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Phosphorylation of the human leukemia inhibitory factor (LIF) receptor by mitogen-activated protein kinase and the regulation of LIF receptor function by heterologous receptor activation

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ABSTRACT **We used a bacterially expressed fusion protein containing the entire cytoplasmic domain of the human leukemia inhibitory factor (LIF) receptor to study its phosphorylation in response to LIF stimulation. The dose- and time-dependent relationships for phosphorylation of this construct in extracts of LIF-stimulated 3T3-L1 cells were superimposable with those for the stimulation of mitogen-activated protein kinase (MAPK). Indeed, phosphorylation of the cytoplasmic domain of the low-affinity LIF receptor α-subunit (LIFR) in Mono Q-fractionated, LIF-stimulated 3T3-L1 extracts occurred only in those fractions containing activated MAPK; Ser-1044 served as the major phosphorylation site in the human LIFR for MAPK both in agonist-stimulated 3T3-L1 lysates and by recombinant extracellular signal-regulated kinase 2 *in vitro*. Expression in rat H-35 hepatoma cells of LIFR or chimeric granulocyte–colony-stimulating factor receptor (G-CSFR)–LIFR mutants lacking Ser-1044 failed to affect cytokine-stimulated expression of a reporter gene under the control of the β-fibrinogen gene promoter but eliminated the insulin-induced attenuation of cytokine-stimulated gene expression. Thus, our results identify the human LIFR as a substrate for MAPK and suggest a mechanism of heterologous receptor regulation of LIFR signaling occurring at Ser-1044.**

Leukemia inhibitory factor (LIF) is a member of the family of multifunctional cytokines capable of stimulating numerous physiological processes in a variety of cells (1, 2). LIF, along with ciliary neurotrophic factor, interleukins (ILs) 6 and 11, and oncostatin M, constitutes a distinct subgroup of the cytokine family (3) in which each member has its own unique α-receptor subunit that associates with the shared receptor subunit gp130 to initiate transmembrane signaling (4–12).

Early studies of LIF signaling have indicated activation of a pathway(s) involving both Tyr and Ser/Thr protein kinases (12, 13). Both the low-affinity LIF receptor α subunit (LIFR) and gp130 associate with and stimulate members of the Jak/Tyk family of nonreceptor protein-tyrosine kinases (14). We (15) and others (16, 17) have shown recently that activation of LIFR by agonist rapidly stimulates several components of the mitogen-activated protein kinase (MAPK) cascade, including MAPK kinase, the MAPK isozymes extracellular signal-regulated kinases (ERKs) 1 and 2, and S6 protein kinase activities against both S6 peptide and 40S ribosomes. In the current study, we show that the human LIFR can be phosphorylated at Ser-1044 by activated MAPK *in vitro* and suggest that this residue mediates a pathway for regulation of LIFR signaling after stimulation of a heterologous receptor system.

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MATERIALS AND METHODS

Materials. Recombinant human LIF was purchased from Preprotech (Rocky Hill, NJ) or, together with granulocyte–colony-stimulating factor (G-CSF), was provided by Immunex. Recombinant human IL-6 derived from COS-1 cells was from Genetics Institute (Cambridge, MA). Insulin and phorbol 12-myristate 13-acetate were from Sigma, and epidermal growth factor (EGF) was from Upstate Biotechnology. All additional supplies or materials were routinely available.

Fusion Protein Construction. The fusion proteins used in these studies were constructed by PCR amplification of the entire or truncated regions of the cytoplasmic domain of the human LIFR (cLIFR). Site-directed mutagenesis was performed using PCR with mutagenic oligonucleotides containing single-base substitutions to replace Ser with Ala. The 5′ and 3′ oligonucleotides also contained restriction sites for *Bam*HI and *Eco*RI, respectively, to facilitate subsequent subcloning of isolated fragments to the C terminus of glutathione *S*-transferase (GST) in the bacterial expression vector pGEX-3X (Pharmacia). Induction and purification of the various constructs from transformed *Escherichia coli* was according to the procedure of Smith and Johnson (18). All fusion protein plasmids were sequenced either by United States Biochemical Sequenase dideoxynucleotide terminator kits according to the manufacturer's instructions or by the University of Washington Molecular Pharmacology Facility on an Applied Biosystems model 373 DNA sequencing system.

Cell Culture and Extract Preparation. Mouse 3T3-L1 preadipocytes were cultured, stimulated, and prepared for determination of protein kinase activities in clarified lysates or in Mono Q (HR 5/5 column; Pharmacia) fractionated extracts as described in Schiemann and Nathanson (15). The procedures for developing the column were as described (15), except that elution was with a linear 0–0.35 M NaCl gradient over 120 min at a flow rate of 0.5 ml/min, and fractions were collected every 2 min.

Kinase Assays. *Myelin basic protein (MBP) assays.* Analysis of MBP phosphorylation both in 3T3-L1 lysates and in Mono Q column fractions was performed as described in Schiemann and Nathanson (15).

Fusion protein assays. Phosphorylation of the cytoplasmic domain of the human LIFR in clarified 3T3-L1 extracts was performed in a final reaction volume of 30 μl containing 1.5 μg of cellular protein and 5 μg of fusion protein or native GST.

Abbreviations: CAT, chloramphenicol acetyltransferase; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; G-CSF, granulocyte–colony-stimulating factor; G-CSFR, G-CSF receptor; GST, glutathione *S*-transferase; IL, interleukin; LIF, leukemia inhibitory factor; LIFR, low-affinity LIF receptor α subunit; cLIFR, cytoplasmic domain of human LIFR; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein.

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Reactions were initiated by addition of 10 μl of 3× assay buffer {buffer AB: final assay concentrations of 25 mM β-glycerophosphate, 0.5 mM dithiothreitol, 50 μM sodium vanadate, 10 mM $MgCl_2$, and 50–100 μM ATP ([γ-^{32}P]ATP, ≈2000 cpm/pmol)} and were incubated for 30 min at 30°C. After addition of 10 μl of 4× sample buffer [200 mM Tris·HCl, 4% SDS, 4% 2-mercaptoethanol, and 40% (vol/vol) glycerol] and boiling for 5 min, fusion protein substrates were separated from other cellular proteins through SDS/10% PAGE. Specific phosphorylation of human LIFR fusion proteins, determined after excision and scintillation counting of the appropriate substrate bands, was calculated by subtracting the radioactivity incorporated into samples incubated in the absence of substrate from those incubated in its presence after subtraction of GST values in matched samples.

Determination of protein kinase activities in Mono Q fractions was performed as above in a final reaction volume of 30 μl containing 15 μl of column fraction in the presence or absence of 2 μg of either fusion protein construct or native GST.

Phosphorylation of the fusion protein constructs by recombinant ERK2 was performed by incubating 0.06–40 μg of fusion protein or native GST with 10–100 ng of ERK2 in a final assay volume of 30 μl, using MBP phosphorylation buffer (15), for 0–180 min at 30°C.

Phosphoamino Acid Analyses. Phosphoamino acid analyses were performed by the method of Kamps and Sefton (19).

Expression and Functional Analysis of Ser-1044 in Rat H-35 Hepatoma Cells. Rat H-35 hepatoma cells were cultured in DMEM and transfected with rat β-fibrinogen promoter–chloramphenicol acetyltransferase (CAT) reporter gene construct pβFB(350)–CAT (10 μg/ml) receptor expression vectors (2.5 μg/ml), and pIE-MUP (an internal marker for transfection efficiency; 2 μg/ml) as described (20). The expression vectors (pDC302) containing the full-length wild-type human LIFR, the chimeric G-CSF receptor (G-CSFR)–LIFR(238), and the C-terminally truncated G-CSFR–LIFR(180) chimera (corresponding to position 1039 in the full-length LIFR) have been described (20). These same expression vectors were used to generate Ala-1044-substituted mutants either in the full-length LIFR or in the chimeric G-CSFR–LIFR, yielding plasmids pLIFR(S1044A) and pG-CSFR-LIFR(S1044A), respectively. IL-6, LIF, and G-CSF were used when indicated at 100 ng/ml. In some experiments, the effects of heterologous receptor activation on LIFR-stimulated gene induction were tested by treating identically prepared H-35 transfectants with 0.5 μM insulin either concomitantly with or 24 hr prior to addition of cytokine. Data are presented as the mean fold simulations ± SD relative to control values, and statistical significance ($P < 0.05$) was analyzed by unpaired Student's t test.

RESULTS

Signal transduction mediated by activated LIFRs requires the dual stimulation of both Tyr and Ser/Thr protein kinases (12–17); gp130, in response to agonist stimulation, contains elevated levels of phosphotyrosine, phosphoserine, and phosphothreonine (21). To determine if cLIFR could be similarly phosphorylated after LIF stimulation, we incubated extracts of 3T3-L1 cells with several GST fusion proteins containing all or various regions of cLIFR and measured their ability to serve as substrates in protein kinase reactions. Treatment of quiescent 3T3-L1 cells with LIF rapidly stimulated phosphorylation of the full-length LIFR fusion protein construct, GST–cLIFR/856–1097, which peaked at 10 min and then declined to basal levels by 60 min (Fig. 1). Phosphotransferase activity against this construct, which occurred predominantly on Ser residues (data not shown), was stimulated in a dose-dependent manner and exhibited half-maximal stimulation (EC_{50}) at ≈15 ng/ml

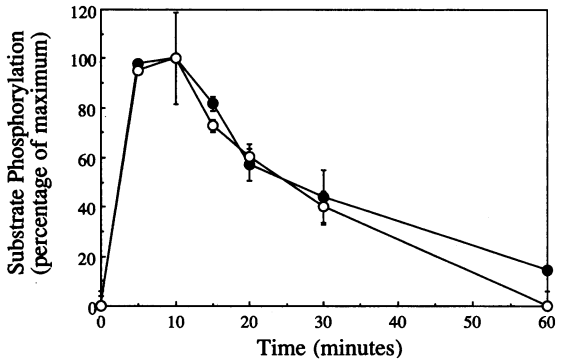

FIG. 1. Time course of LIF-stimulated MBP and GST–cLIFR/856–1097 phosphotransferase activities in 3T3-L1 lysates. Quiescent 3T3-L1 cells were incubated with a maximal concentration of LIF (100 ng/ml) for 0–60 min at 37°C. Phosphotransferase activities against MBP (●) and GST–cLIFR/856–1097 (○) were performed as described in *Materials and Methods.* Values, which are expressed as the percentage of maximal phosphotransferase activity against each substrate, are the means ± SD of duplicate cultures from a representative experiment that was repeated twice with identical results.

(0.75 nM; data not shown). Interestingly, the time course (Fig. 1) and dose–response (data not shown) curves for LIF-stimulated phosphorylation of GST–cLIFR/856–1097 were indistinguishable from those for MBP. Because we have shown previously that phosphorylation of MBP in LIF-stimulated lysates correlates with activation of the MAPK isozymes ERK1 and ERK2 (15), these results suggested that some of the phosphorylation of the LIFR construct in 3T3-L1 extracts may be mediated by activated MAPK.

Stimulation of 3T3-L1 cells with insulin, EGF, or phorbol 12-myristate 13-acetate, which activate MAPK in these cells (15), also resulted in the activation of phosphotransferase activity against GST–cLIFR/856–1097 (Fig. 2, lanes 1). Truncation of the 60 C-terminal amino acids blocked agonist-stimulated phosphorylation of the LIFR (Fig. 2; GST–cLIFR/856–1037; lanes 2), while constructs containing all (Fig. 2; GST–cLIFR/1005–1097; lanes 3) or part (Fig. 2; GST–cLIFR/856–1063; lanes 4) of this region were phosphorylated as ef-

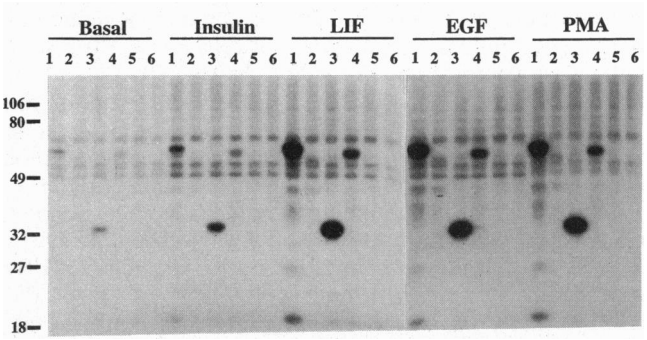

FIG. 2. Comparison of agonist-stimulated fusion protein phosphorylation in 3T3-L1 extracts. Quiescent 3T3-L1 cells were incubated for 15 min at 37°C with diluent (PBS; Basal), insulin (100 ng/ml), LIF (100 ng/ml), EGF (100 ng/ml), or phorbol 12-myristate 13-acetate (PMA; 100 nM) as indicated. Agonist-stimulated phosphotransferase reactions were performed by incubating 1.5 μg of clarified extract per lane in the presence (lanes 1–5) or absence (lane 6) of fusion protein (5 μg per lane) for 30 min at 30°C. Quenched reaction mixtures were fractionated through SDS/10% PAGE and were processed as described in *Materials and Methods.* Shown is a representative autoradiograph (≈15-hr exposure at −70°C) of a single experiment of agonist-stimulated phosphorylation of GST–cLIFR/856–1097 (lane 1; ≈55 kDa); GST–cLIFR/856–1037 (lane 2; ≈48 kDa), GST–cLIFR/1005–1097 (lane 3; ≈38 kDa), GST–cLIFR/856–1063 (lane 4; ≈51 kDa), native GST (lane 5; ≈27 kDa), and substrate blanks (lane 6). This experiment was repeated twice with identical results.

fectively as the full-length fusion protein. These results suggested that a growth factor/mitogen-stimulated protein kinase phosphorylates one or more sites localized within the 60 C-terminal amino acids of the cytoplasmic domain of the LIFR.

We fractionated extracts from unstimulated and LIF-stimulated 3T3-L1 cells over the anion-exchange resin Mono Q to confirm that activated MAPK could phosphorylate GST–cLIFR/856–1097. Stimulation of 3T3-L1 cells with LIF resulted in three distinct peaks of MBP kinase activity (Fig. 3). In accord with our previous results (15), immunoreactivity against anti-MAPK-7884 antibody confirmed the presence of the MAPK isozymes ERK2 and ERK1 in peaks one and two, respectively (data not shown). Interestingly, phosphorylation of GST–cLIFR/856–1097 is detectable only in those fractions containing LIF-stimulated ERK2 and ERK1 (Fig. 3). Thus, activated MAPK represents a major protein kinase activity against the human LIFR in LIF-stimulated lysates.

We also found that *in vitro*-activated recombinant ERK2 readily phosphorylates GST–cLIFR/856–1097, with the enzyme incorporating 0.90 ± 0.36 (mean ± SD; $n = 2$) mol of phosphate per mol of fusion protein with an apparent affinity (K_m) of 3.86 ± 1.72 μM (0.21 mg/ml; mean ± SD; $n = 3$). Phosphorylation of GST-cLIFR/856–1097 occurred almost exclusively on Ser residues, and, as in agonist-stimulated lysates, truncation of the 60 C-terminal amino acids blocked the ability of recombinant ERK2 to phosphorylate the LIFR (Fig. 4).

The MAPK phosphorylation consensus site is Pro-Xaa-Ser/Thr-Pro (22), but the Pro at position −2 is not absolutely required for MAPK-mediated phosphorylation. By these criteria, there are four potential MAPK phosphorylation sites contained within the 60 C-terminal amino acids of the human LIFR, located at Ser-1041, Ser-1044, Ser-1059, and Ser-1077. We mutated, both in singular and in multiple combinations, these Ser residues to Ala and then tested their ability to be phosphorylated both by LIF-stimulated lysates and by recombinant ERK2. Only Ala substitution at Ser-1044 significantly diminished LIFR phosphorylation by MAPK, blocking ≈80% and ≈70% of wild-type (GST–cLIFR/1097) phosphorylation in LIF-stimulated extracts and by recombinant ERK2, respectively (Fig. 5). Furthermore, GST–cLIFR(Ala-1044) exhibited

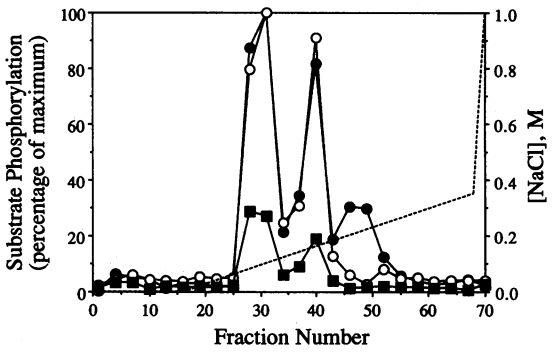

FIG. 3. MBP and GST–cLIFR/856–1097 protein kinase activities in LIF-stimulated Mono Q-fractionated 3T3-L1 extracts. Extracts of LIF-stimulated 3T3-L1 cells (100 ng/ml for 10 min at 37°C) were fractionated over the anion-exchanger Mono Q. The dashed lines (----) approximate the linear NaCl gradient (0–350 mM) used to elute bound proteins collected every 2 min in 1-ml volumes. Determination of phosphotransferase activity against MBP (●), GST–cLIFR/856–1097 (○), and GST–cLIFR(Ala-1044) (■) was carried out as described in *Materials and Methods*. Protein kinase activities are plotted as the percentage of maximal peak height for each substrate, except for GST–cLIFR(Ala-1044), which was normalized to GST–cLIFR/856–1097. Data are representative of a single column run, which was repeated three times with similar results, except for activities against GST–cLIFR(Ala-1044), which was performed once.

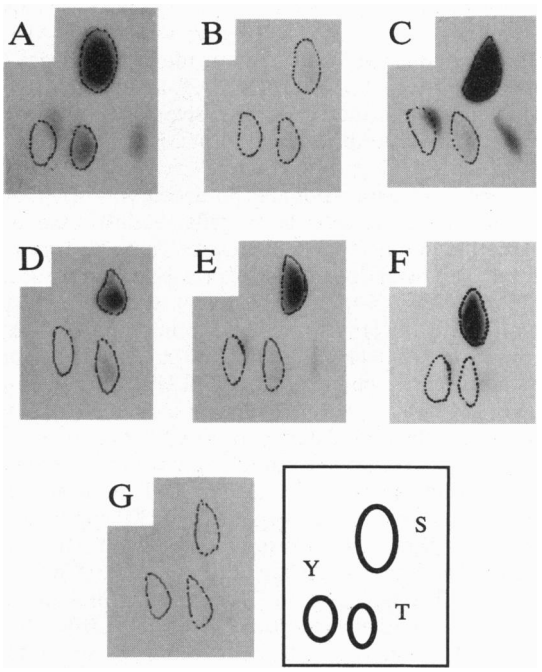

FIG. 4. Phosphoamino acid analysis of human LIFR fusion proteins phosphorylated *in vitro* by recombinant ERK2. Active recombinant ERK2 (≈100 ng per tube) was incubated in the presence of 5 μg of GST–cLIFR/856–1097 (*A*), GST–cLIFR/856–1037 (*B*), GST–cLIFR/1005–1097 (*C*), GST–cLIFR/856–1063 (*D*), GST–cLIFR/1005–1063 (*E*), GST–cLIFR/1054–1097 (*F*), or native GST (*G*) for 15 min at 37°C as indicated. Quenched samples were fractionated through SDS/10% PAGE and transferred electrophoretically to poly(vinylidene difluoride). Fusion protein substrates were excised, hydrolyzed, and separated by two-dimensional electrophoresis as described in *Materials and Methods*. Data are the resulting autoradiographic phosphoamino acid spots from a representative experiment that was repeated at least once for each construct.

<30% of total ERK phosphorylation (≈30% of ERK2 and ≈23% of ERK1 phosphorylation, respectively) of the LIFR when compared to wild-type levels in Mono Q-fractionated 3T3-L1 extracts (Fig. 3). Thus, the major phosphorylation site in the LIFR for active MAPK, both in LIF-stimulated extracts and by recombinant ERK2, is Ser-1044.

We then tested the role of Ser-1044 in LIFR signaling by transiently cotransfecting rat H-35 hepatoma cells either with

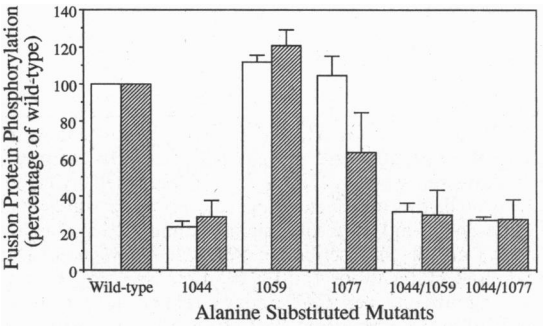

FIG. 5. Effects of site-directed mutagenesis on fusion protein phosphorylation in LIF-stimulated extracts and by recombinant ERK2. Three micrograms of wild-type (GST–cLIFR/856–1097) or Ala-substituted mutant human LIF receptor fusion proteins were incubated at 30°C in the presence of 1.5 μg of LIF-stimulated 3T3-L1 extracts (100 ng/ml for 10 min at 37°C; open bars) per tube or ≈100 ng of recombinant ERK2 (hatched bars) per tube for 30 min or 10 min, respectively. Values, which are expressed as the percentage of wild-type phosphorylation, are the mean ± SD of four (extracts) or three (ERK2) independent experiments.

the wild-type LIFR or the chimeric G-CSFR–LIFR(238), which contains the entire cLIFR, or with their Ala-1044-substituted counterparts to compare their capabilities to induce the expression of pβFB(350)–CAT, a CAT reporter gene construct containing the IL-6-responsive promoter of the rat β-fibrinogen gene. Addition of IL-6 or LIF to wild-type H-35 cells produced a 41 ± 6-fold or 13 ± 1-fold, respectively, induction of CAT expression (mean ± SD; $n = 2$). Transfection of human LIFR into H-35 cells reconstituted a LIF-stimulated induction of CAT expression that was comparable to that induced by endogenously expressed IL-6 receptors (56 ± 9-fold versus 53 ± 4-fold, respectively), and neither response was significantly different from that stimulated by LIF in cells expressing LIFR(S1044A) mutants (57 ± 11-fold). Similarly, expression of either chimeric G-CSF–LIFR(238) or G-CSF–LIFR(S1044A) was equally effective in inducing CAT expression in H-35 cells stimulated with G-CSF (Table 1). These results suggest that cytokine stimulation, which should lead to phosphorylation of the LIFR at Ser-1044, is insufficient in altering, either positively or negatively, LIFR-mediated gene induction occurring at IL-6-responsive elements. In addition, extended culture times did not alter the extent of G-CSF-mediated CAT expression in H-35 cells expressing either wild-type or Ala-1044-substituted G-CSFR–LIFR chimeras (Table 1), suggesting that phosphorylation at Ser-1044 does not dramatically alter LIFR turnover.

Last, we investigated whether activation of a heterologous receptor system could modulate LIFR signaling through effects at Ser-1044. Coadministration of insulin (0.5 μM) to H-35 transfectants significantly reduced the extent of LIF-stimulated CAT expression (Table 1). Furthermore, this insulin-induced attenuation of LIFR signaling was even greater in cells expressing chimeric G-CSFR–LIFR(238) receptors (Table 1). The inhibitory effects of insulin on G-CSF-stimulated CAT expression were completely lost in G-CSFR–LIFR(S1044A)-expressing cells (Table 1), suggesting that insulin-mediated inhibition of LIFR-stimulated CAT induction occurs at Ser-1044. Consistent with this hypothesis is our finding that activation of G-CSFR–LIFR(180), which lacks amino acids 1040–1097 of the LIFR, was also insensitive to the inhibitory effects of insulin (Table 1). These results suggest that Ser-1044 of the LIFR may serve as a site for regulation of LIFR signaling after heterologous receptor activation.

Table 1. Activity of chimeric G-CSFR–LIF receptors in H-35 cells

Treatment	Pretreatment with serum-free medium, hr	Receptor expression vector: G-CSFR–LIFR-(238)	G-CSFR–LIFR-(S1044A)	G-CSFR–LIFR-(180)
No addition		1	1	1
IL-6	0	31 ± 8	32 ± 5	34 ± 6
	72	30 ± 5	33 ± 2	28 ± 6
LIF	0	11 ± 3	9 ± 4	12 ± 1
	72	10 ± 1	9 ± 1	9 ± 3
LIF + insulin	0	4 ± 1*	3 ± 2	5 ± 2*
	72†	6 ± 1	5 ± 1	6 ± 0
G-CSF	0	28 ± 10	22 ± 5	20 ± 1
	72	17 ± 3	18 ± 3	15 ± 3
G-GSF + insulin	0	9 ± 3‡	18 ± 4	26 ± 3
	72†	7 ± 1‡	19 ± 2	20 ± 4

Rat H-35 cells were transfected with a plasmid DNA mixture consisting of pβFB(350)–CAT (10 μg/ml), pIE-MUP (2 μg/ml), and receptor expression vectors (2.5 μg/ml) as indicated. After a 16-hr recovery period, the transfected cell cultures were subdivided into appropriate dishes and were subsequently stimulated and assayed for specific CAT activity as described in *Materials and Methods.* Data are the mean fold stimulations (±SD) of three separate experiments measuring agonist-induced CAT activity relative to control treated cells (defined as 1) after normalization of all values to correct for transfection efficiency against the coexpressed major urinary protein (MUP) marker. Values for LIF plus insulin treatment at 72 hr are the mean fold stimulation ± range of duplicate determinations from a single experiment.

*Significant reduction from matched LIF treatment ($P < 0.05$).

†Cells were treated with insulin (0.5 μM) for 24 hr prior to addition of cytokines.

‡Significant reduction from matched G-CSF treatment ($P < 0.05$) and significant reduction from mutant chimeric G-CSFR–LIFR values determined in the presence of insulin ($P < 0.05$).

DISCUSSION

The results presented here show that the human LIFR can be phosphorylated at Ser-1044 by activated MAPK *in vitro* and that this residue is required for regulation of LIFR signaling after activation of a heterologous receptor system. Because activated LIF receptors can be considered upstream stimulators of MAPK, it is tempting to speculate that MAPK-mediated phosphorylation of LIFRs may modulate their activity after LIF binding. However, expression in rat H-35 hepatoma cells of human LIFR or chimeric G-CSFR–LIFR Ala-1044-substituted mutants failed to alter the magnitude of cytokine-mediated gene regulation. Due to extremely low levels of transfection efficiency and receptor expression in H-35 cells (data not shown), we have been unable to compare the receptor binding properties of wild-type human LIFRs with those containing Ala-1044 substitutions. Nevertheless, the failure of Ala-1044 to alter the extent of CAT expression in response to cytokine suggests that phosphorylation of Ser-1044 will not greatly affect the binding of LIF to its receptor. While more studies are clearly needed to clarify the effects of phosphorylation at Ser-1044 on additional LIF-inducible genes and LIFR binding properties, our results suggest that MAPK-mediated phosphorylation of the human LIFR after its activation by LIF is not required for homologous receptor regulation of reporter gene expression in H-35 cells.

Unlike the effects of Ser-1044 during homologous receptor regulation, Ser-1044 is required during heterologous receptor regulation of human LIFR signaling. Treatment of H-35 cells with insulin significantly attenuated LIF-stimulated CAT expression, and this effect was even more pronounced in cells expressing chimeric G-CSFR–LIFR constructs, most likely resulting from receptor signaling through homodimers composed solely of LIFR cytoplasmic domains rather than the customary heterodimers consisting of LIFR and gp130 (i.e., effects due to changes in the cytoplasmic domain of the human LIFR would be amplified in chimeric receptors lacking gp130). The finding that Ala-1040-substituted chimeric G-CSFR–LIFR mutants or C-terminally truncated chimeric G-CSFR–LIFR constructs lacking Ser-1044 were completely resistant to the inhibitory effects of insulin on cytokine-stimulated CAT expression in H-35 cells indicates that, in addition to its effects at the level of transcription (23), the major site of action for insulin-mediated inhibition of LIF-stimulated CAT induction occurs at the level of the LIFR itself.

Because both insulin (24) and LIF (15–17) can activate MAPK, it was surprising that LIF-mediated activation of MAPK does not attenuate its ability to stimulate CAT expression in H-35 cells expressing wild-type LIFRs. It is possible that Ser-1044 is accessible to phosphorylation by MAPK only during the inactive state of the human LIFR, a state mimicked by the GST–LIFR fusion proteins. Indeed, after LIF binding and subsequent association of the LIFR with gp130, Ser-1044 may be sequestered within the heterodimeric receptor complex to protect against LIF-induced homologous receptor desensitization.

The intracellular domain of the LIFR and gp130 have three conserved cytoplasmic sequence motifs (20), designated boxes 1, 2, and 3, which function in a cooperative manner to regulate LIF-mediated gene induction in both hepatic and neuronal cell

lines (20). These regions in heterodimeric LIFR–gp130 complexes stimulate at lest two types of gene-inductive signals, one targeting cytokine responsive elements generated from boxes 1 and 2, and a second targeting IL-6-responsive elements involving the actions of boxes 1, 2, and 3 (25). Ser-1044 lies ≈40 amino acids C-terminal to the end of box 3. The ability of insulin to inhibit LIF-stimulated pβFB(350)–CAT expression, which requires the cooperative interaction of boxes 1, 2, and 3 for maximal induction, raises the possibility that phosphorylation of the human LIFR by MAPK may uncouple LIF-mediated gene induction from IL-6 response elements but not necessarily those from cytokine response elements. Alternatively, phosphorylation at Ser-1044 of the human LIFR may alter the substrate specificity of its associated nonreceptor protein-tyrosine kinases or modulate, either positively or negatively, the coupling of activated LIFRs to various activators of the MAPK cascade.

Finally, while Ala-1044-containing chimeric G-CSFR–LIFR constructs are insensitive to the inhibitory effects of insulin, the responsiveness to insulin of wild-type or LIFR(S1044A) receptors on Hep3B cells was less dramatic than those determined for their chimeric counterparts on H-35 cells (data not shown). Although the varying sensitivity of these receptors to insulin could reflect specific cell or reporter gene (rat haptoglobin promoter) differences, this effect might also be explained by the presence and functioning of gp130 within the heterodimeric receptor complexes. Indeed, we have recently found that gp130 is phosphorylated on Ser residues C-terminal to box 3 in response to LIF stimulation by a protein kinase distinct from MAPK (W.P.S. and N.M.N., unpublished observation). Thus, phosphorylation and regulation of human LIFRs may require the actions of two distinct protein kinases, each targeting specific phosphorylation sites in the LIFR (MAPK at Ser-1044) and in gp130 (unknown protein kinase).

In summary, we have shown that Ser-1044 of the human LIFR is a substrate for activated MAPK. Moreover, Ser-1044 is required for heterologous receptor regulation of LIFR signaling in H-35 cells. Although additional studies will be required to fully elucidate the role of Ser-1044 in LIFR signaling, its localization places this residue adjacent to conserved intracellular cytokine receptor sequence motifs known to function in regulating growth and differentiation signals.

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