The Mannose 6-Phosphate/Insulin-like Growth Factor II Receptor Is a Nanomolar Affinity Receptor for Glycosylated Human Leukemia Inhibitory Factor

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Comparison of the binding properties of non-glycosylated, glycosylated human leukemia inhibitory factor (LIF) and monoclonal antibodies (mAbs) directed at gp190/LIF-receptor β subunit showed that most of the low affinity (nanomolar) receptors expressed by a variety of cell lines are not due to gp190. These receptors bind glycosylated LIF produced in Chinese hamster ovary cells (CHO LIF) (Kd = 6.9 nM) but not Escherichia coli-derived LIF or CHO LIF treated with endoglycosidase F. CHO LIF binding to these receptors is neither affected by anti-gp190 mAbs nor by anti-gp130 mAbs and is specifically inhibited by low concentrations of mannosyl-6-phosphate (Man-6-P) (IC50 = 40 μM), suggesting that they could be related to Man-6-P receptors. The identity of this LIF binding component with the Man-6-P/insulin-like growth factor-II receptor (Man-6-P/IGFII-R) was supported by several findings. (i) It has a molecular mass very similar to that of the Man-6-P/IGFII-R (270 kDa); (ii) the complex of LIF cross-linked to this receptor is immunoprecipitated by a polyclonal anti-Man-6-P/IGFII-R antibody; (iii) this antibody inhibits LIF and IGFII binding to the receptor with comparable efficiencies; (iv) soluble Man-6-P/IGFII-R purified from serum binds glycosylated LIF (Kd = 43 nM) but not E. coli LIF. The potential role of Man-6-P/IGFII-R in LIF processing and biological activity is discussed.

Leukemia inhibitory factor (LIF) is a multifunctional, highly glycosylated soluble protein belonging to the interleukin-6 (IL-6) subfamily of helical cytokines (also including IL-11, oncostatin M (OSM), ciliary neurotropic factor (CNTF), and cardiothrophin-1 (CT-1)) (1–3). Signal transduction by these cytokines is proposed to result from cytokine-mediated homodimerization of the gp130 signal transducer (4) or heterodimerization of gp130 with another signal transducing subunit. IL-6 first binds with low affinity (nanomolar) to a specific IL-6Ra subunit and the complex then recruits and homodimerizes two gp130 subunits for signaling. A final hexamer complex of two molecules each of IL-6, IL-6Ra, and gp130 has been proposed (5). Similarly, specific IL-11Ra subunits have also been identified (6, 7) and the IL-11/IL-11Ra complex is also proposed to induce homodimerization of gp130 for signaling. Other members of this cytokine family can induce heterodimerization of gp130 with gp190, a signaling molecule initially identified as the low affinity (nanomolar) LIF receptor (or LIF-Rβ) (8). CNTF recruits gp130 and gp190 together with a third cytokine specific receptor chain (CNTF-Rα). LIF and OSM have been proposed to require only gp130 and gp190 to form a common signaling complex designated type I OSM receptor. Another type of OSM receptor, not used by LIF, has been described (type II OSM receptor). It involves the recruitment of gp130 with a recently identified gp180 transducing molecule also called OSM-Rβ (9). Cross-linking studies have also suggested that another receptor subunit besides gp130 and gp190 might participate in the structure of the LIF receptor (10). Similarly, a cytokine specific receptor chain has been described by cross-linking in the case of CT-1 (11).

Most of the cytokines of this subfamily have been shown to exert biological activities on various cell types, both within and outside of the hematopoietic system (1, 2). Due to the sharing of common transducing receptor subunits, these cytokines also show overlapping activities. In agreement with the multiple activities elicited by LIF, high affinity (picomolar) LIF receptors have been demonstrated on a number of cell lines (12). In addition, several cell lines have been described to express only low affinity (nanomolar) LIF receptors, and it was postulated that this was linked to the expression of gp190 (LIF-Rβ) in the absence of gp130 (12). Evaluation of this hypothesis was, however, hampered by the lack of anti-LIF-Rβ antibodies. In this study, with the recent availability of such anti-LIF-Rβ mAbs (13, 14), we show that the low affinity LIF receptor expressed by a number of cell lines is unrelated to gp190. This receptor binds LIF through its carbohydrate moieties and displays biochemical, immunochemical, and functional features, indicating that it is identical to the mannosyl-6-phosphate/insulin-like growth factor II receptor (Man-6-P/IGFII-R).
EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Cytokines—The human myeloma U266 cell line and the human choriocarcinoma JAR cell line were obtained from the American Type Culture Collection (ATCC, Rockville, MD). U266 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), and JAR cells were cultured in Dulbecco's modified Eagle's medium supplemented with 8% FCS. CHO cells stably transfected with the full-length gp190 DNA (10) were cultured in RPMI 1640 containing 10% FCS. The anti-human gp190 mAbs have been recently raised against a soluble form of the human LIF-R (gp190) and their initial characterizations were described elsewhere (13, 14). Polyclonal antibodies against gp190 or gp130 were purchased from R&D Systems (Minneapolis, MN). Anti-gp130 mAb BR3 was a kind gift from Dr John Wijdenes (Diaclone, Besançon, France). LIF (CHO LIF) was purified from the full-length conditioned medium of CHO cells transfected with a full-length cDNA encoding for human LIF as described (12). Recombinant Escherichia coli-derived human LIF (E. coli LIF) was obtained from PeproTech, Inc. (Rocky Hill, NJ). Recombinant SF21-derived human IL-9 (insect IL-9) was from R&D Systems. Natural MG63-derived human IL-6 (osteosarcoma IL-6) was from Sigma. A natural form of LIF was purified from A575 melanoma cells by affinity chromatography on concanavalin A-Sepharose and polyclonal antibodies coupled to agarose beads (Affi-Gel, Bio-Rad). Its concentration was determined by a specific enzyme-linked immunosorbent assay as described (15). Monosaccharides from Sigma. Affinity-purified rabbit polyclonal antibodies against Man-6-P/IGFII-R (extracytoplasmic domain) were purified from bovine serum by affinity chromatography on phosphonomannan as described (16).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis—Total RNA was extracted from human cell lines using a guanidinium thiocyanate/phenol method (17). The first cDNA strand was synthesized using total RNA (2 μg) at 4 °C for 30 min in a reaction mixture containing 50 mM Tris-HCl (pH 8.3), 60 mM KCl, 10 mM dithiothreitol, 5 mM MgCl₂, 50 units of RNase inhibitor (Boehringer, Mannheim, Germany), 1 mM amounts of each deoxynucleotidetriphosphate (dNTP), 600 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.), and 2 μg of oligo(dT) 15-mer 1 μl of the reaction mixture was made up to 25 μl using Taq polymerase buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂) containing 10 pmol of each primer and 0.6 unit of Ampli-Taq DNA polymerase (Pharmacia Biotech, Uppsala, Sweden). Amplifications were performed using a thermal cycler for 35 cycles under the following conditions: denaturation at 1 min for 94 °C, annealing for 45 s at 59 °C, and elongation for 1 min at 72 °C using Ampli-Taq polymerase (2 units/ml). PCR products were separated in a 1% agarose gel and autoradiographed as described above.

Radioiodination of LIF and Antibodies—The different mAbs were radiolabeled as described (14) according to a procedure using IODOGEN as a catalyst. The specific radioactivities obtained were in the range of 450–900 Ci/nmol. CHO LIF and E. coli LIF were iodinated according to the chloramine T method as described (12). LIF was labeled at a specific radioactivity of around 1,800 Ci/nmol for binding studies under low affinity conditions. CHO LIF and E. coli LIF were iodinated according to a procedure using Iodo-Gen as a catalyst. The specific radioactivities obtained were in the range of 450–900 Ci/nmol. CHO LIF and E. coli LIF were iodinated according to the chloramine T method as described (12). LIF was labeled at a specific radioactivity of around 1,800 Ci/nmol for binding studies under low affinity conditions.

Binding and Competition Assays—CHO or JAR cell monolayers were incubated with PBS containing 0.05% (w/v) trypsin to detach the cells in suspension, washed with FCS containing culture medium, and resuspended in PBS containing 0.5% bovine serum albumin (PBS-BSA). U266 cells were washed prior to resuspension in PBS-BSA. Binding experiments were carried out in PBS-BSA as described previously (12). Cells (1 × 10⁵/well in 96-well round-bottomed plates) were incubated with increasing concentrations of labeled LIF or anti-gp190 mAb for 90 min at 4 °C (equilibrium conditions) at 4 °C. Cell bound (B) and unbound (F) fractions were separated by centrifugation through a layer of dibutylphthalate (90%) and paraffin oil (10%). Regression analysis of the binding data was accomplished using a one- or two-site equilibrium binding equation (Grafit, Erithacus Software, Staines, United Kingdom).

RESULTS

U266 cells Express a LIF Receptor That Does Not Involve gp190—In the course of studies investigating the expression of gp190, gp130 and LIF receptors by various cell lines, a surprising observation was the absence of gp190 from cell line. U266 expressed LIF receptors but did not bind anti-gp190 mAbs. Indeed, as shown in Fig. 1A, U266 cells display a single class of low affinity binding sites for glycosylated LIF, CHO-derived LIF (24,200 sites/cell, K_D = 6, 9 nM) but no detectable binding of the anti-human gp190 mAb 1B4. This is in sharp contrast with Fig. 1B, which shows that CHO cells transfected with human gp190...
bind CHO LIF and 1B4 mAb with similar maximal binding capacities (11,500 and 11,700 sites/cell, respectively). Five other anti-gp190 mAbs, which have previously been shown to react with separate epitopes in addition to the one recognized by 1B4 (13, 14), also bound to gp190-CHO cells without detectable binding to U266 cells (data not shown). In agreement with previous results (18), U266 cells expressed 790 binding sites for CHO LIF and 1B4 mAb with similar maximal binding capacities (11,500 and 11,700 sites/cell, respectively). Five other anti-gp190 mAbs, which have previously been shown to react with separate epitopes in addition to the one recognized by 1B4 (13, 14), also bound to gp190-CHO cells without detectable binding to U266 cells (data not shown). In agreement with previous results (18), U266 cells expressed 790 binding sites for the anti-gp130 mAb BR3 (Kd = 0.68 nM, Fig. 1A).

Lack of gp190 expression by U266 cells was further confirmed at the transcriptional level. RT-PCR (Fig. 2) showed the absence of detectable human gp190 transcripts in U266 cells, using a cDNA probe that was positive on CHO-190 cells and on the gp190-positive choriocarcinoma JAR cell line.

In order to further document this finding, competition experiments were performed. Fig. 3A shows that glycosylated LIF binding to U266 cells was not affected by the anti-gp130 mAb BR3, a mAb that has previously been shown to block high affinity binding of all the cytokines using the gp130 molecule (19), nor by the combination of anti-gp190 mAbs 1B4 and 1C7, two antibodies that have been shown to synergize for inhibiting the binding of glycosylated LIF to high affinity LIF receptor (14) as well as the binding of glycosylated LIF to soluble gp190 molecule (13). Control experiments (Fig. 3B) showed that the combination of 1B4 and 1C7 did inhibit CHO LIF binding to gp190-CHO-transfected cells.

Together, these data indicated that U266 cells expressed a low affinity LIF receptor unrelated to the gp190/LIF-Rβ component or to the gp130 signal transducer.

The LIF Receptor on U266 Binds Glycosylated LIF but Not Non-glycosylated LIF—Another surprising observation was that U266 cells did not bind non-glycosylated, E. coli-derived LIF (Fig. 1A). This again was in sharp contrast to gp190-transfected CHO cells, which bound the non-glycosylated cytokine with a stoichiometry (11,200 sites/cell) similar to that for the glycosylated species (11,700 sites/cell) (Fig. 1B). Additional experiments showed that treatment of glycosylated LIF with Endoglycosidase F, an enzyme that removes the N-linked carbohydrates, completely abrogated LIF binding to U266 cells, while leaving its binding to gp190-CHO-transfected cells unaffected (data not shown).

We then analyzed whether E. coli LIF could compete for CHO LIF binding. As indicated in Fig. 3A, CHO LIF binding to U266 cells was not affected by excess amounts of E. coli LIF, whereas similar amounts of E. coli LIF completely abolished CHO LIF binding to gp190-CHO cells (Fig. 3B). Fig. 3C shows dose-response curves for inhibition of CHO LIF binding to U266 cells. E. coli LIF has no effect on this binding over a wide concentration range. As expected, unlabeled CHO LIF competes with its labeled counterpart with an IC50 (12 nM) in agreement with the Kd of CHO LIF binding to U266 cells (Kd = 6.9 nM; see Fig. 1A). A glycosylated form of LIF immunopurified from cell culture supernatants of the A375 human melanoma cell line also competed with CHO LIF binding with efficiency similar to that for unlabeled CHO LIF. In contrast, natural glycosylated human IL-6 (purified from MG63 osteosarcoma cells) or glycosylated recombinant human IL-9 derived from insect cells were without effect on CHO LIF binding. These data reinforce the notion that the LIF receptor of U266 cells is unrelated to gp190. They further demonstrate that it specifically binds glycosylated forms of LIF, and suggest N-linked carbohydrate involvement in this binding.

A Large Number of Cell Lines Express Low Affinity LIF Receptors Similar to Those Expressed by U266 Cells—The JAR cell line has been previously shown in our laboratory to express both low and high affinity receptors for glycosylated LIF (10, 12, 14). As shown in Fig. 4A, the binding of glycosylated CHO LIF to JAR cells is characterized by a curvilinear Scatchard plot, which is resolved into two linear components: 2,400 high affinity sites with a Kd of 140 pM and 40,000 low affinity sites with a Kd of 14.2 nM. In contrast, non-glycosylated E. coli-derived LIF was found to bind to JAR cells with a linear Scatchard plot indicating the presence of a single class of high affinity binding sites (Kd = 112 pM) with a maximal capacity (2,300 sites/cell) being comparable to that of high affinity CHO LIF binding sites. Similarly, the anti-gp190 mAbs 1B4 bound to JAR cells with a single class of binding sites (Kd = 0.22 nM) and a maximal binding capacity (2,330 sites/cell) comparable to the number of E. coli LIF binding sites or CHO LIF high affinity binding sites and far less than the number of low affinity CHO LIF binding sites. Similar results were found with other anti-gp190 antibodies directed at separate epitopes (data not shown). These results indicated that most of the low affinity
LIF receptors on JAR cells were not able to bind E. coli LIF and were different from gp190. The number of gp130 molecules on JAR cells, as measured by the binding of BR3 mAb, was in excess of the number of anti-gp190 mAb binding sites (8,200 sites/cell, \(K_D = 0.77 \text{ nM}\)).

Competition experiments on LIF binding to JAR cells were performed either with labeled E. coli LIF at 0.25 nM (Fig. 4D) or with labeled CHO LIF used either at 50 pM to label mainly the low affinity component (Fig. 4C), or at 5 nM to label mainly the high affinity component (Fig. 4B). Results show that CHO LIF competed with E. coli LIF binding and, conversely, E. coli LIF competed with CHO LIF binding to its high affinity component. In contrast, E. coli LIF only displayed a low inhibitory effect (about 25%) on the CHO LIF low affinity component. In addition, whereas the anti-gp190 mAbs almost completely inhibited E. coli LIF and CHO LIF high affinity component (Fig. 4, D and C), they had little inhibitory effect (30%) on the CHO LIF low affinity component (Fig. 4B).

Together, these data indicate that the low affinity component on JAR cells behaves like the LIF receptor on U266 cells: no relation to gp190 and specific recognition of the glycosylated form of LIF.

The effects of the anti-gp130 mAb BR3 were also analyzed (Fig. 4); as already observed on U266 cells, it had no significant effect on CHO LIF low affinity component, an observation that reinforces the similarity between U266 and JAR cells low affinity LIF receptors. On the contrary, BR3 displayed similar partial inhibitory effects on CHO LIF high affinity binding (about 50%) and E. coli LIF binding (about 65%).

In a first attempt to investigate the spectrum of expression of such LIF receptors, a number of cell lines were evaluated for their binding capacities of CHO LIF and E. coli LIF. Table I shows that a number of cell lines, including U266, expressed fairly large numbers of CHO LIF binding sites in the absence of detectable (<50 sites/cell) binding sites for E. coli LIF. Other cell lines, including JAR cells, expressed CHO LIF binding sites in large excess to the number of E. coli binding sites. The

![Image](https://example.com/image1.png)

**Fig. 4.** A, Scatchard plots of the binding of CHO LIF (●), E. coli LIF (○), mAb BR3 anti-gp130 (□), and 1B4 anti-gp190 (◇) to JAR cells. B–D, competition binding studies on JAR cells. 125I-Labeled CHO LIF was used at 5 nM (LA, low affinity) (B) or 50 pM (HA, high affinity) (C) or 125I-labeled E. coli LIF used at 250 pM (D). Unlabeled competitors were used at concentrations as in Fig. 3, A and B.

Epstein-Barr virus-transformed DAB lymphoblastoid B cell line was receptor-negative for both types of LIF.

**Biochemical and Immunoochemical Identification of the LIF Receptors**—In order to identify the molecular features of the LIF receptors expressed by U266, CHO-190, and JAR cells, cross-linking experiments were carried out with iodinated CHO LIF. The cross-linked species were immunoprecipitated with anti-LIF, anti-gp130, or anti-gp190 antisera and resolved by SDS-PAGE under reducing conditions (Fig. 5).

Cross-linking of CHO LIF to U266 cells yielded one band at 310 kDa, which corresponds to the complex of one molecule of LIF (molecular mass of about 40 kDa) with a molecular component of 270 kDa. This material was immunoprecipitated by an anti-LIF antisera but could not be immunoprecipitated with an anti-gp130 antisera or with an anti-gp190 antisera. This band was clearly different from the 225-kDa band immunoprecipitated from gp190-CHO cells, which corresponds to the LIF-gp190 cross-linked complex.

Cross-linking of CHO LIF to JAR cells yielded three bands that were precipitated with the anti-LIF antisera. 1) The lower band (185 kDa) was immunoprecipitated with the anti-gp130 antisera and likely corresponds to a LIF-gp130 cross-linked species. 2) The intermediate band (230 kDa) was immunoprecipitated with the anti-gp190 antisera and migrated similar to that immunoprecipitated on gp190-CHO cells. It therefore likely corresponds to a LIF-gp190 cross-linked complex. The anti-gp190 antisera also immunoprecipitated the lower band, suggesting either that this antisera cross-reacts with gp130 or that it can co-immunoprecipitate a gp130/gp190 complex. 3) The higher cross-linked band on JAR cells had a molecular mass (300–320 kDa) identical to the band identified on U266 cells, and similarly could not be immunoprecipitated either with anti-gp130 or with anti-gp190 antisera.

**Glycosylated LIF Binding to U266 Cells and Low Affinity Binding to JAR Cells Are Inhibited by Mannose and Mannose 6-Phosphate**—The fact that LIF receptors expressed by U266 cells were able to bind glycosylated LIF but not its non-glycosylated (E. coli or deglycosylated (endoglycosidase-F-treated) isoforms, prompted us to investigate the potential competing effects of monosaccharides on this binding.

Fig. 6A shows that CHO LIF binding to U266 cells is completely and dose-dependently inhibited by D-mannose, D-mannose 6-phosphate (Man-6-P), and D-glucose 6-phosphate (Glc-6-P), whereas it remains insensitive to similar concentrations of D-galactose and D-glucose. N-Acetylgalactosamine was also inactive (not shown). The effect of Man-6-P was much more pronounced than that of mannose or Glc-6-P; its half-maximal inhibitory effect was observed at a concentration of 40 \(\mu\text{M}\) as compared with 58 and 14 \(\mu\text{M}\) for mannose and Glc-6-P, respectively. Control experiments showed that CHO LIF or E. coli

| Tissue type                  | Cell line | E. coli-LIF binding sites/cell | CHO-LIF binding sites/cell |
|-----------------------------|-----------|-------------------------------|---------------------------|
| Placental choriocarcinoma   | JAR       | 2500                          | 42,000                    |
| Hepatocarcinoma             | HepG2     | 100                           | 30,000                    |
| Neuroblastoma               | SKNSH     | 500                           | 11,000                    |
| Keratinocyte                | SVK14     | 800                           | 7000                      |
| Myeloma                     | LP1       | 100                           | 5000                      |
| Osteosarcoma                | MG63      | ND                            | 40,000                    |
| Myeloma                     | U266      | ND                            | 24,000                    |
| Erythroleukemia             | K562      | ND                            | 25,000                    |
| Malignant melanoma          | A375      | ND                            | 6500                      |
| Acute monocytic leukemia    | U937      | ND                            | 3000                      |
| B cell line                 | DAB       | ND                            | ND                        |

*ND, not detectable.
LIF binding to gp190-CHO cells were not affected by any of the monosaccharides (Fig. 6B and data not shown). The effects of monosaccharides were also investigated on JAR cells (Fig. 6C). In the presence of galactose (1 mM), the curvilinear Scatchard plot characteristic of CHO LIF high and low affinity binding was not modified. In sharp contrast, the presence of 1 mM Man-6-P induced the complete disappearance of the low affinity component, leaving the high affinity component essentially unaffected. E. coli LIF binding to JAR cells was not affected by any of the monosaccharides (not shown).

Phosphate Groups on Glycosylated LIF Are Required for Binding to U266 Cells—In view of the high inhibitory effect of Man-6-P as compared with mannose on LIF binding to U266 cells, we investigated whether CHO LIF contained phosphate groups that could be involved in binding. This was evaluated by competition experiments. Fig. 6D shows that treatment of unlabeled CHO LIF with alkaline phosphatase completely inhibited its ability to compete with iodinated CHO LIF binding to U266 cells. In contrast, alkaline phosphatase-treated CHO LIF retained a strong (>80%) inhibitory effect on iodinated CHO LIF binding to gp190-CHO cells.

The U266 LIF Receptor Is Biochemically and Immunologically Related to the Man-6-P/IGFII Receptor—The high inhibitory effect of Man-6-P on LIF binding to U266 cells as well as the high molecular mass of the receptor in cross-linking studies prompted us to investigate whether the LIF receptor on these cells could be related to the cation-independent mannose 6-phosphate receptor (CI-MPR), a type I membrane glycoprotein with an $M_r$ of 275,000. This receptor is also a receptor for insulin-like growth factor II (IGFII) (20). In Fig. 7A, it is shown that affinity-purified immunoglobulins raised against the Man-6-P/IGFII-R are able, as does anti-LIF antiserum, to immunoprecipitate the radiolabeled LIF-receptor cross-linked complex solubilized from U266 cells.

U266 cells were found to express about 30,000 binding sites for radioiodinated IGFII ($K_d$ 52 nM) (data not shown), a figure similar to the number of LIF binding sites. We next analyzed whether anti-CI-MPR could compete with LIF and IGFII binding to U266 cells, and whether the two factors (LIF and IGFII) could compete for binding (Fig. 7, B and C). The anti-Man-6-P/IGFII-R antibody was able to inhibit CHO LIF binding to U266 cells. The dose-response curve obtained suggests that antibody concentrations higher than 100 μg/ml are required to reach full inhibition. This inhibitory effect was similar to the one observed on the binding of iodinated IGFII to U266 cells. However, IGFII was unable to affect LIF binding and conversely.
Soluble Man-6-P/IGFII-R Purified from Serum Binds Glycosylated but Not Non-glycosylated LIF—In order to provide a direct proof that the Man-6-P/IGFII-R is a receptor for LIF, a natural soluble form of this receptor purified from bovine serum was covalently coupled to a dextran matrix and assayed for its ability to bind LIF by the technique of surface plasmon resonance. Fig. 8 shows clear association and dissociation curves for IGFII and CHO LIF (500 nM). Experiments performed at different concentrations (between 5 nM and 1 μM) of the factors showed that the increases in resonance signals were dose-dependent (data not shown). In sharp contrast, no association or dissociation could be observed. Furthermore, Man-6-P (5 mM) strongly inhibited the ability of CHO LIF to interact with immobilized Man-6-P/IGFII-R, whereas it did not affect binding of IGFII (not shown). The kinetic and equilibrium parameters derived from analysis of the sensograms depicting IGFII and CHO LIF binding to immobilized soluble Man-6-P/IGFII-R are shown in Fig. 8. The association phase starts at zero time, and the dissociation phase is initiated at 600 s. On the lower panel are gathered the kinetic and equilibrium parameters calculated from the sensograms ($k_a$, and $k_d$, kinetic association and dissociation constants; $K_d$, equilibrium dissociation constant).

FIG. 8. The upper panel shows the sensograms depicting the binding of CHO LIF, E. coli LIF, or IGFII binding to immobilized soluble Man-6-P/IGFII-R. Each factor was tested at a concentration of 500 nM. The association phase phase starts at zero time, and the dissociation phase is initiated at 600 s. On the lower panel are gathered the kinetic and equilibrium parameters calculated from the sensograms ($k_a$, and $k_d$, kinetic association and dissociation constants; $K_d$, equilibrium dissociation constant).

|  | $k_a$ (M-1s-1) | $k_d$ (s-1) | $K_d$ (M) |
|---|---|---|---|
| IGFII | 3.6 × 10^5 | 4.9 × 10^-4 | 1.4 × 10^-9 |
| CHO-LIF | 6.7 × 10^4 | 3.1 × 10^-4 | 4.6 × 10^-9 |

This paper demonstrates the existence of a novel type of low affinity receptor for LIF. This receptor is different from gp190 (also designated as the low affinity LIF receptor) or gp130. It binds glycosylated LIF but not E. coli-derived LIF, and this binding involves N-linked carbohydrates containing Man-6-P residues. Its identity with the Man-6-P/IGFII-R is strongly supported by biochemical, functional, and immunological findings.

Binding of CHO LIF to the Man-6-P/IGFII-R was not due to a special glycosylation state of LIF produced in the non-human CHO cell line, as glycosylated LIF naturally produced by the human melanoma cell line A375 was also found to react with the Man-6-P/IGFII-R. It also appeared to be cytokine-specific, as IL-9, a cytokine that, like LIF, is also heavily glycosylated (21), as well as natural human IL-6, also a glycosylated protein (22), did not interfere with CHO LIF binding to Man-6-P/IGFII-R when tested at similar concentrations.

Mannose, Man-6-P, and Glc-6-P, but not galactose, glucose, or N-acetylgalactosamine, inhibited LIF binding to Man-6-P/IGFII-R in a dose-dependent fashion. Whereas the inhibitory effects of mannose and Glc-6-P developed at non-physiological concentrations, Man-6-P was active at much lower concentrations (IC50 of 40 μM), an observation that has initially raised the hypothesis that the LIF receptor on U266 cells could be a Man-6-P receptor (MPR). Two distinct MRPs have been characterized and their cDNAs cloned (23, 24). One is a type I integral membrane glycoprotein with a molecular mass of 275 kDa. This receptor binds Man-6-P-containing ligands independent of divalent cations and has thus been called the cation-independent (CI)-MPR. This receptor has subsequently been demonstrated to bind IGFII (25) and is now referred to as the Man-6-P/IGFII-R. The other receptor is also a type I integral membrane glycoprotein with a molecular mass of 46 kDa. Unlike the first one, it requires divalent cations for optimal binding of Man-6-P-containing ligands and is referred to as the cation-dependent (CD)-MPR. Strong experimental evidence suggests that the LIF receptor expressed by U266 cells is identical to the Man-6-P/IGFII-R. (i) A polyclonal anti-LIF antibody immunoprecipitates a 310-kDa band corresponding to one molecule of LIF cross-linked to a receptor of 270 kDa; (ii) a polyclonal antibody raised against Man-6-P/IGFII-R immunoprecipitates a similar 310-kDa LIF-receptor complex; (iii) this
anti-Man-6-P/IGFII-R antibody inhibits binding of CHO LIF to U266 cells with an efficiency comparable to its ability to inhibit IGFII binding. Finally, a direct proof for the LIF binding capacity of Man-6-P/IGFII-R is provided by the demonstration that purified soluble Man-6-P/IGFII-R binds CHO LIF with an affinity (K_d = 4.6 nM) similar to that measured on U266 cells (K_d = 6.9 nM), while being unable to bind E. coli LIF.

We have previously described the expression of high and low affinity receptors for CHO LIF by various cell lines (12). Subsequent reports have shown that gp190, when expressed alone, behaves as a low affinity LIF receptor (8, 26). It was therefore assumed that low affinity LIF receptors expressed by numerous cell lines reflected gp190 molecules in excess to gp130 at the cell membrane. In this study, we show that most of the low affinity LIF receptors expressed by various tumor cell lines do not correspond to excess gp190 molecules but behave like the Man-6-P/IGFII/LIF receptor identified on U266 cells. This was demonstrated in detail with the JAR cell line. This cell line was found to express low affinity LIF receptors (over 40,000) far in excess of the number of gp190 (2,500) as well as gp130 (8,000) molecules. These low affinity LIF receptors displayed the same characteristics as the U266 LIF receptors with respect to CHO LIF versus E. coli LIF binding, inhibition by anti-gp130 and anti-gp190 mAbs, inhibition by monosaccharides, LIF cross-linking, and reactivity with the anti-Man-6-P/IGFII-R antibodies.

Human LIF contains seven potential N-glycosylation sites (27), of which four have been shown to be functional (28). In LIF purified from the human HS62 T lymphoma cell line, N-linked carbohydrates have been described to account for about 20 kDa in the molecular mass (43 kDa) of the cytokine. O-Linked glycosylations were also demonstrated, which accounted for about 1–2 kDa (29). There are three main types of N-linked sugar chains in glycoproteins: those of the oligomannosidic type with two to nine linked mannose, those of the N-acetyllactosaminic type with terminal sialic acid residues, and those of the mixed type (30). Our results suggest that oligomannosidic and/or mixed type sugar chains on the LIF molecule are specifically involved in the interaction with the Man-6-P/IGFII-R, and indicate that at least some of these side chains contain terminal Man-6-P residues.

Crystallization of LIF and site-directed mutagenesis studies have allowed a three-site model for receptor binding to be proposed (31, 32). In this model, site II formed by the N-terminal part of helix A and the C-terminal part of helix C contacts the cytokine receptor module of gp130 molecule, whereas site I (beginning of AB loop and C-terminal half of helix D) and site III (C terminus of CD loop) contact, respectively, the C-terminal and N-terminal cytokine receptor modules of gp190. In this model, it can be noticed that none of these sites contain potential N-glycosylation sites. Most of the N-glycosylation sites are distributed on parts of the LIF molecule that are exposed to solvent (1 site near N terminus, 1 site on helix A, 2 sites on end of AB loop, 2 sites on helix B, 1 site on N terminus of helix C). This observation raises the possibility that the Man-6-P/IGFII-R could interact with carbohydrates on glycosylated LIF even if it is bound to gp130 and gp190.

The extracellular region of the Man-6-P/IGFII-R is composed of 15 homologous repeat units with an average length of 147 amino acids (25). Whereas repeats 1–3 and 7–9 each contain one Man-6-P binding determinant involved in binding of Man-6-P-containing ligands (33, 34), the binding site of IGFII has been localized on repeat 11 (35), indicating that IGFII and Man-6-P binding sites are distinct. Some investigators have shown that high molecular weight Man-6-P-containing lysosomal enzymes can compete with IGFII for binding to the Man-6-P/IGFII-R (36). These results could suggest some overlap between the binding sites or merely reflect steric hindrance. The present work shows that whereas Man-6-P abrogates glycosylated LIF binding, glycosylated LIF and IGFII do not compete for binding, in agreement with the notion of non-overlapping binding sites.

One major question that arises from this work concerns the role of the Man-6-P/IGFII-R in LIF biological action. Diverse functions have been attributed to the Man-6-P/IGFII-R (20). The majority (90%) of this receptor is expressed within endosomal compartments, where its first role is to bind the newly synthesized lysosomal enzymes via their Man-6-P-containing N-linked oligosaccharides and to divert these phosphorylated ligands from the secretory pathway for subsequent sorting to endosomes and lysosomes. This receptor is also present at the plasma membrane where it endocytoses secreted lysosomal enzymes. It has also been shown to have a role in the clearance and activation of hormones and growth factors. The Man-6-P/IGFII-R is able to internalize IGFII, resulting in lysosomal degradation of this factor (37). It binds the precursor form of TGF-β (latent TGF-β) through Man-6-P residues, leading to the activation of this precursor form into biologically active TGF-β (38, 39). It also binds the Man-6-P-containing hormone prolactin, a prolactin-related murine protein (40), as well as porcine thyroglobulin (41) and may participate in the capture and degradation of these hormones in lysosomes. By this ability to activate TGF-β, a potent growth inhibitor for most cell types, to promote degradation of IGFII, a potent growth factor, and to endocytose proteolytic enzymes involved in extracellular matrix degradation, the Man-6-P/IGFII-R has been considered as a tumor suppressor, and recent findings have strengthened this concept by showing that Man-6-P/IGFII-R allelic loss is an early event in the etiology of cancer (42). LIF is a growth and differentiation factor that has been shown to be produced by various cell types and to be active on a large spectrum of cellular targets. In addition to its demonstrated roles in hematopoiesis, inflammation, lipid metabolism, bone homeostasis, and embryogenesis, LIF can also regulate tumor growth and metastasis (1, 2). By its capacity to bind to the Man-6-P binding sites, glycosylated LIF could modulate TGF-β activation, lysosomal enzyme trafficking, and extracellular matrix degradation.

Glycosylated LIF, as already suggested for other Man-6-P-containing ligands (43) (44), might as well modulate the activity of other Man-6-P/IGFII-R binding hormones such as IGFII itself and prolactin. Proliferin has been shown to promote neovascularization (45, 46), whereas LIF inhibits endothelial cell proliferation (47). Competition between these two factors at the level of the Man-6-P/IGFII-R could be the basis of these opposite effects.

In view of its large range of biological effects, there is a need to tightly regulate the production and circulating levels of LIF. Our data suggest that the Man-6-P/IGFII-R could participate in this process by its ability to recapture LIF, leading to internalization and lysosomal degradation. Indeed, preliminary data indicate that LIF bound to Man-6-P/IGFII-R is internalized and degraded at 37 °C but not at 4 °C.

Another function for the Man-6-P/IGFII-R could be to capture LIF molecules at the cell surface and favor its interaction with functional, high affinity LIF receptors containing gp130 and gp190. Such high affinity receptors have usually a low cell surface density. In contrast, low affinity Man-6-P/IGFII/LIF receptors have a much higher cell density (see Table I) and therefore could serve to increase the local concentration LIF at

\[ \text{F. Blanchard, unpublished data.} \]
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the cell surface and increase the probability of interaction of LIF with high affinity receptors. Such a mechanism has already been proposed in the case of growth factors that bind with low affinity to cell surface proteoglycans such as fibroblast growth factor and TGF-β (48).

The cytoplasmic domain of the Man-6-P/IGFII-R is short and devoid of tyrosine kinase activity. However, a number of reports have suggested that the Man-6-P/IGFII-R may couple to heterotrimeric G proteins (51, 52), although this finding is controversial (53, 54). Finally, a recent report has shown that either proliferin or IGFII binding to Man-6-P/IGFII-R induces endothelial cell chemotaxis through a G protein-coupled, mitogen-activated protein kinase-dependent pathway (46). Whether glycosylated LIF is also able to activate signal transduction through the Man-6-P/IGFII-R remains to be investigated.

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