoprecipitated from the same ES cell lysates using an Hck specific antiserum (Fig. 3A). To investigate whether the phosphorylated 56-kDa and 59-kDa products observed in the Hck and Jak1 immunoprecipitates were the same, the two species of radiolabeled bands were recovered from the gels and subjected to partial digestion with V8 protease. As shown in Fig. 3B, the pattern of V8-generated products was identical suggesting that in ES cells p56/59hck exists in the
same immunological complex as Jak1, possibly utilizing gp130 as a molecular bridge. Intriguingly, the anti-Hck antiserum did not co-immunoprecipitate Jak1 suggesting the possibility that the amino-terminal epitope recognized by the anti-Hck antiserum may be involved in the formation of the protein complex between Hck and Jak1.

Jak1 Activation Is Required for Maintaining ES Cell Pluripotentiality—To gauge the extent to which the biological effect of LIF/IL6 stimulation of ES cells could be attributed to activated Jak1 kinase, we generated ES cell lines expressing Jak1 antisense RNA under the control of the interferon α-inducible human 6–16 promoter. Following a preincubation period of 6 days in the absence or presence of INFα (3000 units/ml), ES cell lines stably expressing the inducible antisense construct showed a suppression of Jak1 expression by approximately 80% as assessed by Western blot analysis using a Jak1 antiserum (Fig. 4). By contrast, INFα had no effect on Jak1 protein levels in mock-transfected ES cells (clone 18) (Fig. 4). This observation most likely reflects a direct involvement of Jak1 in signal transduction from the LIFR-gp130 receptor complex that contributes to maintenance of the stem cell characteristics of ES cells in vitro.

The ras/MAP Kinase Pathway Is Involved in gp130-mediated Signaling—While the signaling cascades initiated through the Jak family of tyrosine kinases are believed to be distinct from those converging at the level of p21ras, it has been suggested that the activation of at least some of the Src kinases results in the activation of Ras guanine nucleotide exchange factor (35). We therefore investigated whether LIF/IL6-dependent inhibition of ES cell differentiation involved the activation of p21ras. In preliminary experiments, we found that LIF stimulation of 32Pi-preloaded, serum-starved ES cells led to a moderate increase in the ratio of [32P]GTP-p21ras to [32P]GDP-p21ras of approximately 45% to 55% (data not shown). The high proportion of GTP-p21ras in unstimulated cells suggested that the majority of serum-starved undifferentiated ES cells still progressed through the cell cycle. In order to investigate the LIF-dependent activation of p21ras in more detail, we determined the GDP/GTP exchange activity on p21ras according to the GTP-loading protocol described by Buday and Downward (32). For this purpose, cultures of undifferentiated ES cells were starved of LIF and FCS for 12 h.
before stimulation with either LIF, IL6 plus sIL6R, or FCS in the presence of [α-32P]GTP. Fig. 5 shows that experimental conditions that prevent ES cell differentiation in vitro (i.e., stimulation with LIF or IL6 plus sIL6R) led to a rapid and transient 3–4-fold increase in nucleotide exchange activity on p21ras. The stimulation of GDP/GTP exchange activity is comparable to the increase observed after mitogenic stimulation of ES cells with FCS, and the onset of nucleotide exchange activity occurred in less than 2 min after stimulation with FCS, LIF, or IL6 plus sIL6R. By contrast, treatment of ES cells with LIF or IL6 plus sIL6R had no effect on p120GAP-mediated hydrolysis of GTP-p21ras (data not shown). These observations suggest that the LIFR-gp130 heterodimeric or gp130 homodimeric receptor complex mediated activation of p21ras reflects increased GDP/GTP exchange activity rather than decreased GTP hydrolysis of p21ras.

Since phosphorylation of the SH2-adaptor protein SHC often provides a functional link between p21ras-dependent signaling cascades and membrane-associated tyrosine kinases, we next investigated the appearance of tyrosine phosphorylation of SHC in response to stimulation of ES cells with LIF or IL6 plus sIL6R. Within 2 min after stimulation, we observed a transient increase in the abundance of the phosphorylated p46/52SHC isoforms (Fig. 6A), while no tyrosine-phosphorylated p66SHC was detected. Therefore, the time course of SHC phosphorylation is consistent with a possible function of SHC upstream of the GDP/GTP exchange factor in mediating gp130-dependent signal transduction in ES cells.

**FIG. 5.** **p21**ras**-specific guanine nucleotide exchange activity in ES cells.** Undifferentiated ES cells were starved of LIF and FCS for 12 h before being permeabilized for 5 min. [α-32P]GTP and LIF (32 nM), IL6 (36 nM) plus sIL6R (64 nM), or FCS (10%) was then added for the indicated period of time after which p21ras was immunoprecipitated. Guanine nucleotides bound to p21ras were separated by thin layer chromatography, and the associated radioactivity was visualized and quantified using a PhosphorImager. The radioactivity associated with [α-32P]GTP is expressed as a percentage of total radioactivity associated with guanine nucleotides, and the exchange activity in cells mock-treated for 2 min was taken as 100%. Results are given as means ± S.E. of triplicate cultures.
Western blotting using an anti-MAP kinase antiserum on a 10% SDS-PAGE gel, and the transferred proteins were analyzed by two intracellular signaling cascades. We propose that one pathway leading to c-Ha-ras is mediated through at least two intracellular signaling pathways that operate in non-overlapping pathways. Indeed, our results suggest that signaling through the Ras/MAP kinase cascade is additive to signaling through Jak kinases, consistent with the notion that these molecules contribute to relieving ES cell dependence on LIF independently of each other. Notwithstanding this observation, our results do not preclude the involvement of molecules, in addition to those operating downstream of Ras/MAP kinase or Jak kinases which are required to elicit a full LIF/IL6 response.

The existence of multiple, often independent intracellular signaling pathways is an emerging theme for many hematopoietic receptors. For instance, the membrane-proximal region of the gp130-related A1C2B chain, the common signal-transducing molecule for the receptors for G-CSF, IL3, and IL5, is essential for c-myc induction by G-CSF, IL3, and IL5, while the distal portion is required for the activation of the Ras/MAP kinase pathway and the induction of c-fos/c-jun activity (15). Similarly, mutational analysis of the IL2R β-chain in factor-dependent BaF cells indicates a membrane-proximal origin for Jak kinase-dependent pathways (16). Our results on LIF signaling in ES cells extend findings that IL6-dependent gene expression depends on the p21ras-dependent MAP kinase cascade for the activation of NF-IL6 (37) and the Jak-dependent activation of the signal transducer and activator of transcription (STAT) 3 complexes (38).

A number of cytokines, including IL6 (39) and LIF (2) activate components of the ras/MAP kinase pathway, notably p21ras, Raf-1, and p42/44 MAPK (40). Furthermore, cytokine-mediated activation of p21ras is often preceded by tyrosine phosphorylation of SHC and its association with Grb2 (41). In an IL6-dependent B-cell line, it has been shown that Grb2 may be constitutively associated with gp130, whereas SHC appears in the same immunological complex as gp130 following stimulation of cells with IL6 (40). While neither gp130, LIFR nor p56/59ck contain the consensus binding sequence pYXY (42), several lines of circumstantial evidence link the activation of p56/59ck to the Ras/MAP kinase pathway. They include the observations that Hck-mediated signaling from the FcRI receptor leads to the activation of p42/44 MAPK (43), that p120GAP can serve as a substrate for Hck in a baculovirus system (44), and that p95ck, a nucleotide exchange factor for p21ras (45), is co-immunoprecipitated with Hck in myelomonocytic U937 cells (43). It is likely that the proliferative response of ES cells in vitro to serum depends on the activation of the Ras/MAP kinase pathway, since we found an increase in GDP/GTP nucleotide exchange activity and of SHC and p42/44 MAPK phosphorylation following serum stimulation. While it could be argued that partial suppression of differentiation by p21ras is a consequence of a decrease in transit time through the cell cycle, no alteration in cell doubling time was detected during the first 96 h.
after withdrawal of LIF. Thus, our results strongly suggest a
dual role for the Ras/MAP kinase pathway in ES cells by
transducing intracellular signals affecting proliferation as well as
differentiation. Hence, the Ras/MAP kinase pathway may play a role in ES cells similar to that reported in the neuronal
PC12 cell line, where EGF-induced mitosis as well as nerve
growth factor-induced differentiation critically depend on the
activation of the Ras/MAP kinase pathway. While SHC has
been shown to associate with Jak2 following stimulation with
erthropoietin, the contribution of Jak kinases to activation
of the Ras/MAP kinase pathway has been shown to associate with Jak2 following stimulation with
erthropoietin (47), the contribution of Jak kinases to activation
dependent tyrosine phosphorylation of intracellular proteins in-
cluding the cytosolic portion of gp130 (52).

In this report we show that LIF, OncM, and IL6 are func-
tionally equivalent in preventing ES cell differentiation in vitro, thereby confirming that activation of gp130 is sufficient for the maintenance of their pluripotentiality. Indeed, the pluripotency of long term cultures of ES cells maintained in
growth medium supplemented with OncM has been demon-
strated (53), and chimeric mice have been produced from ES
cells propagated in the presence of CNTF (54) or a combination
of IL6 plus sIL6R (33). However, these studies have not inves-
tigated possible quantitative differences in the signaling capacity
between the LIFR-gp130 heterodimeric receptor core complex (in the case of LIF, OncM, CNTF) and the gp130
homodimer receptor core complex (in the case of IL6). Our data
on ES cell renewal, tyrosine phosphorylation of Hck, Jak1,
Jak2, Tyk2, and SHC, and the activation p21^ras and MAP
kinase strongly suggests that the LIFR binding chain in het-
erodimeric receptor complexes may produce an amplification of the intracellular signal(s) over those generated by the gp130
homodimer receptor complex. The suggestion of an independ-
ent signaling contribution of the LIFR binding chain is consistent.

The search for ligand-dependent association and/or activa-
tion of cytoplasmic tyrosine kinases is a classical biochemical
method for identifying components of non-tyrosine kinase recep-
tor complexes. Out of the eight currently recognized families of non-receptor kinases, the ligand-dependent phos-
phorylation and/or physical association of gp130 has been reported for members of the Src (10), Jak (13), Tec/Btk (50), and
Fes families (51) of kinases. We have previously provided evidence for the physical and functional involvement of Hck in the
gp130-mediated suppression of ES cell differentiation. Using Jak1 antisense RNA experiments, we now present strong
and direct evidence for the functional involvement of Jak1
activation in LIF-dependent prevention of ES cell differen-
tiation. Surprisingly, the decreased levels of Jak1 protein in these
cells did not affect the ligand-dependent activation of Jak2 (data not shown). While these results are consistent with the
finding that the absence of one Jak kinase does not prevent
topolasmic kinases, which leads to their reciprocal transphosphorylation and activation. As a consequence, tyrosine residues within distinct regions of gp130 and the LIFR become phosphorylated, thereby providing SH2 domain binding sites for STAT and other intracellular signaling proteins that may be selectively required for the generation of cell-type specific responses. For instance, the membrane proximal Box1 and Box2 regions found in most hematopoietin receptors are required for the activation of receptors with Jak kinases as well as for the generation of proliferative signals (11, 17, 56). In contrast, a membrane distal domain (Box3) is unique to gp130, the LIFR, and the G-CSFR receptor, and failure to promote ES cell self-renewal is not generated from receptor truncations lacking Box3. Consistent with these observations, we have also demonstrated that activation of the transfected receptor for G-CSFR partially prevents ES cell differentiation. 2 Conversely, the introduction and activation of the related thrombopoietin receptor that contains Box1 and Box2 while lacking Box3 failed to promote ES cell self-renewal despite the ligand-dependent activation of Jak kinases. 2 Similarly, the stimulation of the INFγ receptor led to activation of Jak1 and Tyk2 in ES cells without affecting their responsiveness to LIF. These results support the notion that the cytokine response of a particular cell type is determined by the binding specificity of signaling proteins to individual phosphotyrosine residues within the receptor as well as the association and activation of one or other Jak kinases (21). Indeed, recent findings have indicated that the C-terminal portions (including Box3) of gp130, the LIFR, and the G-CSFR are required for the activation of STAT3 (57). Thus, the appearance of STAT3-containing DNA binding activity is readily observed following G-CSF, LIF, or IL6 stimulation, while the extent of STAT3 activation is much less pronounced after engagement of the INFγ receptor (19). It is tempting to speculate that Jak kinase-mediated STAT3 activation may be a critical component in the intracellular signaling cascade activated in ES cells undergoing self-renewal.

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