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An Antagonist for the Leukemia Inhibitory Factor Receptor Inhibits Leukemia Inhibitory Factor, Cardiotrophin-1, Ciliary Neurotrophic Factor, and Oncostatin M*

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The leukemia inhibitory factor receptor (LIF-R) is activated not only by LIF, but also by cardiotrophin-1, ciliary neurotrophic factor with its receptor, and oncostatin M (OSM). Each of these cytokines induces the hetero-oligomerization of LIF-R with gp130, a signal-transducing subunit shared with interleukin-6 and interleukin-11. The introduction of mutations into human LIF that reduced the affinity for gp130 while retaining affinity for LIF-R has generated antagonists for LIF. In the current study, a LIF antagonist that was free of detectable agonistic activity was tested for antagonism against the family of LIF-R ligands. On cells that express LIF-R and gp130, all LIF-R ligands were antagonized. On cells that also express OSM receptor, OSM was not antagonized, demonstrating that the antagonist is specific for LIF-R. Ligand-triggered tyrosine phosphorylation of both LIF-R and gp130 was blocked by the antagonist. The antagonist is therefore likely to work by preventing receptor oligomerization.

Functional overlap among cytokines often derives from shared receptor components. Interleukin-6 (IL-6),[1] IL-11, leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), ciliary neurotrophic factor (CNTF), and OSM (oncostatin M) have biological activities in common, and all require gp130 as a signal-transducing subunit (for review see Ref. 1). LIF (2–6), CT-1 (7), CNTF (8, 9), and OSM (6, 10, 11) activate gp130 in complexes with LIF receptor (LIF-R). LIF-R is a transmembrane signaling subunit that is structurally related to gp130 and belongs to the same hematopoietin receptor family (4). Ligand binding drives the heterodimerization of LIF-R and gp130, which results in inter alia activation and tyrosine phosphorylation of the Jak-Tyk cytoplasmic tyrosine kinases (12, 13). The Jaks in turn phosphorylate tyrosine residues in the cytoplasmic domains of both LIF-R and gp130 (8), allowing the recruitment of substrates with Src homology 2 domains including STAT3 and protein tyrosine phosphatase PTP1D (14–17) (for review see Refs. 18 and 19). CNTF weakly activates LIF-R-gp130, becoming a potent agonist once first bound to a non-signal-transducing subunit, CNTFRα. In addition to LIF-R-gp130, OSM also activates a receptor complex unique to OSM made up of OSM-R and gp130 (20). OSM-R has recently been identified as a transmembrane signaling subunit related to LIF-R and gp130 (20). Thus, LIF-R participates in a subset of gp130-mediated responses that includes all known LIF, CT-1, and CNTF responses, as well as some responses to OSM.

LIF-R is moderately expressed in testis, eye, skeletal muscle, ovary, uterus, thymus, brain, and fat and is highly expressed in liver and placenta (21). Activation of LIF-R-gp130 complexes regulates the differentiation and proliferation of a variety of cell lines as well as influencing the behavior of cultured neurons, hepatocytes, and adipocytes (for review see Ref. 22). Gene knockout experiments have demonstrated roles for LIF-R in development. LIF-R−/− and CNTFRα−/− mice show a substantial loss of motor neurons (23, 24). LIF-R−/− mice have also been reported to suffer poor placentation, severe bone abnormalities, metabolic defects, and a reduction in the number of astrocytes (25). LIF-R−/− and CNTFRα−/− mice die perinatally. LIF-R ligands have also been implicated in postnatal pathology, including breast cancer (26), rheumatoid arthritis (27), inflammation (28, 29), giant cell arteritis (30), liver (31) and muscle regeneration (32), glial responses to central nervous system lesions (33, 34), and peripheral nerve injury (35–40). Determining the precise role of LIF-R activation in each of these processes is of considerable clinical interest.

An antagonist for all LIF-R ligands would be useful in defining the function of LIF-R *in vivo*, especially in situations in which the LIF-R ligand has not been identified or more than one is present. Neutralizing the currently known ligands may be insufficient, because novel LIF-R ligands are likely to be identified in the future (23). A LIF-R antagonist would also be useful in dissecting OSM responses according to receptor usage and might have therapeutic applications in pathologies such as multiple myeloma (41).

One approach to designing a LIF-R antagonist is to block the heterodimerization of LIF-R and gp130. Mutant LIF molecules with reduced gp130 binding will occupy LIF-R but will be impaired in receptor complex formation. We recently described the generation of LIF antagonists with reduced affinity for gp130 (42). Using binding assays to the purified extracellular domains of human gp130 (hgp130) and human LIF-R (hLIF-R), we showed that the antagonists bound to hLIF-R with wild-type affinity (K_D = ~1 nM (4, 43)) but did not bind detectably to hgp130 (wild type affinity for gp130 is 100–1000-fold lower than to LIF-R (42, 44)), a finding that is consistent with topologically discrete receptor binding sites. One of the antagonists, hLIF-05, which carries the simultaneous substitution of four residues in the A helix and five residues in the C helix (A117E, D120R, I121K, G124N, S127L, Q25L, S28E, Q32A, and S36K),

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[1] The abbreviations used are: IL, interleukin; LIF, leukemia inhibitory factor; m, murine; h, human; LIF-R, LIF receptor; CNTF, ciliary neurotrophic factor; CNTFRα, CNTF receptor α; CT-1, cardiotrophin-1; OSM, oncostatin M; OSM-R, OSM receptor; G, glutamine; P, penicillin; S, streptomycin; 293T, 293/tsA1609neo; HRV3C, human rhinovirus protease 3C; β-Gal, β-galactosidase; VIP, vasoactive intestinal polypeptide.

is free of any residual agonistic activity. In proliferation assays on the Ba/F3-hLIF-R-hgp130 cell line, hLIF-05 showed no stimulatory activity when presented alone and successfully antagonized hLIF when presented at excess to hLIF (42). Results presented here demonstrate that hLIF-05 is a true LIF-R antagonist that will antagonize responses to all currently identified LIF-R ligands, including hLIF, murine CT-1 (mCT-1), human CNTF (hCNTF), and human OSM (hOSM). We further show that hLIF-05 discriminates between hLIF-R and human OSM-R (hOSM-R). Receptor phosphorylation assays demonstrate that hLIF-05 blocks tyrosine phosphorylation of hLIF-R and hgp130, which suggests that hLIF-05 blocks receptor activation and signal transduction.

EXPERIMENTAL PROCEDURES

Cells, Cytokines, and Antibodies—Ba/F3-hLIF-R-hgp130 cells are Ba/F3 cells stably transfected with the transmembrane forms of hgp130 and hLIF-R (45). They were grown in RPMI (Life Technologies, Inc.), supplemented with glutamine (2 mM), penicillin (50 IU/ml) streptomycin (50 mg/ml), 10% fetal calf serum, and 20 ng/ml hLIF. The HepG2 cell line, obtained from the William Dunn School of Pathology (Oxford), and the IMR 32 neuroblastoma cell line, obtained from the European Collection of Animal Cell Cultures, were cultured in Dulbecco's modified Eagle's medium/F-12 1:1, glutamine, penicillin, streptomycin, 10% fetal calf serum. 293/tsA1609neo (293T) cells are a transformed epithelial kidney cell line that produces SV40 large T antigen (46). 293T cells were cultured in Dulbecco's modified Eagle's medium, glutamine, penicillin, streptomycin, 10% fetal calf serum.

hLIF and hLIF-05 were produced in *Escherichia coli* JM109 as described previously (42, 45, 47). hOSM was produced as a glutathione *S*-transferase fusion protein that has a recognition site for human rhinovirus protease 3C (HRV3C) (48, 49) inserted in place of the thrombin site.[2] hOSM was purified in the same manner as hLIF, except that in place of thrombin, HRV3C tagged with six tandem histidine residues was applied as the protease. The HRV3C tagged with histidine was produced in the expression vector pTrcHisA (Invitrogen) and purified on nickel-chelating resin. mCT-1 was a gift from Diane Pennica (Genentech, Inc). Recombinant hCNTF produced in bacteria was a gift from Ralph Laufer (Istituto di Ricerche di Biologia Molecolare P. Angeletti). A serum-free culture medium of baculovirus-infected Hi-5 insect cells expressing c-*myc*-tagged soluble CNTFRα (~2 mg/liter) (50) was kindly provided by Giacomo Paonessa (Istituto di Ricerche di Biologia Molecolare P. Angeletti).

Rabbit anti-LIF-R-Fc was raised against a portion of the extracellular domain of human LIF-R (amino acids 2–538) fused to the Fc region of human IgG1 (42). Antibodies were purified on protein A-Sepharose (Pharmacia Biotech Inc.) and used directly or coupled to protein A-Sepharose with dimethylpimelimidate (51). Sheep anti-LIF-R was generated by The Binding Site Limited. The immunogen was the same as for the rabbit serum except that the receptor was purified by cleavage away from the Fc region via a HRV3C site that had been inserted between the LIF-R and the Fc region by expressing the fusion in a modified pIG-1 vector.[2] Goat anti-human gp130 was purchased from R & D Systems. Rabbit anti-human gp130 and anti-phosphotyrosine (clone 4G10) were purchased from Upstate Biotechnology Incorporated. The secondary antibodies, donkey anti-rabbit Ig peroxidase-linked species-specific $F(ab')_2$ fragment and sheep anti-mouse Ig peroxidase-linked species-specific $F(ab')_2$ fragment were purchased from Amersham Corp. Affinity purified donkey anti-Sheep IgG, peroxidase-linked, was purchased from The Binding Site Limited.

Biological Assays—Proliferation assays on Ba/F3-hLIF-R-hgp130 were carried out as described previously except that 4-fold successive dilutions of the cytokines were applied to the washed cells (45). Cell proliferation was assessed by 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide (Sigma) staining (52).

HepG2 cells were stimulated in triplicate as described except that for the first 24-h period the induction medium contained 1 μM dexamethasone (Sigma) and 1% heat-inactivated fetal calf serum. Recombinant factors were presented during the second 24-h period in the same medium. Acute-phase protein production was assessed by an enzyme-linked immunosorbent assay for secreted haptoglobin (53).

IMR 32 cells were transiently transfected by the calcium phosphate method (54) with pVIPCAT1 (55) (a kind gift from Richard H. Goodman; Tufts New England Medical Center) and Rous sarcoma virus-β-Gal in an assay analagous to that described by Johnson and Nathanson (56). After transfection, the cells were washed, and medium containing the factors was added. Following 24 h of stimulation, the cells from triplicate wells were combined and lysed. Chloramphenicol acetyltransferase and β-Gal in the lysates were measured by enzyme-linked immunosorbent assay (Boehringer Mannheim).

Phosphorylation Assays—293T cells were transiently transfected by calcium phosphate (54) with hLIFR subcloned into PXMT2, a derivative of pXM (57) and with hgp130 (from T. Kishimoto; Osaka University Medical School) subcloned into pcDNA3.1 (Invitrogen). Transfected cells were washed and allowed 48 h for expression. Following serum starvation for 4 h, 293T cells were treated with medium alone, with medium containing hLIF (0.5 nM), with hLIF (0.5 nM) plus hLIF-05 (500 nM), or with hLIF-05 (500 nM) alone for 15 min. Cells were lysed using the buffer described by Bellido *et al.* (58), except that 50 mM Tris, pH 8.0, was substituted for 20 mM, pH 7.5, and 0.5% Nonidet P-40 (Boehringer Mannheim) was substituted for 1% Brij96. Lysates were cleared of insoluble material and then divided into two portions. One portion was precleared on protein A-Sepharose and then incubated overnight at 4 °C with rabbit anti-LIF-R-Fc coupled to protein A-Sepharose. The second portion was precleared on protein G-Sepharose (Pharmacia) and then incubated with protein G-Sepharose coated with goat anti-human gp130.

Immunoprecipitated protein/Sepharose beads were washed in lysis buffer. Bound protein was eluted in electrophoresis sample buffer and separated on SDS-polyacrylamide gels (4–15% precast gel, Bio-Rad Laboratories Inc.), immunoblotted onto Immobilon P (polyvinylidene difluoride) (Millipore), and blocked as described (58). SDS-polyacrylamide gels were run in duplicate so that each sample could be analyzed for both phosphotyrosine containing proteins and receptor recovery. Sheep anti-LIF-R and rabbit anti-gp130 were used as probes for receptor recovery. Immunoblots were developed with SuperSignal Substrate (Pierce).

HepG2 cells were handled in the same manner as the 293Ts except for a few changes. The hepatoma cells were not transfected and were 90% confluent when stimulated. To obtain a strong signal, 2.5 nM of hLIF or OSM were applied to the cells. The concentration of hLIF-05 was increased slightly to 625 nM. Rabbit anti-LIF-R-Fc was not covalently bound to the protein A- Sepharose, because coupling resulted in higher backgrounds. Immunoprecipitated proteins were separated on 7.5% gels.

RESULTS

hLIF-05 Antagonizes All LIF-R Ligands on Human Cell Lines—Because hLIF-05 antagonized hLIF stimulated proliferation of the Ba/F3-hLIF-R-hgp130 cell line (42), we tested hLIF-05 for antagonism of hOSM. In the experiment shown, 15 nM hLIF-05 achieved 50% inhibition of hLIF (Fig. 1*A*), compared with 10 nM for 50% inhibition of hOSM (Fig. 1*B*). Taking into account that more hOSM was applied to reach the baseline stimulation, the ratio of antagonist to agonist required for 50% inhibition was higher for hLIF than for hOSM (10,000-fold *versus* 200-fold). Full antagonism of both hLIF and hOSM was achieved with ~100 nM hLIF-05. hLIF-05 showed no agonistic activity, even at high concentrations. The ratio of antagonist to agonist required for 50% inhibition varied within a 10-fold range among experiments, according to the sensitivity of the cells; higher ratios were required when the EC_{50} values for hLIF and hOSM were lower ($n = 3$). Nonetheless, hLIF-05 was always more efficient at antagonizing hOSM than hLIF. The antagonism by hLIF-05 could not be attributed to nonspecific toxicity because it was easily overcome by higher agonist concentrations (Fig. 1, *C* and *D*). The observed shift in the dose-response curve to higher concentrations of agonist in the presence of hLIF-05 is consistent with hLIF-05 competing with hLIF for LIF-R occupancy.

A good test of the specificity of hLIF-05 for LIF-R is to challenge hLIF-05 with the related OSM-R. The hepatoma cell line HepG2 is well suited to this experiment because both hOSM and hLIF elicit acute phase responses. The maximal response to hOSM, however, is much greater than to hLIF, a reflection of the greater number and/or potency of hOSM-R in

[2] K. R. Hudson, unpublished data.

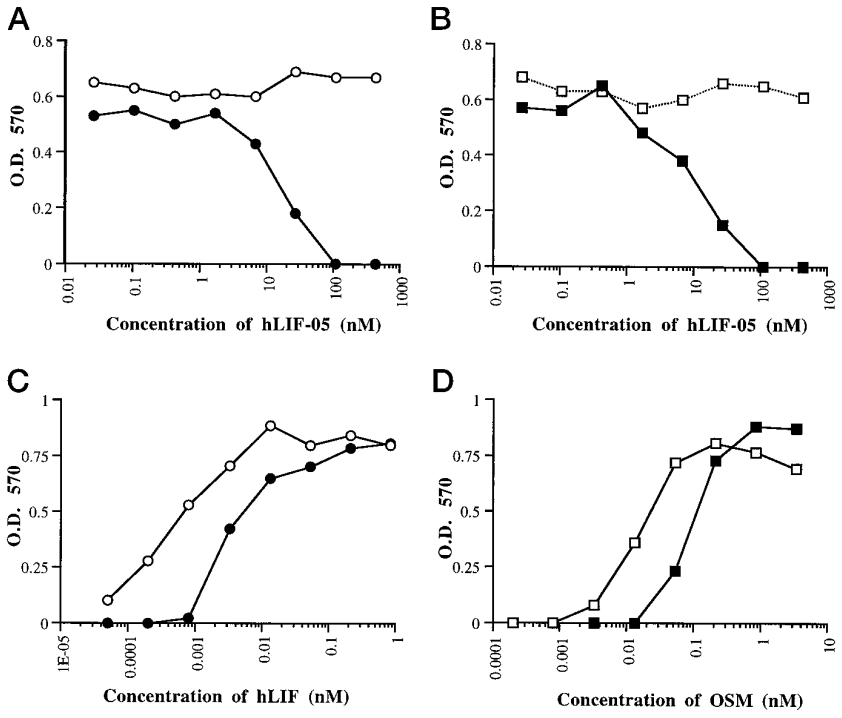

FIG. 1. **The proliferation of the Ba/F3-hLIF-R-hgp130 cell line in response to hLIF and hOSM is inhibited by hLIF-05.** A constant concentration of agonist was applied in the presence (*filled symbols*) or the absence (*open symbols*) of increasing concentrations of hLIF-05. *A*, hLIF (1.5 pM). *B*, hOSM (50 pM). The antagonism is overcome by increasing concentrations of agonist. Agonist was titrated in the presence (*filled symbols*) or absence (*open symbols*) of a constant concentration of hLIF-05 (54.5 nM). *C*, hLIF. *D*, hOSM. Results are expressed as the A_{570} value of cells assayed for proliferation by 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide. Values represent the mean of triplicate samples. The S.E. for all points was less than 10% of the mean.

this cell line (10, 20, 53). mCT-1 and hCNTF/soluble CNTFRα elicit maximal responses similar to those obtained with hLIF, a result that is consistent with LIF-R dependence (data not shown). CNTF on its own is effective only at 10,000-fold higher concentrations (data not shown and Ref. 53).

As in the Ba/F3-hLIF-R-hgp130 cells, hLIF-05 antagonized the response to hLIF in HepG2 cells (Fig. 2). Because hLIF responses were relatively small, a maximal dose of hLIF (50 pM), which provided a robust response, was chosen for antagonism assays. The acute phase response was measured by haptoglobin accumulation in the culture supernatants. About 10 nM hLIF-05 (200-fold excess) was required for 50% inhibition of the hLIF response and about 100 nM for full inhibition. hLIF-05 also antagonized mCT-1 and CNTF-soluble CNTFRα with 50% inhibition at 10-fold lower concentrations of hLIF-05 than required for inhibition of hLIF. The lower concentrations of hLIF-05 required may reflect a lower affinity of mCT-1 for hLIFR-hgp130 and limitations of adding soluble CNTFRα. In contrast, hLIF-05 did not significantly diminish responses to hOSM or hIL-6 (Fig. 2). The failure to inhibit hOSM and hIL-6 demonstrates that hLIF-05 is specific for LIF-R. Including hLIF-05 throughout the dose-response curves for IL-6 and OSM also failed to reveal inhibition (data not shown). hLIF-05 on its own did not stimulate an acute phase response.

CNTFRα-mediated responses are likely to be an important subset of LIF-R responses, to judge by the similar phenotypes observed in CNTFRα−/− and LIF-R−/− mice (23, 24). In many neurons, LIF-R ligands stimulate the production of vasoactive intestinal polypeptide (VIP) (35, 59, 60), a neuropeptide that may serve as an autocrine growth factor (61). hLIF-05 was tested for inhibition of VIP induction in IMR 32, a human neuroblastoma cell line that makes glycosylphosphatidylinositol-linked CNTFRα. hLIF-05 inhibited VIP induction by both hLIF (Fig. 3*A*) and hOSM (Fig. 3*B*). CNTF responses were also antagonized but only to a modest extent (Fig. 3*C*).

hLIF-05 Blocks LIFR-gp130 Phosphorylation—The biological assays described above measure downstream responses occurring over a 24–72-h incubation period. Measuring receptor phosphorylation provides a more direct assessment of receptor activation. Receptor tyrosine phosphorylation was assayed in 293T cells transfected with hgp130 and hLIF-R. The cells were stimulated with hLIF in the presence or the absence of an excess of hLIF-05. Receptors were immunoprecipitated from cell lysates with anti-LIF-R or anti-gp130 antibodies and then immunoblotted and probed with anti-phosphotyrosine antibodies. Exposing the cells to hLIF resulted in the tyrosine phosphorylation of LIF-R (190 kDa; *top left*, Fig. 4*A*). The smaller protein (145 kDa) that co-immunoprecipitated is likely to be tyrosine phosphorylated gp130 because the protein co-migrated with the major component immunoprecipitated by the anti-gp130 antibodies. Including an excess of hLIF-05 blocked the majority of the tyrosine phosphorylation of LIF-R. No phosphorylated LIF-R was observed in response to hLIF-05 alone (*top left*, Fig. 4*A*). As in the case of LIF-R, gp130 was tyrosine phosphorylated in response to hLIF (145 kDa; *top right*, Fig. 4*A*). A small amount of phosphorylated LIF-R (190

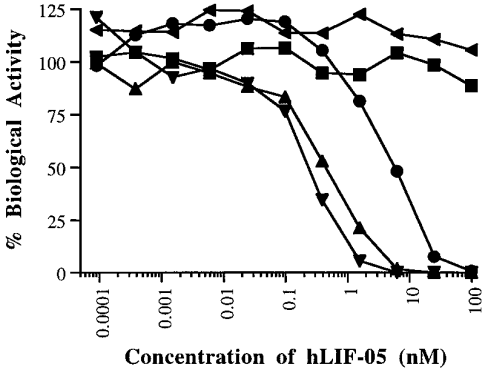

FIG. 2. **The production of haptoglobin by HepG2 cells in response to hLIF, mCT-1, and hCNTF-CNTFRα is inhibited by hLIF-05 but not the response to hIL-6 or hOSM.** A constant concentration of agonist (50 pM) was applied in the presence or the absence of increasing concentrations of hLIF-05. CNTFRα was applied as a 40-fold dilution of serum-free culture medium containing c-*myc* tagged soluble CNTFRα. Results are expressed as a ratio of haptoglobin produced in the presence of hLIF-05 divided by the haptoglobin produced in the absence. Values represent the mean of three independent experiments. The S.E. of the mean was less than 15%. ●, hLIF; ▼, mCT-1; ▲, hCNTF-CNTFRα; ◀, IL-6; ■, hOSM.

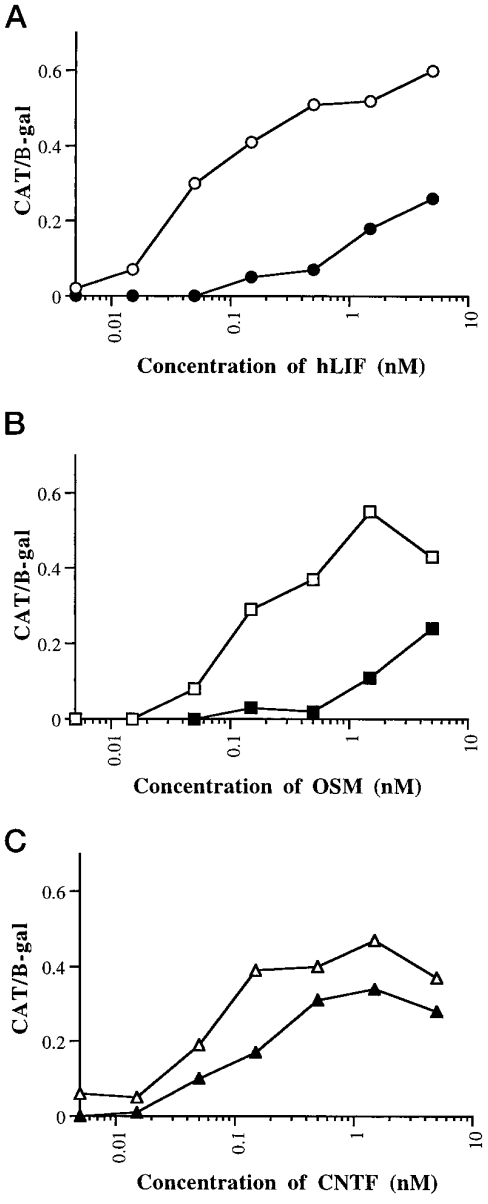

FIG. 3. **The induction of VIP expression by IMR 32 cells in response to hLIF, hOSM, and hCNTF is inhibited by hLIF-05.** Agonist was titrated in the presence (*filled symbols*) or absence (*open symbols*) of a constant concentration of hLIF-05 (100 nM). *A*, hLIF. *B*, hOSM. *C*, hCNTF. IMR32 cells were co-transfected with VIPCAT1 and Rous sarcoma virus-β-Gal, stimulated with cytokines, and assayed for reporter expression. Results are presented as the ratio of chloramphenicol acetyltransferase expressed under the control of the VIP promoter to β-Gal expressed under the Rous sarcoma virus promoter. Chloramphenicol acetyltransferase and β-Gal were measured by enzyme-linked immunosorbent assay. One of two experiments is shown.

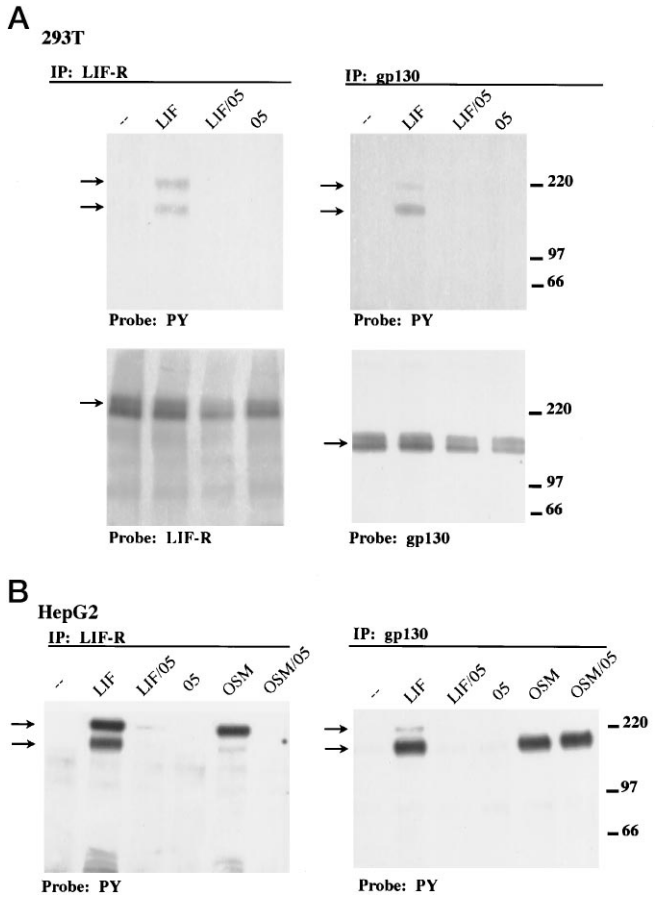

FIG. 4. **Receptor phosphorylation is inhibited by hLIF-05.** 293Ts transfected with LIF-R and gp130 (*A*) were incubated for 15 min in the absence (–) or in the presence of hLIF (*LIF*), hLIF-05 (*05*), or hLIF+hLIF-05 (*LIF/05*) at the concentrations indicated under "Experimental Procedures." HepG2s (*B*) were also stimulated with OSM or OSM+hLIF-05 (*OSM/05*). Cells were lysed with Nonidet P-40, and the lysates were immunoprecipitated with anti-LIF-R antibodies (*left-hand side*) or anti-gp130 (*right-hand side*). Immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. Immunoblots were probed with the following antibodies. *A* (*top row*) and *B*, anti-phosphotyrosine. *A* (*bottom left*), anti-LIF-R. *A* (*bottom right*), anti-gp130. LIF-R is positioned at 190 kDa. gp130 is positioned at 145 (*A*) or 155 kDa (*B*).

kDa) was co-immunoprecipitated. Stimulation with hLIF and an excess of hLIF-05 blocked the tyrosine phosphorylation of gp130. hLIF-05 on its own did not result in detectable phosphorylation of gp130 (*top right*, Fig. 4*A*). Similar quantities of receptors were recovered in each immunoprecipitation, as evidenced by the signals observed when portions of the immunoprecipitates were probed for LIF-R or gp130 (*bottom half*, Fig. 4*A*).

Receptor phosphorylation was next measured in HepG2 cells because they present both LIF-R and OSM-R. As in the transfected 293T cells, stimulating HepG2 cells with hLIF and immunoprecipitating receptors with anti-LIF-R antibodies revealed tyrosine phosphorylated LIF-R (190 kDa) and co-immunoprecipitated tyrosine phosphorylated gp130 (155 kDa in HepG2s). Excess hLIF-05 was able to block the majority of the tyrosine phosphorylation of LIF-R (*left side*, Fig. 4*B*). Stimulation by OSM also resulted in the appearance of tyrosine phosphorylated LIF-R. Very little gp130 was co-immunoprecipitated, a result that perhaps reflects a faster dissociation of the OSM-LIF-R-gp130 complex. As for hLIF, an excess of hLIF-05 blocked the phosphorylation of LIF-R stimulated by OSM (*left side*, Fig. 4*B*). The receptors immunoprecipitated by the anti-gp130 antibodies proved more revealing. hLIF stimulated the tyrosine phosphorylation of gp130 (155 kDa) and a small amount of co-immunoprecipitated LIF-R (190 kDa). Again, the phosphorylations were blocked by an excess of hLIF-05. hOSM also stimulated the phosphorylation of gp130 (155 kDa). However, hLIF-05 did not block the phosphorylation of gp130 stimulated by OSM (*right side*, Fig. 4*B*), despite having blocked the phosphorylation of LIF-R by OSM. The failure to block agrees with the Ba/F3-hLIF-R-hgp130 and HepG2 bioassays above, which demonstrated that hLIF-05 blocks OSM responses mediated by LIF-R-gp130 but not those mediated by OSM-R-gp130.

DISCUSSION

Antagonists for a variety of four-helical bundle cytokines have been created by allowing high affinity binding to the first receptor subunit while destroying low affinity binding to a sequentially assembled receptor subunit. Examples include both homodimerizing ligands such as growth hormone (62) and hetero-oligomerizing ligands such as IL-4 (63), or granulocyte macrophage colony stimulating factor (64). Within the subgroup of gp130-dependent cytokines, IL-6 antagonists with reduced binding to gp130 (65, 66) and CNTF antagonists with reduced binding to LIF-R (67) have been described. IL-6 and CNTF antagonists show narrow specificities because the antagonists retain binding to their respective specificity subunits but do not sequester shared subunits (68). The broad specificity of hLIF-05 makes it a novel reagent for this family.

The high ratio of hLIF-05 to LIF-R ligand required for effective antagonism is not unusual. Although the EC_{50} for hLIF varied on Ba/F3-hLIF-R-hgp130, the average was 2.5 pM (n = 12), making the cells as sensitive as XG-1 myeloma cells, which have been used as a rigorous test for residual agonism in IL-6 antagonists (68). The ratios reported here for Ba/F3-hLIF-R-hgp130 are similar to those required of IL-6 antagonists with reduced gp130 binding (68, 69). Subsequently, IL-6 antagonists with very high potency were obtained by adding mutations that enhance IL-6 receptor binding (65, 66, 69). A similar strategy may be applicable to hLIF-05.

The EC_{50} for hLIF on HepG2s (3–4 pM) was within the range observed for Ba/F3-hLIF-R-hgp130 cells, yet a much lower ratio of antagonist to agonist was required for inhibiting hLIF responses on HepG2 cells. Similar cell type-dependent potencies have been documented for other cytokine antagonists (64, 69). The complete sparing of the OSM response in HepG2s suggests that activating OSM-R is sufficient for the acute phase response. HepG2 cells may form three times as many OSM-R-gp130 complexes as LIF-R-gp130 complexes (10). Further experiments with hLIF-05 on cell types that are limiting in OSM-R might reveal situations in which both OSM-R and LIF-R are needed for a full response.

Although many cytokine antagonists have been described, the immediate receptor events have rarely been documented. A murine IL-4 antagonist has been shown to inhibit the tyrosine phosphorylation of JAK3 and STAT6 in Ba/F3 cells (70). LIF-R and gp130 each has five tyrosine-based motifs that are distal to the membrane-proximal Jak binding domain (71). The most distal four of gp130 and the most distal three of LIF-R are all capable of mediating STAT3 tyrosine phosphorylation (71). As demonstrated here, hLIF-05 inhibits the tyrosine phosphorylation of LIF-R and gp130, so hLIF-05 will block STAT3 recruitment, activation by phosphorylation, and translocation to the nucleus. Blocking STAT3 activation fits with the antagonism observed in both HepG2 and IMR 32 cells. STAT3 plays a key role in the acute phase response, including the stimulation of haptoglobin expression (17, 72) and in the induction of VIP by LIF-R ligands (73). Altbough we cannot rule out receptor-phosphorylation-independent signaling, the lack of any detectable agonism by hLIF-05 in the three cell types examined argues that hLIF-05 does not itself activate primary signal transduction pathways. The inhibition of receptor phosphorylation further demonstrates that hLIF-05 prevents activation of a major signal transduction pathway for LIF-R ligands and is consistent with a block in receptor hetero-oligomerization.

The broad specificity of hLIF-05 and its lack of intrinsic signaling make hLIF-05 a powerful reagent. As for other soluble antagonists, tailoring the dosage of hLIF-05 for a particular cell type allows flexibility in setting the degree of inhibition. hLIF-05 has been used successfully to demonstrate that LIF-R activation is required for the arrest of rod differentiation in cultures of mouse retinal cells (74). In the future, hLIF-05 may prove useful in defining a role for LIF-R in receptor complexes for newly identified ligands. Antagonists specific to IL-4 receptor α also antagonized IL-13 responses, revealing receptor subunits shared between IL-4 and IL-13 (63, 75, 76). Application of hLIF-05 should lead to a better understanding of the role of LIF-R in a variety of homeostatic and disease processes.

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