Mannose 6-Phosphate/Insulin-like Growth Factor II Receptor Mediates Internalization and Degradation of Leukemia Inhibitory Factor but Not Signal Transduction*

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Leukemia inhibitory factor (LIF) is a multifunctional cytokine belonging to the interleukin-6 subfamily of helical cytokines, all of which use the glycoprotein (gp) 130 subunit for signal transduction. The specific receptor for LIF, gp190, binds this cytokine with low affinity and is also required for signal transduction. We have recently reported that glycosylated LIF produced by transfected Chinese hamster ovary cells also binds to a lectin-like receptor, mannose 6-phosphate/insulin-like growth factor II receptor (Man-6-P/IGFII-R) (Blanchard, F., Raher, S., Duplomb, L., Vusio, P., Pitard, V., Taupin, J. L., Moreau, J. F., Hoflack, B., Minvielle, S., Jacques, Y., and Godard, A. (1998) J. Biol. Chem. 273, 20886–20893). The present study shows that (i) mannose 6-phosphate-containing LIF is naturally produced by a number of normal and tumor cell lines; (ii) other cytokines in the interleukin-6 family do not bind to Man-6-P/IGFII-R; and (iii) another unrelated cytokine, macrophage-colony-stimulating factor, is also able to bind to Man-6-P/IGFII-R in a mannose 6-phosphate-sensitive manner. No functional effects or signal transductions mediated by this lectin-like receptor were observed in various biological assays after LIF binding, and mannose 6-phosphate-containing LIF was as active as non-glycosylated LIF. However, mannose 6-phosphate-sensitive LIF binding resulted in rapid internalization and degradation of the cytokine on numerous cell lines, which suggests that Man-6-P/IGFII-R plays an important role in regulating the amounts of LIF available in vivo.

Leukemia inhibitory factor (LIF) is a multifunctional soluble protein belonging to the interleukin-6 (IL-6) subfamily of helical cytokines (which also includes IL-11, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and cardiotoxin-1 (CT-1)), all of which have a similar three-dimensional structure, use the common receptor subunit gp130, and elicit overlapping physiological responses (1–3). Among its various biological activities, LIF is important for embryo implantation, conversion of sympathetic neurons from the adrenergic to cholinergic phenotype, maintenance of hematopoietic stem cells, induction of acute phase protein synthesis in liver cells, and proliferation of certain cancer cells (1–6).

One peculiarity of LIF relates to its high glycosylation state. Human LIF contains seven potential N-glycosylation sites (7), six of which are functional (8, 9). In LIF purified from the human HSBl T lymphoma cell line, N-linked carbohydrates account for about 20 kDa in the molecular mass (43 kDa) of the cytokine (10) and O-linked glycosylations for about 1–2 kDa (10). Other glycosylated cytokines include macrophage-colony-stimulating factor (M-CSF), stem cell factor (six N-glycosylation sites), IL-9 (four sites), and IL-6 and OSM (two sites), whereas cytokines such as IL-1β, IL-2, IL-11, CNTF, or CT-1 have no N-glycosylation sites.

Internalization of LIF and signal transduction are thought to result from LIF binding to the low affinity (nA) LIF receptor (gp190 or LIF-Rβ) and subsequent heterodimerization of gp190 with the gp130 signal transducer (2). LIF binding to gp190/gp130 induces the activation of receptor-associated Janus kinases, which in turn phosphorylate tyrosine residues in both receptor intracellular domains and Janus kinases. This process induces at least two distinct signal transduction pathways as follows: the SHP-2/RAS/MAPK pathway and activation of the signal transducers and activators of transcription (STAT) STAT3 (2).

We have recently identified a new low affinity LIF receptor that is abundant in numerous cell lines and not related to gp190 (11). This receptor binds LIF through its carbohydrate moieties and displays biochemical, immunochemical, and functional features indicating that it is identical to the mannose 6-phosphate/insulin-like growth factor II receptor (Man-6-P/IGFII-R). The extracellular region of Man-6-P/IGFII-R is composed of 15 homologous repeat units with an average length of 147 amino acids (12). Whereas repeats 1–3 and 7–9 each contain one Man-6-P binding determinant involved in the binding of Man-6-P-containing ligands (lysosomal enzymes, latent TGF-β, prolinin, and LIF) (13), the binding site of IGF-II has been localized on repeat 11 (14). More recently, it has been reported that retinoic acid and urokinase-type plasminogen linked immunosorbent assay; MPR, Man-6-P receptor.
activator receptor bind to Man-6-P/IGFII-R at a site or sites different from those involved in Man-6-P or IGF-II binding (15, 16). Man-6-P/IGFII-R is mainly expressed within endosomal compartments where its major role is to divert Man-6-P-containing ligands from the secretory pathway for subsequent sorting to endosomes and lysosomes (12). This receptor is also present at the plasma membrane where it endocytoses secreted lysosomal enzymes, mediates internalization and subsequent degradation of growth factors such as IGF-II (17) or proliferin (18), and potentiates the activation of the precursor form of TGF-β (latent TGF-β) into biologically active TGF-β (19). Man-6-P/IGFII-R is considered to be a tumor suppressor because of its ability to activate TGF-β (a potent growth inhibitor), promote degradation of the growth factor IGF-II, and regulate localization of lysosomal enzymes implicated in extracellular matrix degradation. Recent findings have shown that Man-6-P/IGFII-R allelic loss is an early event in the etiology of cancer (20) and that TGF-β activation is deficient and IGF-II expression excessive in these Man-6-P/IGFII-R mutant tumors (21). Moreover, mouse mutants lacking Man-6-P/IGFII-R are (partly) rescued from perinatal lethality in an IGF-II null background, which indicates the physiological importance of the Man-6-P/IGFII-R turnover mechanism for degradation of IGF-II during embryogenesis (22, 23).

The discovery by Morgan et al. (24) that the Man-6-P receptor and the IGF-II receptor are the same protein suggested that this receptor could function in two diverse biologic processes, i.e. protein trafficking/turnover and transmembrane signaling. However, it has been difficult to establish a role in signal transduction since IGF-II also binds to the IGF-I receptor, a tyrosine kinase receptor that transmits signals across the plasma membrane after IGF-I and IGF-II binding (12). Nevertheless, many reports have suggested that Man-6-P/IGFII-R may also function as a transmembrane signaling molecule (12), although these findings are controversial (25, 26). For example, it has been shown that induction of calcium influx in Balb/c 3T3 cells (27) or CHO cells (28) and of the proliferation of Balb/c 3T3 cells (27), K562 cells (29), or OPM2 cells (30) by IGF-II is mediated by Man-6-P/IGFII-R. A recent report has shown that either proliferin or IGF-II binding to Man-6-P/IGFII-R induces endothelial cell chemotaxis through a G protein-coupled, mitogen-activated protein kinase (MAPK)-dependent pathway (31).

In the context of these possible multiple functions for growth factor IGF-II, our studies concerned the role of Man-6-P/IGFII-R in the physiology of the cytokine LIF, i.e. LIF trafficking/turnover and transmembrane signaling. Our results suggest that secretion of Man-6-P-containing LIF is a natural phenomenon in vitro and that other cytokines such as M-CSF can also bind to Man-6-P/IGFII-R. Moreover, LIF, after binding to Man-6-P/IGFII-R, was rapidly internalized and degraded, even though no signal transduction was detected on cells expressing Man-6-P/IGFII-R, but not LIF receptor gp190, and glycosylated LIF had biological activities identical to those of non-glycosylated LIF. It is concluded that LIF (and possibly other cytokines such as M-CSF) is regulated by Man-6-P/IGFII-R in mammalian cells, a situation very similar to that observed with IGF-II.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Antibodies, and Cytokines**—All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA), except NIH3T3 murine fibroblasts, a kind gift from Dr. Sylvie Hermout and Dr. Isabelle Corre (Inserm U463, Nantes, France). All cell lines were cultured in their respective medium (11, 32, 33), supplemented with 8% fetal calf serum (FCS), except NIH3T3 fibroblasts which were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum (CS). Human IGF-II and polyclonal anti-gp190 antibodies against gp190 or gp130 were from R & D Systems (Minneapolis, MN). Phosphotyrosine antibodies (PY20) were from Transduction Laboratories (Lexington, KY) and anti-phospho-STAT3 (Tyr-705) from New England Biolabs (Beverly, MA). Polyclonal anti-MAPK anti-serum (specific for p44/p42 MAPK) was kindly provided by Dr. Gérard Meijer (National Institute of Metrology, NKI, Amsterdam, The Netherlands). A affinity purified rabbit polyclonal antibodies against Man-6-P/IGFII-R by Dr. Stuart Kornfeld (Washington University, St Louis, MO). LIF (CHO-LIF) was purified from serum-free conditioned medium of CHO cells transfected with a full-length cDNA encoding for human LIF (32). Recombinant Escherichia coli-derived human LIF (E. coli-LIF) was obtained from PeproTech, Inc. (Rocky Hill, NJ). M-CSF was a kind gift from Genetics Institute (Cambridge, MA). Mannose 6-phosphate was from Sigma.

**Affinity Purification of LIF and Cytokine Binding to U266 Cells**—Supernatants of cultured cells were obtained as follows. Peripheral blood lymphocytes (PBL) were sorted (34) and cultured for 2 days in RPMI 1640 supplemented with human serum (5%), phospholipid 12-myristate 13-acetate (1 ng/ml) (Sigma), and calcium ionophore A23187 (250 ng/ml) (Sigma). Kit 225 cells were cultured in medium containing rIL-2 (100 U/ml), MG63 cells in medium containing 1 ng/ml IL-1β, A375 and NIH3T3 cells were cultured without added cytokine. COS and CHO cells were transfected with a full-length cDNA encoding LIF, IL-6, or OSM (7, 32, 35).

All forms of LIF (Fig. 1A) were purified by affinity chromatography on polyclonal anti-LIF antibodies coupled to agarose beads, and their concentrations were determined by a specific ELISA (11). For endoglycosidase F/N-glycosidase F treatment, 100 ng of all forms of LIF were first treated at 95 °C in the presence of 0.1% SDS for 5 min prior to incubation for 18 h at 37 °C with or without 480 milliunits of endoglycosidase F/N-glycosidase F (Sigma) in 50 mM potassium phosphate buffer, pH 7, containing 25 mM EDTA, 1% Nonidet P-40, and 1% mercaptoethanol. Immuno blotting of LIF was performed using the polyclonal anti-LIF antibody after loading on a 10% SDS-polyacrylamide gel.

Mannose 6-phosphate-sensitive binding of LIF was determined in U266 cells. Briefly, U266 cells (20 × 10^6 cells) were incubated with 100 ng/ml LIF (2 ng/ml) in 1 ml of PBS, 50 μM Hepes, and 0.5% bovine serum albumin (PBS/Hepes/BSA) for 1 h at 4 °C with or without mannose 6-phosphate (5 mM). After one wash, cell surface-bound LIF was recovered and quantified as follows: 100 μl of PBS/Hepes/BSA containing 5 mM mannose 6-phosphate was added to the cell pellet and incubated for 15 min at 4 °C; after centrifugation, the LIF concentration in each supernatant was determined by a specific ELISA (36).

In Fig. 1B, cytokine binding was detected with the same procedure, except that crude supernatants of transfected COS cells were used and IL-6 and OSM concentrations were determined by a specific ELISA (BIOSOURCE, Fleurus, Belgium, and R & D Systems, respectively).

**Surface Plasmon Resonance Studies**—These experiments were performed with the BIAcore 2000 optical biosensor (BIACore, Uppsala, Sweden) on a dextran flow cell coupled with soluble Man-6-P/IGFII-R (11). The association was monitored for 10 min before the dissociation phase was initiated for another 10 min.

**Radiiodination of LIF, Internalization, and Degradation**—CHO-LIF was iodinated according to the chloramine-T method (32), and LIF was labeled at a specific radioactivity of around 1,800 Ci/nmol.

For internalization studies, 4 × 10^6 U266 cells were incubated with 5 nM labeled CHO-LIF in 200 μl of RPMI 1640, 8% FCS, and 50 mM Hepes with or without mannose 6-phosphate (5 mM) for 1 h at 4 °C. Cell internalization was initiated by switching the temperature to 37 °C. At regular intervals, cell surface-bound LIF was removed by incubating cells at 4 °C for 10 min in the presence of Man-6-P (5 mM), and internalized LIF (Man-6-P-nondissociable fraction) was determined by centrifuging cells through a layer of dibutylphthalate (90%) and paraffin oil (10%). Total LIF binding (internalized LIF + membrane-bound LIF) was determined using the same procedure, except that cells were incubated at 4 °C for 10 min in the absence of Man-6-P.

**Kinetics**—was performed for degradation studies in the same way as for internalization studies, and supernatants and cell lysates were then subjected to SDS-PAGE and trichloroacetic acid precipitations (10% final). Cell lysates were obtained by incubating 4 × 10^6 cells in 10 ml of lysis buffer, pH 7.4, containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide, and 0.5% BSA. In some experiments (Fig. 2D), cells were incubated for 6 days with 0.5 mM radiolabeled CHO-LIF with or without 5 mM Man-6-P in culture conditions (cell concentrations below 10^5 cells/ml or monolayers before confluence; medium supplemented with 50 mM Hepes and 8% FCS), and trichloroacetic acid precipitations were performed on supernatants.

**Binding Capacity Studies**—Binding was performed as described pre-
physiological relevance of LIF binding to Man-6-P/IGFII-R

viously (32); 0.2 × 10^6 to 8 × 10^6 U266 or CHO cells stably transfected with the full-length gp190 cDNA (CHO-190 cells) (37) were incubated with 5 nM radiolabeled CHO-LIF in 50 μl of PBS containing 0.5% BSA for 90 min at 4 °C. Nonspecific binding was evaluated by including a 100-fold excess of unlabeled CHO-LIF. Cell-bound and unbound fractions were separated and counted (32).

Determination of [Ca^{2+}]/[Ca^{4+}] was determined by fluorescence measurements on cells loaded with fluo-3 AM (Molecular Probes, Eugene, OR) (38). Briefly, cells were incubated for 30 min at 37 °C in RPMI containing 20 μM fluo-3 AM and then washed twice in RPMI containing 0.5% BSA and 2 mM CuCl2. Cells were resuspended at a concentration of 1.5 × 10^6 cells/ml and incubated with Leuca (1 μg/ml) or CHO-LIF (50 nM). Fluorescence (F) measurements were done at various times using a FACSscan apparatus (Becton Dickinson, Mountain View, CA). Average autofluorescence was measured on non-loaded cells. Maximum fluorescence (F_max) was obtained by stimulating cells with 2 μg/ml calcium ionophore for 2 min and minimum fluorescence (F_min) by subsequent incubation with 2 mM MnCl2 for 10 min. [Ca^{2+}] was calculated as shown in Equation 1,

\[ [Ca^{2+}]_{\text{RM}} = K_d \frac{F - F_{\text{min}}}{(F_{\text{max}} - F)} \]  

(1)

where \( K_d \) is the equilibrium dissociation constant of fluo3 (400 nm).

Immunoprecipitations and Immunoblotting—JAR, CHO-190, and NIH3T3 cells from an approximately 80% confluent 28-cm^2 dish were starved overnight in serum-free medium, treated with the indicated cytokine (CHO-190 cells) (37) and lysed on ice in a lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM NaF, 1 mM Na_3VO_4, 0.25% deoxycholic acid, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfon fluoride, and 1% Nonidet P-40. Insoluble material was pelleted in a centrifuge (13,000 rpm, 15 min, 4 °C) and the protein concentration in the supernatant was determined with the BCA colorimetric assay from Pierce (using BSA as standard).

For immunoprecipitations, a part of the lysates (750 μl) was incubated with 50 μl of protein A-Sepharose for 1 h, under stirring, at 4 °C (pre-clearing), and then centrifuged at 13,000 rpm (15 min, 4 °C). Antibodies (anti-gp130, anti-gp190, and anti-CIMPR) were added to the supernatant at a concentration of 2% (antibody/protein). After overnight incubation, antibodies were captured with 100 μl of protein A-Sepharose. Pellets were washed 5 times in lysis buffer and eluted with SDS protein sample buffer.

For immunoblotting, samples were run on polyacrylamide gel and electrophoretically transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA). The membrane was blocked for 1 h in Tris-buffered saline containing 1% BSA and 0.1% Tween and then immunoblotted with the appropriate antibody. Incubation with the primary antibody was followed by washing, incubation with the appropriate horseradish peroxidase-conjugated antibody, and visualization using ECL. For reprobing, the membrane was stripped by incubation with Tris-buffered saline containing 100 mM β-mercaptoethanol and 0.04% SDS for 30 min at 50 °C.

For MAPK, activation was studied using a mobility shift assay. Lysate proteins (20 μg) were loaded on a 10% SDS-polyacrylamide gel (ratio of acrylamide/bisacrylamide in 30% stock solution, 29.7/0.3), and run on a 10% SDS-polyacrylamide gel. Lysate proteins (20 μg) were loaded on a 10% SDS-polyacrylamide gel and electrophoretically transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA). The membrane was blocked for 1 h in Tris-buffered saline containing 1% BSA and 0.1% Tween and then immunoblotted with the appropriate antibody. Incubation with the primary antibody was followed by washing, incubation with the appropriate horseradish peroxidase-conjugated antibody, and visualization using ECL. For reprobing, the membrane was stripped by incubation with Tris-buffered saline containing 100 mM β-mercaptoethanol and 0.04% SDS for 30 min at 50 °C.

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the internalization of Man-6-P-containing ligands and IGF-II, we investigated the disappearance of radiolabeled CHO-LIF from the indicated cell line was immunopurified as described under “Experimental Procedures,” incubated with U266 cells in the presence (open bars) or absence (closed bars) of 5 mM Man-6-P, eluted and subjected to a specific ELISA. Inset, indicated LIF preparations were treated with or without endoglycosidase F/N-glycosidase F and subjected to Western blot analysis. B, the same protocol as in A but using crude supernatants of transfected COS cells. C, the upper panel shows sensorgrams depicting the binding of CHO-LIF and CHO-M-CSF to immobilized purified soluble Man-6-P/IGF II-R. CHO-LIF was tested at a concentration of 100 nM and M-CSF at 400 nM. Binding was inhibited by Man-6-P (5 mM). The kinetic and equilibrium parameters calculated from the sensorgrams are grouped in the lower panel.

**Fig. 1.** Man-6-P-sensitive binding of different forms of LIF (A) and M-CSF (C) but not IL-6 and OSM (B) to Man-6-P/IGFII-R. A, glycosylated LIF from the indicated cell line was immunopurified as described under “Experimental Procedures,” incubated with U266 cells in the presence (open bars) or absence (closed bars) of 5 mM Man-6-P, eluted and subjected to a specific ELISA. Inset, indicated LIF preparations were treated with or without endoglycosidase F/N-glycosidase F and subjected to Western blot analysis. B, the same protocol as in A but using crude supernatants of transfected COS cells. C, the upper panel shows sensorgrams depicting the binding of CHO-LIF and CHO-M-CSF to immobilized purified soluble Man-6-P/IGF II-R. CHO-LIF was tested at a concentration of 100 nM and M-CSF at 400 nM. Binding was inhibited by Man-6-P (5 mM). The kinetic and equilibrium parameters calculated from the sensorgrams are grouped in the lower panel.

Cell-mediated degradation of LIF was then monitored by trichloroacetic acid precipitations and SDS-PAGE on U266 cell lysates and supernatants. As shown in Fig. 2B, the percentage of precipitable radioactivity from cell lysates declined, reaching 50% after 160 min. Electrophoresis and autoradiography confirmed a strong intracellular degradation at that time, with half of the radioactivity recovered at the migration front (Fig. 2C). The percentage of precipitable radioactivity also declined in the supernatant, suggesting that the degradation products were secreted (Fig. 2B). However, 75% of the radioactivity remained trichloroacetic acid-precipitable, confirming that only a minority of CHO-LIF contained Man-6-P. Similar Man-6-P-dependent degradation of LIF was observed in the supernatant of other cell lines expressing Man-6-P/IGFII-R (Fig. 2D). Longer cell culture periods were required for certain cells, possibly because of lower Man-6-P/IGFII-R density at the plasma membrane and/or lower cell concentration.

Taken together, these results strongly suggest that Man-6-P/IGFII-R is active after LIF binding, producing rapid inter-
nalization and degradation of this Man-6-P-containing cytokine.

**CHO-LIF Binding to Man-6-P/IGFII-R Does Not Appear to Mediate/Modulate Signal Transduction**—As some reports have provided evidence of signal transduction mediated by Man-6-P/IGFII-R after IGF-II or prolierin binding (29, 31, 40), we investigated a possible biological action of glycosylated LIF mediated by this receptor.

U266 and Jurkat T lymphoma cells expressed Man-6-P/IGFII-R but not LIF receptor gp190 (11), and Jurkat cells responded to IGF-II via Man-6-P/IGFII-R (40). However, CHO-LIF (or IGF-II) induced no detectable protein tyrosine phosphorylation on these two cell lines and did not activate MAPK (data not shown). Moreover, CHO-LIF applied at 100 nM on Jurkat cells (Fig. 4A) and U266 cells (data not shown) did not increase cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_i\)], whereas LeucoA had a marked effect (Fig. 4A). CHO-LIF did not modulate thymidine incorporation by U266 cells (Fig. 4B) or Jurkat cells (data not shown) in a chemically defined medium without serum (Fig. 4B) or in a medium containing various concentrations of FCS (0.1 to 10%, data not shown). Similar negative results were obtained with IGF-II (data not shown), whereas exogenous IL-6 had a slight stimulatory effect on the U266 cell line, as previously reported (41), with a 40% increase in thymidine incorporation and an EC\(_{50}\) ~20 pM (Fig. 4B). The erythroleukemia cell line K562, which like U266 and Jurkat cells expresses Man-6-P/IGFII-R but not gp190 (11), responded to IGF-II by enhanced proliferation, possibly via Man-6-P/IGFII-R (29). However, this cell line gave negative results with CHO-LIF or IGF-II (tyrosine phosphorylation, MAPK activation, Ca\(^{2+}\) influx, and thymidine incorporation; data not shown).

A comparison of the protein tyrosine phosphorylations induced by CHO-LIF and *E. coli*-LIF (5 nM) in JAR cells expressing Man-6-P/IGFII-R and gp190 indicated that the two factors induced the same rapid phosphorylation of two proteins with apparent molecular masses of 140 and 185 kDa (Fig. 5A). Similar results were observed with a higher cytokine concentration (50 nM, data not shown), thus ruling out the involvement of Man-6-P/IGFII-R in this activity. Immunoprecipitation and rehybridization experiments using polyclonal antibodies against Man-6-P/IGFII-R, gp190, and the signal transducer gp130 revealed that the two phosphorylated proteins were gp130 and gp190, respectively (Fig. 5A). Immuno precipitation experiments also showed that Man-6-P/IGFII-R was not co-precipitated with the gp190-gp130 complex when anti-gp130 antibody was used and, conversely, that gp190/gp130 was not co-precipitated with Man-6-P/IGFII-R (Fig. 5A). This indicates that no interaction occurred between Man-6-P/IGFII-R and gp190/gp130, or that affinity was too low for co-immunoprecipitation experiments. Activation of the downstream intracytoplasmic substrates STAT3 and MAPK was not detected on JAR cells but on CHO-190 cells, which expressed around 5–10-fold more gp190 than JAR cells. Nevertheless, both CHO-LIF and *E. coli*-LIF strongly activated gp130 and

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**Fig. 2.** Internalization (A), degradation, and excretion (B and C) of CHO-LIF. A, after U266 cells were incubated with radiolabeled CHO-LIF at 4 °C, in the presence or not of 5 mM Man-6-P, the temperature was switched to 37 °C for the indicated time. Cell surface-bound LIF was removed by Man-6-P washing. Supernatants and cell lysates of the same experiment were subjected to trichloroacetic acid precipitation (B) or SDS-PAGE electrophoresis (C). D, the indicated cell lines were cultured for 6 days in the presence of 0.5 nM radiolabeled CHO-LIF with or without 5 mM Man-6-P (in duplicate), and supernatants were subjected to trichloroacetic acid precipitation. The results take the control values obtained without cells into account.

**Fig. 3.** Binding capacity of CHO-LIF to U266 cells (●) and CHO-190 cells (○). Cells (0.2 to 8 \(\times\) 10⁵) were incubated with 5 nM radiolabeled CHO-LIF, and bound/unbound fractions were determined.
Man-6-P/IGFII-R does not modulate gp190-dependent LIF bioactivity or IGF-II action.

**DISCUSSION**

Our results showed that all forms of glycosylated natural human LIF (from tumor cell lines or activated lymphocytes) or recombinant human LIF (from CHO and COS cells) bound to Man-6-P/IGFII-R and that other glycosylated cytokines such as M-CSF bound to this receptor in a Man-6-P-sensitive manner. The Man-6-P-containing cytokine LIF was rapidly internalized and degraded by all cells expressing Man-6-P/IGFII-R at the plasma membrane, which suggests that this receptor could be biologically active after LIF binding and implicated in its trafficking/turnover. However, no activation of MAPK or calcium influx and no induction of tyrosine phosphorylation or proliferation were observed after incubation with glycosylated LIF in cells that expressed Man-6-P/IGFII-R but not LIF receptor gp190. Moreover, no difference between glycosylated and non-glycosylated LIF was noted in various LIF biological tests as follows: induction of gp130/gp190 tyrosine phosphorylation, activation of STAT3 and MAPK, induction of proliferation, or induction of haptoglobin secretion by HepG2 cells. In addition, LIF binding to Man-6-P/IGFII-R did not alter IGF-II bioactivity on NIH3T3 fibroblasts (MAPK activation and induction of proliferation).

As reported for other secretory Man-6-P-containing ligands such as DNase I (45), our results suggest that only a minority of LIF molecules (15–25%), and probably of M-CSF molecules, is phosphorylated on mannose. As the natural forms of LIF studied here bound 4–8-fold less to Man-6-P/IGFII-R, it is likely that non-recombinant LIF in vitro contains less than 5% of Man-6-P. Direct analysis of the phosphorylated oligosaccharides of LIF is needed to provide definitive conclusions. It is noteworthy that phosphorylation of Asn-linked oligosaccharides is mediated by the enzyme N-acetylglucosamine-1-phosphotransferase (phosphotransferase), which recognizes a conformation-dependent protein determinant involving specific lysine and arginine residues (45, 46). Although phosphotransferase acts primarily on lysosomal hydrolases, a few secretory glycoproteins such as DNase I acquire Man-6-P moieties. Newly synthesized Man-6-P-containing glycoproteins are then captured in the Golgi apparatus by Man-6-P/IGFII-R or CD-MPR, another Man-6-P receptor (47). Ligands complexed to MPRs are subsequently translocated via clathrin-coated vesicles (interaction with AP-1 clathrin adapter complex) (48) to endosomes where acidic pH induces a dissociation between the ligand and MPRs. The ligand is then packaged into lysosomes, and the receptor can return to the Golgi apparatus for another cycle (12, 49). As LIF, like DNase I, would appear to be a relatively weak substrate for phosphotransferase, oligosaccharides in most of the molecules are not (or poorly) phosphorylated. Therefore, most of the newly synthesized LIF is secreted rather than being captured in the Golgi apparatus by MPRs. In culture, a weak base such as chloroquine induces enhanced secretion of Man-6-P-containing proteins. In the case of A375 cells, a 25% increase in secreted LIF occurred repeatedly in the presence of chloroquine, suggesting that Man-6-P-containing LIF molecules are well diverted to lysosomes. Direct analysis of the intracellular location of LIF is needed to confirm this hypothesis.

Man-6-P/IGFII-R is also present at the plasma membrane where it mediates internalization and delivery of IGF-II (50) and Man-6-P-containing ligands (lysosomal enzymes, prolif-
erin, and latent TGF-β (51) to the endocytic compartment. This receptor utilizes clathrin-coated vesicles via the AP-2 clathrin adapter complex for endocytosis (52–53). LIF (and probably other factors such as M-CSF) is rapidly internalized and degraded by all cells expressing Man-6-P/IGFII-R at the plasma membrane, which indicates that this receptor is meta-

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**Physiological Relevance of LIF Binding to Man-6-P/IGFII-R**

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**Fig. 5.** A and B, analysis of protein phosphorylation (PY20 monoclonal antibody), STAT3 phosphorylation, and MAPK activation after CHO-LIF (5 nM) or *E. coli*-LIF (5 nM) stimulations for 10 min on JAR and CHO-190 cells. When indicated, cell lysates were subjected to immunoprecipitation with anti-gp130 or anti-CIMPR antibodies before analysis of protein phosphorylation. The same membranes were also reprobed with anti-CIMPR, anti-gp190, or anti-gp130 antibodies. C, proliferation of OPM2 cells (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide-based assay) in the presence of increasing concentrations of CHO-LIF (●), *E. coli*-LIF (○), or a fixed saturating concentration (10 nM) of *E. coli*-IL-6 (■). D, haptoglobin production by HepG2 cells in the presence of increasing concentrations of CHO-LIF (●), *E. coli*-LIF (○), or a fixed saturating concentration (1 nM) of *E. coli*-IL-6 (■).
bolically active after LIF binding. It is noteworthy that the tumor cell lines studied here for their secretion of Man-6-P-containing LIF all expressed physiological amounts of Man-6-P/IGFII-R and CD-MPR (11). The low percentage of Man-6-P-containing LIF could have been due to various post-translational regulatory events, including dephosphorylation, lysosome targeting, and internalization/degradation (LIF preparations obtained without added antiphosphatase, chloroquine, or Man-6-P).

Man-6-P/IGFII-R is known to bind multiple ligands as follows: lysosomal enzymes (more than 50), soluble factors such as IGF-II, proliferin, latent TGF-β (see Ref. 12 for a review), retinoic acid (16), LIF, and M-CSF (11, and this paper), as well as some receptors such as epidermal growth factor receptor (54) or urokinase-type plasminogen activator receptor (15). It remains to be determined whether other glycosylated cytokines can also bind to Man-6-P/IGFII-R. However, within the IL-6 family of cytokines, LIF seems to be the only member containing Man-6-P in its carbohydrate moieties, or Man-6-P).

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The cytoplasmic domain of Man-6-P/IGFII-R is devoid of tyrosine kinase activity. However, some reports have suggested that Man-6-P/IGFII-R may function as a transmembrane signaling molecule. For example, IGF-II interaction with Man-6-P/IGFII-R has been reported to induce proliferation of Balb/c 3T3 fibroblasts (27), K652 cells (29), or OPM2 cells (30). More recently, other reports have shown that Man-6-P/IGFII-R couples to heterotrimeric G proteins (55, 56), activates phospholipase C (inositol 1,4,5-trisphosphate production) (57), and stimulates calcium influx (27, 28), although these findings are controversial (25, 26). Finally, a recent report has shown that either proliferin or IGF-II binding to Man-6-P/IGFII-R induces endothelial cell chemotaxis through a G protein-coupled, mitogen-activated protein kinase (MAPK)-dependent pathway (31). At present, there is no evidence for such LIF-mediated activation. Further experiments are needed to investigate IP₃ production and ADP-riboseylation of G protein.

Some reports have described a potentiation of IGF-II mitogenic activity by Man-6-P (43, 58) and indicated that endogenous Man-6-P-containing ligands can inhibit IGF-II bioactivity (44). Man-6-P has also inhibited various biological activities not investigated in this study, such as activation of latent TGF-β (19), lysosomal enzyme trafficking, and adhesion of myeloma cells to stromal cells (30). It cannot be excluded that Man-6-P-containing LIF modulates some of these functions. However, LIF did not modulate IGF-II activity on NIH3T3 cells, even when they were washed with Man-6-P prior to incubation with IGF-II (56), possibly because the biological activity of IGF-II on NIH3T3 cells is mediated by IGF-I receptors or a third unknown receptor for IGF-II (59).

It is conceivable that a LIF molecule can bind one molecule each of gp190, gp130, and Man-6-P/IGFII-R, insofar as the respective binding sites on LIF do not seem to overlap (11). Man-6-P/IGFII-R could therefore regulate LIF bioactivity, for example, by stabilizing high affinity receptors. However, no significant difference in biological activity has yet been observed between glycosylated and non-glycosylated LIF, although other biological tests need to be evaluated. The low percentage of Man-6-P-containing LIF in the preparations studied (2–20%) might also explain why no involvement of Man-6-P/IGFII-R was observed. Assessment of this issue implies a comparison of purified Man-6-P-containing LIF with non-glycosylated LIF. Preliminary experiments with a LIF preparation containing more than 95% of molecules able to bind to Man-6-P/IGFII-R seem to confirm that phosphorylated LIF is as active as E. coli-LIF or CHO-LIF on DA-1a cells and HepG2 cells.

In conclusion, the parallel between LIF and lysosomal enzymes or the growth factor IGF-II is intriguing. Man-6-P/IGFII-R is not able to transduce a biological signal after LIF binding on numerous cell lines but could be an important regulator of LIF metabolism and bioavailability. Some physiological circumstances have been described in which the functions of Man-6-P synthesizing enzymes or MPRs are impaired, i.e. in mucolipidosis II where a phosphotransferase defect is responsible for enhanced secretion of lysosomal enzymes in different body fluids (60), and in certain tumor cells where defective expression of Man-6-P/IGFII-R (allelic loss and point mutations) has been observed (20, 61) in correlation with enhanced concentrations of IGF-II and latent TGF-β (21). LIF is able to enhance proliferation on some myeloma cells (42, 62) or breast cancer cells (6, 63), but very little is known about its role in carcinogenesis. The results presented here suggest that some tumor cells, through decreased expression of Man-6-P/IGFII-R, can increase endogenous LIF secretion while reducing its degradation. This enhanced local concentration of LIF (as for IGF-II and latent TGF-β) could contribute to increased proliferation, thereby reinforcing the tumorigenic property of LIF. However, a more intriguing observation concerns embryogenesis. Man-6-P/IGFII-R-deficient mice have increased serum and tissue levels of IGF-II and Man-6-P-containing ligands and exhibit overgrowth, organomegaly, and perinatal death (64, 65). This phenotype is caused primarily by an excess of IGF-II overstimulating IGF-I-R (22, 23). However, LIF and M-CSF may also participate in this phenotype. LIF inhibits the differentiation and supports the proliferation of undifferentiated embryonic stem cells (2) and is necessary for embryo implantation (4), whereas M-CSF is important for successful pregnancy (66). The secretion and turnover of Man-6-P-containing LIF in cells from Man-6-P/IGFII-R and CD-MPR-deficient mice (67) is currently being investigated in our laboratory.

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