The Box-1 Region of the Leukemia Inhibitory Factor Receptor α-Chain Cytoplasmic Domain Is Sufficient for Hemopoietic Cell Proliferation and Differentiation*

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Leukemia inhibitory factor (LIF) is a pleiotropic cytokine that acts on a variety of cell types and regulates cell proliferation and differentiation. The functional receptor for LIF is composed of LIFR α-chain (LIFRα) and gp130 both of which are shared in the functional receptors for oncostatin M, ciliary neurotrophic factor, and cardiotrophin-1. By using stable transfection of wild-type or cytoplasmic deletion mutants of LIFRα together with full-length gp130 into Ba/F3 cells, we found that cells expressing gp130 and an extensively deleted mutant LIFRα containing only the box-1 region were capable of proliferating in response to LIF, although LIF-dependent long term growth of these cells was seriously impaired. Using a similar strategy to generate WEHI-3BD+ cells expressing gp130 and wild-type or truncation mutants of LIFRα, studies revealed that the box-1 region of the LIFRα was also sufficient for LIF-dependent induction of different aspects of differentiation, including up-regulation of macrophage surface marker expression, morphological change, and cell migration in agaur culture. However, the C-terminal region of the LIFRα, although not essential for intracellular signaling, was important for efficient receptor-mediated ligand internalization. In summary, the membrane-proximal box-1 region plays a dominant role in LIF-induced signal transduction of both proliferation and differentiation.

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The abbreviations used are: LIF, leukemia inhibitory factor; LIFRα, LIF receptor α-chain; IL, interleukin; OSM, oncostatin M; CT-1, cardiotrophin-1; CNTF, ciliary neurotrophic factor; G-CSF, granulocyte colony-stimulating factor; CSF-1, granulocyte colony-stimulating factor receptor; EGFR, epidermal growth factor receptor; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; STAT, signal transducer and activator of transcription; h, human; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; mIL-3, murine IL-3.

normal embryonic stem cells; stimulation of acute phase protein synthesis in hepatocytes; conversion of sympathetic neurons from the adrenergic to cholinergic phenotype, and the blocking of lipid accumulation in adipocytes (1–3).

LIF exerts its biological activities through a specific cell-surface receptor, which consists of two components, LIFR α-chain (LIFRα) that binds LIF with low affinity and gp130 that can convert a low affinity receptor complex to a high affinity receptor complex and then lead to intracellular signaling. The signal transducing subunit, gp130, is shared among the receptor complexes for interleukin-6 (IL-6), interleukin-11 (IL-11), oncostatin M (OSM), cardiotrophin-1 (CT-1), ciliary neurotrophic factor (CNTF), and LIF, which may explain some shared functions of this family of cytokines (4–10). Activation of IL-6 and IL-11 receptors and subsequent signaling requires gp130 homodimerization (11, 12). In contrast, initiation of signal transduction by binding of ligands like LIF, OSM, CT-1, and CNTF drives the heterodimerization of LIFRα with gp130, although other receptor subunits may also be required (13–16). Recently, an alternative OSM receptor component (OSMR β-chain) was identified, which can form a unique functional receptor complex for OSM by heterodimerization with gp130 (17, 18).

Although all the known ligand-binding components of the receptors for LIF-related cytokines (including IL-6, IL-11, CT-1, CNTF, OSM, and LIF) belong to the same hemopoietic receptor family, LIFRα is a transmembrane signaling subunit with a long cytoplasmic tail and structurally most related to gp130 and G-CSF receptor (19). After ligand binding to the cell-surface receptor, LIFRα dimerizes with gp130, which results in tyrosine phosphorylation and activation of members of the Jak family of cytoplasmic tyrosine kinases. The activated Jaks in turn phosphorylate tyrosine residues in the cytoplasmic domains of both gp130 and LIFRα, which leads to recruitment of substrates containing Src homology 2 domains (SH2-domain) and a cascade of downstream signal transduction events and eventually alteration in cellular behavior (12, 20–23).

Reconstitution experiments have demonstrated that both LIFRα and gp130 are required to transduce a proliferative signal in Ba/F3 cells in response to LIF (24, 25). In both rat and human hepatoma cells, transfection of LIFRα was able to activate cytokine-mediated transcription of acute-phase plasma protein genes in the context of LIFRα/gp130 heterodimer (26, 27). A mutant LIFRα lacking the cytoplasmic domain, when expressed in the same cell line, however, was inactive (26). The separate contribution of the LIFRα and gp130 cytoplasmic domains to LIF-induced signal transduction has been investigated using chimeric receptors in which the LIFRα cytoplasmic domain was connected to the extracellular domain of the G-CSF receptor (G-CSFR-LIFR). It was found that the G-CSFR-
LIFRα chimeras when expressed in embryonic stem cells were capable of suppressing differentiation in the presence of G-CSF, independent of gp130 activation, but the same chimeric receptors when expressed in either Ba/F3 or M1 cells were unable to signal after G-CSF stimulation (28). This suggests that, although the chimeric receptors were able to form homodimers driven by G-CSF binding, the signal transduction potential of the LIFRα cytoplasmic domain was dependent on the type of tissues or cells stimulated. In addition, analysis of deletion mutants of the cytoplasmic domain of the chimeric receptors (G-CSFR-LIFRα) has shown that the membrane-proximal region containing box-1 and box-2 was required for tyrosine phosphorylation of receptor components, Jak kinases, and STAT3 (signal transducer and activator of transcription-3) (29). A similar region of the LIFRα cytoplasmic domain when expressed in hepatoma cells as a G-CSFR-LIFRα chimera was able to activate STAT3, STAT1, and STAT5b in both gp130-dependent and -independent receptor systems (30). These data suggest that the cytoplasmic domain of the LIFRα plays a role in LIF-induced signal transduction although the contribution of each receptor component, LIFRα and gp130, might not be equivalent.

Although activation of the Jak-STAT biochemical signaling pathway mapped to specific regions of the cytoplasmic domain of the LIFRα, there is very little known about the capacity of specific regions of the LIFRα cytoplasmic domain to mediate different types of biological activities, particularly in hemopoietic cells and in the context of a LIFRα/gp130 heterodimer. In the present study, we investigated the signal transduction capacity of the LIFRα cytoplasmic domain in LIF-induced cellular proliferation and differentiation in hemopoietic cells. We have defined the membrane proximal box-1 region in the LIFRα cytoplasmic domain as essential for LIF-dependent proliferation and differentiation in hemopoietic cells and the membrane-proximal box-1 and box-2 regions as being required for LIF-dependent long term cell survival, in the context of a heterodimer with gp130. The C-terminal membrane distal region of the LIFRα cytoplasmic domain, although not essential for altering cell behavior, was important for efficient receptor-mediated ligand internalization.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Growth Factors—**WEHI-3BD+ cells (31) were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal calf serum (FCS). Ba/F3 cells (32) were grown in RPMI 1640 medium containing 10% (v/v) FCS and 10% (v/v) WEHI-3BD—conditioned medium as a source of IL-3. Cells were maintained in a humidified atmosphere of 10% CO2 in air and passed twice weekly.

Recombinant human LIF (hLIF) was a gift from Sandoz/Novartis, and human G-CSF (hG-CSF) was from Amgen (Thousand Oaks, CA). Recombinant human gp130 was a gift from BD Biosciences/PharMingen. Recombinant human LIFRα (27) and human G-CSF (hG-CSF) was from Amgen (Thousand Oaks, CA).

**Expression Constructs—**A cDNA fragment containing the entire coding region of human LIF receptor was cloned into the mammalian expression vector pEF-BOS (33) using BstXI adapters (Invitrogen, NV Leek, The Netherlands). An Ala → Thr silent mutation was introduced at nucleotide 2740 (nucleotide numbers according to the published sequence of Gearing et al. (19)) to create a unique SpeI restriction enzyme site. This vector, pEF-BOS/hLIF-RSpe, was then digested with SpeI and BamHI (nucleotide 3557) to remove the cytoplasmic domain. Using the polymerase chain reaction, DNA fragments containing an in-frame SpeI site and a 3' BamHI site were inserted back into pEF-BOS/hLIF-RSpe. Expression construct pEF-BOS/hLIFRmut1 encodes the hLIF receptor with only 4 amino acids of the cytoplasmic domain.

**Signal Transduction through the Cytoplasmic Domain of LIFRα**

**Cell Transfection—**Cells were stably transfected by electroporation. Briefly, 5 x 10⁵ cells were centrifuged and resuspended in 1 ml of normal culture medium. Aliquots of 4 x 10⁵ cells were transferred into 4-mm electroporation cuvettes containing 20 μg of expression plasmid and 2 μg of plasmid encoding genes for selectable markers. DNA and cells were incubated at room temperature for 5–10 min before electroporating at 270 V and 960 microfarads in a Bio-Rad Gene Pulser (Bio-Rad). Cells were then incubated for a further 5 min before resuspending in 100 ml of culture medium and aliquoting 4 ml into four 6-well tissue culture plates. After 2 days, selection was commenced by adding geneticin (Life Technologies, Inc.) at a concentration of 0.6 mg/ml or puromycin (Sigma) at a concentration of 20 μg/ml. Clones of proliferating cells were visible after 10–14 days of selection and were expanded to test for receptor expression. Individual clones were obtained by picking up individual colonies from semi-solid agar cultures.

**Binding Studies—**Transfected cells (0.5–5 x 10⁶ cells) were incubated with radio-labeled hLIF with or without unlabeled hLIF (2 μg/ml final concentration) in 100 μl of RPMI 1640 medium supplemented with 10 mM Hepes buffer and 10% FCS) at 4°C for at least 3 h. Cell-associated and free 125I-hLIF were separated by rapid centrifugation through 200 μl of FCS and quantitated in a Packard γ-counter. Specific binding was determined as the difference in counts between parallel sets of tubes with or without unlabeled hLIF. The data were analyzed by the LIGAND program (34) for both 1-site and 2-site binding interactions and presented as Scatchard plots (specific binding/free hLIF versus bound hLIF). The two-site model was used only if it produced a significantly better fit to the experimental data (p < 0.05).

125I-hLIF internalization in both Ba/F3 and WEHI-3BD+ cells was performed at 37°C. Transfected cells (0.8–5 x 10⁶ cells) were incubated with absence or presence of unlabeled hLIF (2 μg/ml final concentration) in RHF (RPMI 1640 medium supplemented with 10 mM Hepes buffer and 10% FCS) at 4°C for at least 3 h. Cell-associated and free 125I-hLIF were separated by rapid centrifugation through ice-cold FCS to separate cell-associated and free 125I-hLIF. The cell pellets were resuspended in 1 ml of ice-cold 3% (v/v) acetic acid in PBS and, after 5–10 min on ice, were centrifuged to separate the cell-associated 125I-hLIF from acid-dissociated 125I-hLIF (supernatant). Both fractions were counted in a γ-counter. The data were fitted using the curve-fitting program of Myers et al. (35), and rate constants were extracted.

**Proliferation Assay—**The proliferation of Ba/F3 cells in response to cytokines was measured in Lux 60 microwell HL-A plates (Nunc Inc., Roskilde, Denmark). Cells were washed three times in DMEM containing 10% FCS and resuspended at a concentration of 2 x 10⁴ cells/ml in the same medium. Aliquots of 10 μl cell suspension were placed in the culture wells with 5 μl of serial 2-fold dilutions of either human LIF or murine IL-3. After 2 days of incubation at 37°C in a fully humidified incubator containing 10% CO2 in air, viable cells were counted using an inverted microscope.

DNA synthesis was measured by [3H]thymidine incorporation. Cells were incubated in quadruplicate in 100 μl of culture medium containing 0.5 ng/ml either hLIF or IL-3 in 96-well plates for either 2 days or 8 days. Six hours before cell harvest, 0.5 μCi of [3H]thymidine was added to each well, and [3H]thymidine incorporation into the washed cells was measured by liquid scintillation counting.

**Long Term Cell Growth and DNA Synthesis Assays—**To determine factor-dependent long term growth potential of the Ba/F3 transfectants, cells were incubated at an initial concentration of 1 x 10⁴ cells/ml in medium containing 0.5 ng/ml either hLIF or IL-3. The medium was replenished every 2–3 days to maintain the cell density at about 1–5 x 10⁵ cells/ml. Viable cells were determined daily using a hemocytometer and eosin exclusion.

**Differentiation Assays—**To assess the differentiation response of
WEHI-3BD+ cells to cytokine stimulation, 100 cells were cultured in 35-mm Petri dishes containing 1 ml of DMEM supplemented with 20% (v/v) FCS, 0.3% (w/v) agar, and 0.1 ml of serial 2-fold dilutions of 10 ng/ml hLIF, 1 µg/ml cIL-6, and 10 ng/ml hG-CSF. After 7 days of incubation at 37 °C in a fully humidified incubator containing 10% CO₂ in air, colonies were scored as differentiated if they were composed of dispersed cells or had a corona of dispersed cells surrounding a tightly packed central core.

Differentiation of WEHI-3BD+ cells was also assessed by analyzing the morphological changes in liquid culture. Cells were cultured in DMEM supplemented with 10% FCS in the absence or presence of either 10 ng/ml hLIF or 10 ng/ml hG-CSF. The medium was replenished every 2–3 days to maintain the cell density at about 0.2–1 × 10⁶ cells/ml. After 11–12 days of culture with cytokine, cells were collected and concentrated on glass slides by cytocentrifugation. Cell morphology was examined after May-Grunwald-Giemsa staining.

Flow Cytometric Analysis of Surface Antigen Expression—WEHI-3BD+ cells, cultured for various days with either 10 ng/ml hLIF or 10 ng/ml hG-CSF, were washed twice in staining buffer (PBS containing 2% (v/v) FCS and 0.02% (v/v) sodium azide). Cells (about 1 × 10⁶) were incubated at room temperature for 10 min with 50 µl of rat anti-Fc receptor-blocking antibody to block nonspecific Fc binding of the test antibody. After washing in staining buffer once, cells were first incubated with 50 µl of staining buffer containing 1 µg of either biotinylated anti-Mac-1 (PharMingen, San Diego, CA) or F4/80 (Caltag Laboratories, South San Francisco, CA) or 1 µg of biotinylated corresponding isotype control antibody (PharMingen, San Diego, CA) at 4 °C for 30 min. Cells were further incubated at 4 °C for 30 min with a 200-fold dilution of streptavidin/phycoerythrin (Caltag Laboratories, San Francisco, CA) in 50 µl of staining buffer after washing twice with staining buffer. Cells were then washed twice and resuspended in 100 µl of staining buffer containing 1 µg/ml propidium iodide to assess viability. Flow cytometry was performed with a Becton-Dickinson FACScan using FACSscan software.

Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays were performed as described previously (36), using the high affinity c-Sis-inducible factor binding site m67 (37). Total cell extracts were prepared from WEHI-3BD+ cells treated with saline or 100 ng/ml hLIF for 10 min at 37 °C. For Ba/F3 cells, cells were starved for 3 h in medium free of serum and IL-3 before being stimulated for 10 min at 37 °C with saline or 100 ng/ml hLIF. Protein extracts (20 µg) were incubated with radiolabeled duplex m67 oligonucleotide. Bound and unbound duplex oligonucleotides were separated on 5% native polyacrylamide gels. Where indicated in the text, extracts were preincubated with antibodies specific for either STAT1 (Transduction Laboratories, South San Francisco, CA) or STAT3 (Upstate Biotechnology Inc.), or with 100-fold molar excess of unlabeled m67 duplex for 20 min at room temperature before the addition of radiolabeled m67 duplex.

Immunoprecipitation and Western Blots—Ba/F3 cells were washed and deprived of mIL-3 and serum for 2–3 h before treatment with fresh medium containing 100 ng/ml hLIF or normal saline at 37 °C for 10 min and then lysed in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 2 mM NaF, 1 mM Na₃VO₄, and Complete™ protease inhibitor mixture (Boehringer Mannheim). After pelleting insoluble material and protein standardization, the supernatants containing about 2 mg of total cellular proteins were incubated with 4 µg of 4G10 anti-phosphotyrosine monoclonal antibody (Upstate Biotechnology Inc.) and protein A-Sepharose beads at 4 °C overnight. The immune complexes were washed in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 2 mM NaF, 1 mM Na₃VO₄, and Complete™ protease inhibitor mixture, eluted from the Sepharose beads by boiling in SDS sample buffer under reducing conditions for 5–10 min before being subjected to 4–15% acrylamide SDS-PAGE and then transferred to polyvinylidene difluoride membrane (PVDF-Plus, Micron Separations Inc.). After blocking with 2% (v/v) bovine serum albumin, 0.1% (v/v) Tween 20 in PBS, the membrane was incubated with anti-human gp130 polyclonal antibody (Santa Cruz Biotechnology) followed by incubation with a goat anti-rabbit immunoglobulin polyclonal antibody conjugated with horseradish peroxidase (DAKO, Denmark). The phosphorylated proteins were visualized by radiography using an ECL system (Pierce). The membranes were stripped with 0.1 M glycine HCl (pH 2.5) for 30–60 min and washed 3 times in PBS containing 0.1% Tween 20 before reprobing with an anti-human LIFR polyclonal antibody (R & D Systems).

RESULTS

Construction and Expression of Wild-type and Mutant Human LIFR and Human gp130 cDNAs—To examine functional regions in the cytoplasmic domain of hLIFR α-chain necessary for transducing LIF-induced proliferation, survival, and differentiation signals in hemopoietic cells, we established Ba/F3 cell lines that stably express either wild-type LIFRα or truncated receptors with progressively longer deletions of the C-terminal region of the LIFRα cytoplasmic domain, as shown in Fig. 1. Expression of receptors in transfected cells was confirmed by testing their ability to bind ¹²⁵I-hLIF, and positive clones were subjected to a second round of transfection to generate cell lines also expressing full-length human gp130. Clones that expressed both components, having the characteristics of slow dissociation and high affinity of hLIF binding, were selected for further studies. The number and affinity of receptors expressed by transfected cells were estimated by performing a saturation binding experiment on 1–5 clones for each transfectant with ¹²⁵I-hLIF. Scatchard transformation of the saturation binding isotherms showed that Ba/F3 cells expressing either wild-type (Fig. 2) or mutant hLIFRα alone (data not shown) displayed a single class of low affinity receptors with a Kₐ value of 300–800 pM and an average receptor number of approximately 1000 per cell (Table I). Cell lines transfected with both wild-type LIFRα and gp130 cDNAs exhibited a single class of high affinity receptors with a Kₐ value of 10–40 pM and a similar receptor number. This is comparable with those detected in the LIF-responsive M1 myeloid leukemic cell line, which expresses both LIFRα and gp130 (38). Cells expressing gp130 and the various cytoplasmic truncated forms of LIFRα showed variable numbers of high and low affinity receptors (Table I), reflecting variability in the relative expression of LIFRα and gp130. Consistently, Western blot experiments showed that the expression level of the various forms of LIFRα was higher than that of gp130 (data not shown).
Essentially, the same procedure was applied to WEHI-3BD+ cells to generate LIF-responsive cell lines. WEHI-3BD+ is a murine myeloid leukemic cell line able to differentiate in response to G-CSF and IL-6, but not LIF (39, 40), although IL-6 was 10-fold less efficient than G-CSF. In order to achieve differentiative responsiveness of WEHI-3BD+ cells to LIF, we first generated a transfected cell line expressing Flag-tagged human gp130 cDNA by electroporation followed by selection, cloning, and then assessment of its expression by flow cytometric analysis using M2 anti-Flag antibody (data not shown). To this Flag-tagged, gp130-transfected cell line, either wild-type or mutant hLIFRα cDNAs were introduced, and doubly transfected cells were tested for their capacity to bind hLIF. Scatchard analysis of the binding of 125I-hLIF to transfected WEHI-3BD+ cells demonstrated that cells expressing wild-type hLIFRα alone displayed both high and low affinity receptors (Table I), suggesting the expression of endogenous gp130. Cells expressing both wild-type hLIFRα and exogenous gp130 exhibited a single class of high affinity receptor. Cell lines expressing cytoplasmic deletion mutants of hLIFRα along with exogenous gp130 showed a single class of hLIF-binding sites with an apparently intermediate affinity (Table I). This apparently intermediate affinity was probably comprised of nearly equal numbers of high and low affinity receptors, which cannot be easily resolved by the curve fitting program.

The Membrane-proximal Region of the LIFR Cytoplasmic Domain Is Sufficient for LIF-dependent Proliferation—The ability of LIF to stimulate proliferation of hematopoietic cells has been established with both murine DA-1a (41, 42) and human TF-1 (43) cell lines. Ba/F3 murine hematopoietic cells are absolutely dependent on IL-3 for proliferation but do not proliferate in response to LIF because they lack endogenous LIFRα-chain and gp130. Using Ba/F3 cell lines stably expressing wild-type or deletion mutant forms of LIFRα along with gp130, any response detected to human LIF could only be due to the presence of a functional LIFR.

The proliferative response of the transfected Ba/F3 cells to hLIF was first measured as an increase in cell number in microwell assays after 48 h culture. It was found that cells expressing wild-type hLIFRα alone were unable to proliferate...
expressed gp130 and an extensively deleted mutant LIFR
viable cells were counted daily. As shown in Fig. 4, most pa-
ing 0.5 ng/ml either hLIF or mIL-3 for a 10-day period, and
factor-dependent long term growth of Ba/F3 cells, both paren-
was still intact (Fig. 3). Half-maximal stimulation (EC50) of h e
hLIF, although their IL-3-dependent proliferation response
completely abrogated the proliferation of cells in response to
medium. Cells expressing wild-type LIFR
shortest mutant LIFR
a

TABLE I
Binding affinities and number of LIF receptors
expressed on cell transfectants

| Parental cell line | Transfectant | $K_a^{a}$ | HA$^{b}$ | LA$^{a}$ | HA$^{b}$ | LA$^{a}$ |
|--------------------|--------------|-----------|---------|---------|---------|---------|
| Ba/F3              | Parental     | ND$^{b}$  | ND$^{b}$| ND$^{b}$| ND$^{b}$| ND$^{b}$|
| LIFR(wt) only$^{a}$| 23           | 357       | 940     | 1159    | 940     | 1159    |
| LIFR(wt)/gp130     | 49          | 1700     | 2200    | 2200    | 2200    | 2200    |
| LIFR150/gp130      | 6.2         | 288      | 1670    | 9226    | 1670    | 9226    |
| LIFR94/gp130       | 1100        | 11100    | 2200    | 650     | 2200    | 650     |
| LIFR68/gp130       | 48          | 3727     | 3727    | 3727    | 3727    | 3727    |
| LIFR34/gp130       | 48          | 1992     | 1992    | 1992    | 1992    | 1992    |
| LIFR4/gp130        | 28          | 838      | 260     | 6459    | 260     | 6459    |
| WEHI-3BD+          | Parental     | ND$^{b}$  | ND$^{b}$| ND$^{b}$| ND$^{b}$| ND$^{b}$|
| LIFR(wt) only$^{a}$| 5.5         | 1685     | 22      | 900     | 22      | 900     |
| LIFR(wt)/gp130     | 28          | 90       | 90      | 90      | 90      | 90      |
| LIFR150/gp130      | (98)$^{e}$  | (2103)   | (2103)  | (2103)  | (2103)  | (2103)  |
| LIFR34/gp130       | (96)$^{e}$  | (2512)   | (2512)  | (2512)  | (2512)  | (2512)  |
| LIFR4/gp130        | (169)$^{e}$ | (5789)   | (5789)  | (5789)  | (5789)  | (5789)  |
| LIFR34/gp130       | (86)$^{e}$  | (1628)   | (1628)  | (1628)  | (1628)  | (1628)  |
| LIFR4/gp130        | (224)$^{e}$ | (3563)   | (3563)  | (3563)  | (3563)  | (3563)  |

$^{a}$ HA and LA refer to high and low affinity binding sites, respectively.
$^{b}$ ND, not detected.
$^{c}$ Analysis was performed on one clone for each cell line.
$^{d}$ Analysis was performed on two to five individual clones for each cell line, and the values of both $K_a$ and receptor number per cell were the mean of the individual clones. All two-site binding profiles, where indicated, were statistically significant ($p < 0.05$) better fits than one-site fits.
$^{e}$ Numbers in parentheses are intermediate affinity LIF receptors.

when cultured with hLIF; however, co-expression of gp130 and
wild-type hLIFRa conferred the ability to proliferate in re-
ponse to hLIF. Analyses of a series of cytoplasmic deletion
mutants of LIFRa in Ba/F3 cells demonstrated that cells that
expressed gp130 and an extensively deleted mutant LIFRa
(LIFRa34/gp130) containing only the membrane-proximal box-1
region were capable of proliferating when cultured with hLIF.
Further truncation of LIFRa to a form that contained only 4
amino acid residues of its cytoplasmic domain (LIFRa4/gp130)
completely abrogated the proliferation of cells in response to
hLIF, although their IL-3-dependent proliferation response
was still intact (Fig. 3). Half-maximal stimulation (EC50) of the
various positive LIFRa/gp130 transfectants occurred at similar
hLIF concentrations between 25 and 110 ng/ml (Fig. 3). Thus,
that the membrane-proximal box-1 subdomain in the
LIFRa cytoplasmic part is a region essential for LIF-in-
duced proliferation in Ba/F3 cells.

To examine whether LIF could replace IL-3 as a stimulus in
factor-dependent long term growth of Ba/F3 cells, both paren-
tal cells and transfected cells were cultured in medium contain-
ing 0.5 ng/ml either hLIF or mIL-3 for a 10-day period, and
viable cells were counted daily. As shown in Fig. 4, most pa-
parental cells and the single-
transfected cells (Fig. 6
and data not shown). This implies
expression of exogenous Flag-tagged gp130 in WEHI-3BD+
cells resulted in enhanced responsiveness to IL-6 (Fig. 6A)
despite very low expression of the Flag-tagged protein
(data not shown).

WEHI-3BD+ cells co-expressing exogenous gp130 and either
wild-type or mutant forms of LIFRa truncated up to a form
containing 34 amino acid residues, including LIFR150,
LIFR136, LIFR94, LIFR68, and LIFR34, were able to respond
not only to G-CSF and IL-6 but also to hLIF (Fig. 6A). In
addition, the response to hLIF was stronger than that to G-CSF
and comparable to that observed to IL-6. Inspection of hLIF-
induced colonies in agar cultures revealed differentiated colo-
nes exhibiting haloes of widely dispersed cells around a tightly
plasmic domain different from those required for LIF-depend-
short term proliferation. The observation that a small pro-
portion of transfected cells from the cell lines LIFR(wt)/gp130,
LIFR150/gp130, and LIFR136/gp130 were still viable after 10
days of culture in the absence of cytokine may suggest that
some dimerization of these longer LIFRa proteins with gp130
could occur even in the absence of LIF, leading to weak signal-
that allowed cell survival but not proliferative expansion.

To assess further the hypothesis that the box-1 region of the
LIFRa cytoplasmic domain is sufficient for LIF-induced prolif-
eration, [3H]thymidine incorporation was measured in trans-
ferred Ba/F3 cells growing in medium containing 0.5 ng/ml
hLIF or mIL-3 for both a short period (2 days) and a relatively
long period (8 days). As shown in Fig. 5, DNA synthesis levels
in the Ba/F3 cells that had been grown in LIF-containing me-
dium for 2 days were essentially similar to those seen in cells
grown in the same medium for 8 days. Thus, it appears that
mutant LIFRa34 when co-expressed with gp130 in Ba/F3 cells
permitted short term LIF-dependent proliferation, although it
was not sufficient for LIF-dependent long term cell survival.

The characteristics of proliferation and survival responses
observed in the Ba/F3 cells define, in the context of gp130, the
box-1 region in the LIFRa cytoplasmic domain as essential
for LIF-dependent proliferation and the membrane-proximal re-
gion including box-1 and box-2 as being required for LIF-dep-
endent long term cell survival.

**LIF Induces Macrophage Differentiation in WEHI-3BD+
Cells Expressing hLIFRa and gp130**—LIF was originally iden-
tified on the basis of its ability to induce differentiation in
murine M1 myeloid leukemic cells. LIF-induced differentiation
of M1 cells is characterized by a reduction in colony number
and size in semi-solid agar culture, distinct morphological
changes, and the increased expression of macrophage-specific
surface markers (39). WEHI-3BD+ cells, however, could be
stimulated to differentiate by G-CSF and displayed a weaker
and slower response than LIF-induced M1 cells. In semi-solid
agar culture, most G-CSF-induced differentiated colonies were
composed of loosely dispersed cells around a tightly packed
central core of cells with no reduction of colony number or size
being observed. In addition, it was found previously that addi-
tion of IL-6 to agar culture resulted in the induction of differ-
entiation in WEHI-3BD+ colonies that exhibited haloes of dis-
persed cells without reduction of either colony number or size
(40).

The ability of WEHI-3BD+ parental cells to respond to IL-6,
but not to LIF, suggests that endogenous gp130 and IL-6 re-
ceptor $\alpha$-chain, but not LIFRa $\alpha$-chain, are present. Transfected
WEHI-3BD+ cells expressing wild-type hLIFRa alone were
able to form differentiated colonies in response to hLIF, similar
to the cells transfected with wild-type hLIFRa along with Flag-
tagged gp130, although both parental cells and the single-
transfected cells were less responsive to IL-6 than the double-
transfected cells (Fig. 6A and data not shown). This implies
expression of exogenous Flag-tagged gp130 in WEHI-3BD+
cells resulted in enhanced responsiveness to IL-6 (Fig. 6A)
despite very low expression of the Flag-tagged protein
packed central core of cells with no reduction of either colony number or size being observed, similar to that seen for IL-6-induced differentiation (Fig. 6B). Similar to what was seen with Ba/F3 proliferative stimulation, WEHI-3BD+ cells expressing a further truncated form of LIFRa (LIFR4) along with exogenous gp130 were completely unable to differentiate in response to hLIF, although their responses to G-CSF and IL-6 were normal (Fig. 6A and B).

Further examination of the extent of macrophage differentiation stimulated by hLIF in WEHI-3BD+ transfectants was carried out by culturing cells in medium containing 10 ng/ml either hLIF, hG-CSF, or saline for a period of time and then assessing their morphology microscopically after May-Grunwald-Giemsa staining. It was found that all of the WEHI-3BD+ cells that responded to hLIF in semi-solid agar differentiation assays gradually changed their morphology, and after 10–12 days of culture in LIF-containing medium, a proportion of the cells displayed the morphological characteristics of mature macrophages, having an increased cytoplasm to nucleus ratio and eccentric nucleus and foamy cytoplasm while some cells were at the stage of monocytes (Fig. 7A). A similar response to hG-CSF, although weaker, was observed with all WEHI-3BD+ cell lines. As expected, WEHI-3BD+ cells expressing the shortest version of mutant hLIFRa and exogenous gp130 (LIFR4/gp130) did not show any significant morphological changes after culture in LIF-containing medium for 11 days, when compared with cells growing in saline. However, morphological changes in the same cell line were observed when growing in G-CSF-containing medium, similar to that seen with WEHI-3BD+ parental cells (Fig. 7A).

To investigate whether expression of macrophage surface markers was up-regulated when the cells were transferred to medium containing either hLIF or hG-CSF, we examined expression of F4/80, a differentiation marker specifically ex-
pressed in murine mature macrophages (44) and Mac-1, whose expression is enhanced specifically during macrophage differentiation (45). Since the basal level of expression of Mac-1 was quite high even on WEHI-3BD parental cells cultured in medium containing saline, consistent with the observation previously made by Smith et al. (46), the fold induction of both Mac-1 and F4/80 expression on cells expressing both exogenous gp130 and either wild-type or various mutant forms of hLIFRα was rather weak. However, after 10 days of culture in medium containing 10 ng/ml hLIF, the expression of both Mac-1 and F4/80 on cells expressing a truncated LIFR (LIFR150) along with exogenous gp130 was significantly up-regulated with approximately 40–50% cells expressing an increased level of F4/80 (Fig. 7B). In cells co-expressing exogenous gp130 and mutant forms of LIFRs such as LIFR34, LIF-induced up-regulation of Mac-1 expression was also observed, but only 5–10% of the cells showed an increased expression of F4/80 (Fig. 7B). Similar results were obtained for cells co-expressing exogenous gp130 and LIFR136, LIFR94, or LIFR68 (data not shown). This indicates that only a small proportion of cells differentiated to

Fig. 4. Factor-dependent long term growth of Ba/F3 parental cells and transfected cells. Ba/F3 cells maintained in medium containing mIL-3 were extensively washed, and $1 \times 10^5$ cells were transferred to medium containing 0.5 ng/ml mIL-3 (▲) or 0.5 ng/ml hLIF (●) or without cytokine (□). Both viable and dead cells were counted in duplicate after eosin exclusion at the indicated times, but only the dead cell number of cell lines, such as LIFR(68)/gp130, LIFR(34)/gp130, and LIFR(4)/gp130, which were cultured in medium containing 0.5 ng/ml hLIF (●) were shown. For the cells expressing LIFR(wt)/gp130, or LIFR(68)/gp130, or LIFR(34)/gp130, these long term cell growth curves are representative of two independent clones examined.
mature macrophages after 10–12 days of culture in hLIF and most of the cells differentiated only to monocytes or immature macrophages, consistent with the observation of morphological changes. A weak up-regulation of Mac-1 expression and no apparent induction of F4/80 expression by hLIF was reproducibly observed in cells co-expressing exogenous gp130 and wild-type hLIFRα (Fig. 7B), which could be explained by the very low number of high affinity LIF receptors expressed in this transfectant (Fig. 3 and Table I). Nevertheless, no LIF-induced up-regulation of Mac-1 and F4/80 expression was detected on the surface of both WEHI-3BD+ parental cells (data not shown) and cells expressing the shortest version of mutant LIFRα along with exogenous gp130 (LIFRα4/gp130), whereas a weak up-regulation of Mac-1 expression on these cells by G-CSF was apparent (Fig. 7B).

Thus, analysis of WEHI-3BD+ cells expressing each of the cytoplasmic deletion mutants of hLIFRα, together with exogenous gp130, showed that, in cooperation with gp130, the box-1 region of the LIFRα cytoplasmic domain was sufficient for LIF-dependent induction of different aspects of differentiation, including the up-regulation of macrophage surface marker expression, the morphological change of blast cells into macrophages, and cell migration in agar culture.

Cytoplasmic Deletion of hLIFR Affects Receptor-mediated LIF Internalization—Whether or not internalization of cytokines or other growth factors bound to their respective receptors is involved in cellular signaling remains unclear. However, it has been well established that interactions of cytokines with their cell-surface receptors involve association and dissociation of ligand with receptor and the internalization of the ligand-receptor complex followed by the release of hydrolyzed ligand from the cells (38, 47, 48). Regardless of its role in intracellular signaling, it is clear that receptor internalization serves an important role in increasing the apparent ligand binding affinity (by removing reaction products) and in terminating signal transduction (by hydrolysis of receptor-ligand complex).

To investigate which cytoplasmic regions of the hLIFRα influence receptor-mediated hLIF internalization, we measured the rate of internalization of ligand-receptor complexes (ke) and the rate of degradation of ligand (k₀). Fig. 8A shows representative hLIF-receptor complex internalization curves measured for Ba/F3 and WEHI-3BD+ cell lines. Both Ba/F3 and WEHI-3BD+ cells co-expressing gp130 and wild-type LIFRα displayed a fast rate of receptor-mediated ligand internalization with an internalization constant (ke) of 0.09 min⁻¹ for Ba/F3 cells and 0.20 min⁻¹ for WEHI-3BD+ cells, respectively. However, Ba/F3 cells co-expressing gp130 and any of the mutant forms of LIFRα exhibited a slower internalization rate with ke values around 0.026 min⁻¹; whereas cells expressing wild-type hLIFRα alone showed a ke value of 0.048 min⁻¹ (top panel in Fig. 8B). With WEHI-3BD+ cells, the influence of cytoplasmic truncation of the LIFRα on receptor-mediated hLIF internalization was slightly different, in that cells expressing wild-type LIFRα and exogenous gp130 gave rise to a ke value of 0.20 min⁻¹. A mutant form of LIFRα such as LIFR150, along with gp130, gave rise to a ke value of 0.10 min⁻¹, whereas further deletion of LIFRα, even to a form only 14 amino acids shorter (LIFR136), resulted in cells having ke values of around 0.05 min⁻¹ (bottom panel in Fig. 8B). Taken together, these data suggest that, in the context of gp130, the C-terminal region of the LIFRα cytoplasmic domain is important for efficient receptor-mediated hLIF internalization in both hemopoietic cell lines although, as described previously (49), cell type also plays a major role in determining the absolute rate of receptor internalization. Thus, the truncated forms of hLIFRα, when co-expressed with gp130, would be able to bind hLIF and efficiently transmit signals but would not be able to be internalized as efficiently as the wild-type LIFRα.

**LIF Induced STAT DNA Binding Activities and Tyrosine Phosphorylation of Receptor Subunits**—It is well known that ligand-induced heterodimerization of LIFRα with gp130 activates the Jak/STAT signaling pathway, resulting in tyrosine phosphorylation of both receptor subunits and activation of STAT DNA binding activities, which is thought to play a central role in LIF-related cytokine signal transduction (20–24, 30). To investigate this event, we first examined LIF-induced STAT DNA binding activities in electrophoretic mobility shift assays, using the high affinity c-Sis-inducible factor binding
sequence, m67, as a probe (37). Three protein-DNA complexes (A, B, and C) were induced in the LIF-responsive cells (Fig. 9, A and B) by LIF treatment, and they were further identified as STAT3 homodimer, STAT3/STAT1 heterodimer, and STAT1 homodimer by the addition of antibodies specific for individual STAT proteins to binding reactions. All protein-DNA interactions were specifically competed by preincubation with an excess of unlabeled m67 DNA duplex with protein extracts. In WEHI-3BD+ cells that express low levels of full-length LIFR (LIFR(wt)/gp130), LIF treatment of cells predominantly in-

Fig. 6. A, dose-dependent differentiative responses of WEHI-3BD+ parental and transfected cells to hLIF, mIL-6, and hG-CSF. WEHI-3BD+ cells were cultured with the designated concentrations of factor as described under “Experimental Procedures.” After 7 days incubation at 37 °C, the proportion of colonies containing differentiated cells (dispersed colonies) was determined. Dose-dependent response is shown as the percentage of colonies displaying a differentiated phenotype versus the indicated dilutions of hLIF (100 ng/ml), or mIL-6 (1 μg/ml), or hG-CSF (100 ng/ml). and thick solid line represents cells responding to LIF (1–2 independent clones being shown); and thin solid line indicates the ability of cells to respond to mIL-6 (only one clone being shown); and dashed line refers to cells in response to hG-CSF (only one clone being shown). B, phenotype of WEHI-3BD+ colonies responding to hLIF or mIL-6 in agar culture. Cells were cultured in semi-solid agar for 7 days in either 10 ng/ml hLIF, or 0.1 μg/ml mIL-6, or saline. par, parental.
duced the STAT3 homodimer complex A formation, whereas the STAT3/STAT1 heterodimer complex B and STAT1 homodimer complex C were only evident in the anti-STAT3 and anti-STAT1 supershift bands (Fig. 9A). A similar pattern was observed with LIF-responsive Ba/F3 cell lines (Fig. 9B). However, the induction of all three protein-DNA complexes on Ba/F3 cells expressing LIFR(34)/gp130 by LIF treatment was weaker than that seen on other LIF-responsive Ba/F3 cell lines. Nevertheless, no LIF-induced protein-DNA complexes were detected in cells expressing LIFR(4)/gp130 for either WEHI-3BD1 or Ba/F3 cell lines.

Next we examined tyrosine phosphorylation of receptor subunits in LIFR α-chain (either wild-type or truncated mutants) and gp130-transfected Ba/F3 cells after LIF stimulation. Considering the variable expression level of gp130 in different transfected Ba/F3 cell lines (Table I and data not shown) and the indistinguishable molecular mass on SDS-PAGE of the truncated LIFRα proteins (such as LIFR(68), LIFR(34), and LIFR(4)) from that of gp130, we performed anti-hLIFRα and anti-hgp130 Western blot analyses on the proteins that had been immunoprecipitated with 4G10 anti-phosphotyrosine antibody. As shown in Fig. 10, LIF-induced tyrosine phosphorylation of both LIFRα and gp130 was observed in cells co-expressing gp130 and either wild-type or mutant forms of LIFRα truncated up to a form containing the box-1 region (LIFR(34)/gp130). However, in parental cells and cells expressing either wild-type LIFRα alone or the shortest mutant form of LIFRα (LIFR4) together with gp130 no LIF-induced receptor tyrosine phosphorylation was observed.

Finally, analysis of SHC tyrosine phosphorylation in WEHI-3BD1 cells with mutant LIF receptors and gp130 failed to detect any activation of SHC in response to LIF for any cell line. In contrast, strong induction of SHC tyrosine phosphorylation was observed in both M1 and WEHI-3BD+ cells expressing human GM-CSF receptor mutants in response to human GM-CSF (data not shown).

**DISCUSSION**

Unlike the related cytokines IL-6 and IL-11 which utilize gp130 homodimerization for functional cell signaling, LIF,
FIG. 8. Receptor-mediated $^{125}$I-hLIF internalization on both Ba/F3 cells and WEHI-3BD+ cells. A, representative receptor-mediated $^{125}$I-hLIF internalization curves measured for Ba/F3 cells (top two panels) expressing gp130 and either wild-type LIFRα (LIFR(wt)/gp130, 0.6 × $10^6$ cells/point) or a mutant LIFR150 (LIFR150/gp130, 1.0 × $10^6$ cells/point), and for WEHI-3BD+ cells (bottom two panels) co-expressing Flag-tagged gp130 and either wild-type LIFRα (LIFR(wt)/gp130, 4 × $10^6$ cells/point) or a mutant LIFR136 (LIFR136/gp130, 1.2 × $10^6$ cells/point). The experiments were performed as described under “Experimental Procedures.” ○, internalized $^{125}$I-hLIF; ■, cell surface-bound $^{125}$I-hLIF. B, comparison of internalization constant ($k_i$) on both Ba/F3 and WEHI-3BD+ cells expressing various forms of LIFRα along with gp130. The values (*) of $k_i$ are means of two to four independent experiments.
CNTF, OSM, and CT-1 utilize heterodimerization of gp130 and LIFR (24–27, 14–16). Indeed the stoichiometry of a solution complex of LIF, LIFR, and gp130 appears to be 1:1:1, suggesting that heterodimerization of the cytoplasmic domains of these two receptor subunits may be sufficient to initiate LIF signal transduction (50). However, because of the ubiquity of expression of both gp130 and LIFR α and the requirement for receptor heterodimerization, it has been difficult to study the separate contribution of each receptor subunit to LIF signal transduction.

Previous studies attempted to overcome these problems by generating receptor chimeras that contain a G-CSF receptor extracellular domain and a series of deletion mutants of the LIFR α cytoplasmic domain. In these experiments homodimerization of the LIFR cytoplasmic domains was driven by G-CSF-induced homodimerization of the extracellular domains. However, by using this system it was found that homodimerization of even full-length LIFR α cytoplasmic domain did not permit proliferative signaling in Ba/F3 cells or differentiative signaling in M1 myeloid leukemic cells, although it did permit signaling in embryonic stem cells (differentiation suppression) and in hepatic and neuronal cell lines (induction of gene expression) (25, 27, 28). In contrast, enforced homodimerization of gp130 cytoplasmic domains or activation of LIFR α/gp130 heterodimerization by LIF in each of these cell lines initiated appropriate functional signaling. These experiments suggest that analysis of the contribution of the LIFR α cytoplasmic domain to intracellular signaling is only meaningful in the context of a LIFR α/gp130 heterodimer.

In the present study we have introduced the wild-type or mutants of LIFR α along with full-length gp130 into cell lines, which do not express endogenous LIFR α but are able to gain full responsiveness to LIF in terms of cell proliferation and cell survival (Ba/F3 cells) or cell differentiation (WEHI-3BD cells). This has allowed us to determine the contribution of different LIFR α cytoplasmic domains to LIF-induced functional signaling in the context of full-length gp130 in a heterodimeric complex.

Ba/F3 cells that were stably transfected with a full-length gp130 cDNA along with wild-type or various mutants of LIFR α truncated up to a form containing only the box-1 of the LIFR α cytoplasmic domain acquired LIF-induced proliferative responsiveness, as determined by counting viable cell numbers or measuring DNA synthesis levels. However, LIF-dependent long term cell survival of transfected Ba/F3 cells required downstream regions including box-2. Similar results obtained in studies of GM-CSF-stimulated signal transduction indicated that the box-1 region of the cytoplasmic domain of the GM-CSF/IL-3/IL-5 receptor common β-chain is essential for GM-CSF-induced proliferative signaling in Ba/F3 and CTLL-2 cells (51), but downstream regions including box-2 were also important, particularly for GM-CSF-dependent long term proliferation and cell survival (46, 52).

LIF-induced differentiation of M1 cells is characterized by
the formation of dispersed colonies with profound clonal suppression of proliferative capacity in agar cultures (40). Tomida (53) had previously found that induction of differentiation by LIF in WEHI-3BD+ cells transfected with LIFRα cDNA was accompanied by suppression of cell proliferation, as determined by measuring their ability to reduce nitro blue tetrazolium. In the present study, LIF-induced differentiation was investigated in WEHI-3BD+ cells transfected with gp130 together with wild-type or mutant forms of LIFRα. In semi-solid agar culture, LIF-stimulated, transfected WEHI-3BD+ colonies were characterized by their large size, unreduced numbers, and a substantial number of migrating cells, suggesting a differentiated phenotype accompanied by persistent proliferation (i.e. no clonal suppression). Previous studies (46) on GM-CSF-induced differentiation in myeloid leukemic cells (M1 and WEHI-3BD+ cells) revealed that distinct regions of the cytoplasmic domain of the common β-chain of GM-CSF/LIF/IL-5 receptors are required for different signaling pathways, which cooperate to generate a fully differentiated phenotype characterized by the formation of dispersed colonies in agar as a measure of macrophage migratory activity, the induction of macrophage cell-surface marker expression, and morphological differentiation. Our results demonstrated that, in WEHI-3BD+ cells, the box-1 region of the LIFRα cytoplasmic domain was sufficient for LIF-dependent induction of most aspects of differentiation, including the up-regulation of macrophage surface marker expression, the morphological change of blast cells into macrophages, and cell migration in agar culture, indicating that differentiative signaling stimulated by LIF requires a smaller region of the LIFRα cytoplasmic domain than is required of the common β-chain of GM-CSF receptors for GM-CSF-induced differentiative signaling.

It has been found previously that overexpression of dominant negative forms of STAT3 in M1 myeloid cells completely blocks terminal myeloid cell differentiation and growth arrest induced by LIF or IL-6 (54). The present study showed a tight correlation of the induction of STAT3 and STAT1 DNA binding activities with the induction of WEHI-3BD+ myeloid cell differentiation, indicating that the activation of both STAT3 and STAT1 might be involved in WEHI-3BD+ cell differentiation in response to LIF. The differences in LIF-induced myeloid differentiation between M1 cells (differentiation accompanied by clonal suppression) and WEHI-3BD+ cells (differentiation accompanied by persistent proliferation) suggest that other signaling pathway(s) may be required for continuous proliferation of WEHI-3BD+ cells during myeloid differentiation.

Internalization of ligand-activated receptors is likely to be an important mechanism for achieving tight control of cellular growth. Internalized receptors may be recycled to the cell surface or may be targeted to lysosomes for degradation that is thought to attenuate growth factor-stimulated signaling. Most growth factor receptors are internalized much more effectively after activation by ligand binding, which is the first step in ligand-induced signal transduction, and are subsequently degraded to terminate signaling (55–59). Our results from the receptor-mediated 125I-lLIF internalization experiments with both hemopoietic cell lines demonstrated that, in the context of a heterodimer of LIFRα/gp130, the C-terminal region of the LIFRα cytoplasmic domain is important for efficient internalization of LIFRα receptor complexes but not essential for LIF-induced cellular signaling in terms of proliferation and differentiation. This suggests that the C-terminal region of LIFRα contains motifs that provide docking sites to recruit proteins involved in receptor endocytosis which, however, are signal-independent. This is consistent with a finding recently made by Thiel et al. (60) that ligand-induced gp130 internalization does not require the activation of the Jak/STAT pathway, which plays an essential role in gp130-mediated signal transduction.

In several cytokine and growth factor receptor cytoplasmic domains dileucine motifs have been implicated in receptor internalization, and these may constitutively signal or, more commonly for growth factor receptors, be activated by nearby phosphorylation on serine residues (61). Such a motif has been defined for gp130 and shown to be important for receptor internalization (62). However, in our studies gp130 contributed only about one-third to the internalization rate constant of bound LIF with LIFRα having a major contribution. The LIFRα does not contain a canonical dileucine internalization motif, but other cytokine receptors have been shown to mediate internalization by different mechanisms, including ubiquitination sites (63) and tyrosine-based motifs (61), as well as others (64, 65). The human LIFRα contains only one tyrosine in the critical region required for internalization (YRPQ), but it remains to be determined if this motif contributes to receptor internalization.

The results indicated that in the context of heterodimerization with gp130, only the box-1 region of the LIFRα cytoplasmic domain was essential for cell proliferation and for the induction of the differentiated phenotype. However, downstream regions were required for long term cell survival (to box-2) and for efficient receptor-mediated LIF internalization in both cell types (C-terminal region of the LIFRα). These data are in partial disagreement with Baumann et al. (27) who found that, using enforced LIFRα homodimers, box-1, box-2, and box-3 (150 amino acid residues in the LIFRα cytoplasmic domain) were all needed for transcriptional activation of a reporter gene in hepatic and neuronal cells. These differences could be due to cell line differences or analysis of different functions or, more likely, to the analysis of LIFRα homodimers rather than heterodimers. The correlation of LIF-induced tyrosine phosphorylation of LIFRα and gp130 and the activation of STAT3 and STAT1 with cell proliferation and differentiation functions in response to LIF suggests that, in the context of LIFRα/gp130 heterodimers, the truncated LIFR(34) was able to interact with Jak(s) that could be activated by ligand-induced receptor heterodimerization and phosphorylate tyrosine residues of gp130 as well as LIFRα. The full-length gp130 cytoplasmic domain, when phosphorylated, may provide docking sites for signaling molecules (such as STAT3), initiating the gp130-mediated cellular functions.

Despite the dominance of the box-1 region of the LIFRα for LIF-induced signaling, we did obtain evidence that other regions of the LIFRα cytoplasmic domain are needed for different functions (cell survival and receptor internalization). This is consistent with studies on other hemopoietic receptor cytoplasmic domains that have revealed a modular design in intracellular signaling pathways (12, 46, 66, 67). A full understanding of LIF receptor signaling pathways will now also require an analysis of gp130 cytoplasmic subdomains in the context of a LIFRα/gp130 heterodimer, and such studies are in progress.

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