Identification and Characterization of Two Distinct Truncated Forms of gp130 and a Soluble Form of Leukemia Inhibitory Factor Receptor α-Chain in Normal Human Urine and Plasma*

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Leukemia inhibitory factor (LIF) is a polyfunctional cytokine known to require at least two distinct receptor components (LIF receptor α-chain and gp130) in order to form a high affinity, functional receptor complex. In this report, we present evidence that there are two distinct truncated forms of gp130 in normal human urine and plasma: a large form with a molecular weight of approximately 100,000, which is similar to a previously described form of soluble gp130 in human serum, and a previously undescribed small form with a molecular weight of approximately 50,000. Using a panel of monoclonal antibodies raised against the extracellular domain of human gp130, we were able to show that the small form of the urinary gp130 probably contained only the hemopoeitin domain. Both forms of gp130 bound LIF specifically and were capable of forming heterotrimERIC complexes with soluble human LIF receptor α-chain in the presence of human LIF. In addition to the soluble forms of gp130, a soluble form of LIF receptor α-chain was also detected in human urine and plasma.

Leukemia inhibitory factor (LIF) is a polyfunctional cytokine that can act on a wide range of cell types including osteoblasts, hepatocytes, adipocytes, neurons, embryional stem cells, and megakaryocytes (1). LIF exerts its multiple biological functions through a specific cell surface receptor system, which consists of at least two membrane-bound glycoproteins, the LIF-binding chain (LIFRα) and gp130. LIF binds first to LIFRα with low affinity (2) and then to gp130 to form a high affinity functional receptor complex leading to activation of downstream signal transduction pathways (3–6). Both LIFRα and gp130 are members of the hemopoietin or cytokine type I family of receptors (7, 8). The extracellular domains of members of this receptor family share common structural features including hemopoietins domains characterized by four conserved cysteine residues and a WSXWS motif and three fibronectin type III (FN III) modules (7, 8). The membrane-bound gp130 was initially defined as the signal transducer of the interleukin-6 (IL-6) receptor system (9, 10) and has been shown subsequently to also be a component of the functional receptor complexes of ciliary neurotrophic factor (CNTF) (4), oncostatin-M (OSM) (3, 11), cardiotrophin-1 (12, 13), and interleukin (IL)-11 (IL-11) (14–16).

In addition to the cell membrane-anchored forms of LIFRα and gp130, it has been reported that naturally occurring soluble forms of these receptor molecules are present in biological fluids and may act as natural inhibitors of LIF activity (17–19). We and others have shown previously that a soluble form of the mouse LIFRα with a molecular weight (Mr) of approximately 90,000–150,000 occurs at high levels in normal mouse serum and is elevated dramatically during pregnancy (17, 18). Recently, we have provided evidence that the soluble form of mouse LIFRα probably arises from an alternative splicing event of the LIFRα mRNA (20). Despite the high levels of soluble LIFRα in mouse serum, its analogue was not detected in human serum (17).

In contrast, a soluble form of gp130 with a M, of 90,000–110,000 has been found in human serum (19). Although gp130 functions as the high affinity converting and signaling subunit in the receptor complexes for IL-6, LIF, OSM, CNTF, cardiotrophin-1, and IL-11, OSM was the only cytokine in this family initially demonstrated to bind to membrane-bound gp130 (3), and subsequently, it has been shown that OSM can bind directly to the soluble form of gp130 with low affinity (21, 22). We and others have recently shown that the soluble form of gp130 was able to bind not only directly and specifically to OSM but also to LIF (22, 23). Using biosensor technology, we were able to determine that the interaction between hLIF and soluble human gp130 was of low affinity, with an equilibrium dissociation constant of approximately 44 nM (23). This low affinity interaction could explain previous failures in detecting direct binding of LIF to the membrane-bound form of gp130. In this study, we present evidence that there are two distinct truncated forms of gp130 in normal human urine and plasma:

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Soluble LIF Receptor and gp130

**EXPERIMENTAL PROCEDURES**

**Reagents—Escherichia coli-expressed hLIF** (a gift from Sandoz Pharmaceutical Co., Hanover, Switzerland) was radiosiodinated using a modified iodine monochloride method (24). Anti-hLIF polyclonal antibodies (mAbs), AM64, GPX22, and GPZ35, which were raised against Chinese hamster ovary cell-expressed extracellular domain of human gp130, were prepared as described previously (10, 25). A goat anti-human LIFR polyclonal antibody raised against the extracellular domain of human LIFR was purchased from R & D Systems.

**Expression and Purification of Soluble Human LIFRα and gp130 in Pichia pastoris—**A soluble form of human gp130 (shgp130), which consists of the Ig-like domain, hemopoietin domain, and three FN III modules, was expressed in the methylotrophic yeast *P. pastoris* with a FLAG™ epitope tag (DYKDDDDK) at its N terminus and purified on an anti-FLAG M2 affinity column by elution with FLAG peptide as described previously (26). A short form of soluble human gp130 (shgp130) was made identically as shgp130 except that the construct lacked all three FN III modules. Protein quantitation for the purified samples was performed by amino acid analysis. To make a soluble form of LIFRα, a cDNA encoding the hLIFRα (26) was altered at its 5’-end to encode an XhoI site and an in-frame 12CA5 epitope (YPYDVPDYA) (27). The sequence at the N terminus of the recombinant LIFRα was GAPVPVPDVYA. The 3’-end was modified to encode an Xhol site and a stop codon was introduced after position 536 (2) so that the recombinant LIFRα contained only the two hemopoietin domains and the intervening Ig-like domain. The cDNA was subsequently cloned into the yeast expression vector pPIC9 and expressed in *P. pastoris* as described (23). The protein was partially purified by gel filtration chromatography and quantified by Scatchard analysis of hLIF binding isotherms (17).

**Human Urine Collection—**Male and female normal human urine was collected from volunteers after informed consent, and 0.2% (v/v) Tween 20 and 0.02% (w/v) sodium azide were added. Small scale urine concentration was carried out using a Centriprep-10 (Amicon), and large scale concentration was performed using the Sartorius EasyFlow Device with a cellulose triacetate membrane (molecular weight cut-off of 20,000). Any precipitating materials in urine occurring before or after concentration were removed by centrifugation.

**Gel Filtration Chromatography—**Concentrated human urine was applied to a Superdex 200 10/30 column (Amersham Pharmacia Biotech), previously equilibrated in 20 mM phosphate-buffered saline (pH 7.0) and added, and the mixtures were incubated for 30 min at 4 °C. Samples were mixed with 7 µl of 4-fold concentrated SDS sample buffer and analyzed by either 7.5% or 10% SDS-PAGE under nonreducing conditions. The gels were dried and visualized by either autoradiography or PhosphorImager analysis (Molecular Dynamics).

**Affinity Chromatography—**The hLIF affinity column was prepared by covalently coupling 1 mg of E. coli-derived hLIF to 1 ml of Affi-Gel 10 (Bio-Rad) according to the manufacturer’s instructions. Normal human urine samples were concentrated 100-fold as described above and incubated with 1 ml of hLIF-Aff-Gel 10 resin for 3–5 h at 4 °C. After unbound proteins were removed by centrifugation, the hLIF affinity beads were washed with 16 × 1 ml of PBS followed by additional washes with 16 × 0.3 ml of 10-fold diluted Actisep elution medium (Sterogene Bioseparations, CA). The bound protein was then eluted with 10 × 0.3 ml of undiluted Actisep elution medium. The affinity column eluates were buffer-exchanged into PBS using NAP-5 columns (Amersham Pharmacia Biotech). Using the same procedures, LIF-binding proteins were enriched and partially purified on the hLIF affinity column from 50 ml of an outdated normal human plasma sample (obtained from the Royal Melbourne Hospital Blood Bank). Aliquots of buffer-exchanged fractions were analyzed for their ability to bind to 125I-hLIF using the cross-linking protocol described above.

**Immunoprecipitation—**Aliquots (20 µl) of the hLIF affinity column eluate, 0.2 µg/ml shLIFRα, or 2 µg/ml shgp130 were incubated with 125I-hLIF (800,000 cpm) in the absence or presence of 50 µg/ml unlabeled hLIF in a final volume of 50 µl for at least 1 h at 4 °C. Then 10 µl of a 12 mM BS3 solution was added, and the mixtures were incubated for 30 min at 4 °C. After adding 1 ml Tris-HCl buffer (pH 7.5) to a final concentration of 50 mM, the cross-linking reactions were incubated for 40 min at room temperature. The cross-linked samples were then mixed with an anti-human LIFRα polyclonal antibody at a concentration of 50 µg/ml. After a 30-min incubation at 4 °C, the mixtures were added to 30 µl of 50% (v/v) protein G-Sepharose gel slurry (Amersham Pharmacia Biotech) previously equilibrated in PBS and incubated for 4 h at 4 °C. The samples were centrifuged, and the protein-G-Sepharose beads were washed with 4 × 0.5 ml of PBS. For elution, the beads were mixed with 30 µl of 2× concentrated SDS sample buffer. The supernatants were then analyzed by 7.5% SDS-PAGE under nonreducing conditions.

**Protein Estimation—**Protein concentrations of pure recombinant soluble receptors were determined by amino acid analysis on a Beckman 6300 high performance amino acid analyzer equipped with a model 7000 data analyzer (Beckman).

**Inhibition of STAT-3 Phosphorylation in M1 Myeloid Cells—**M1 cells (10^6 cells/sample) were stimulated for 5 min at 37 °C with either 1 ng of hLIF or saline together with either soluble hLIF receptor or soluble hgp130 and then lysed in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 2 mM NaF, 1 mM Na3VO4, and 1 mM dithiothreitol in the presence or absence of 2 µg of protein G-Sepharose beads. After inhibiting insoluble proteins by chemical cross-linking, approximately 100 µg of total cellular proteins were subjected to 4–15% acrylamide SDS-PAGE under reducing conditions and then transferred to a prewetted polyvinylidene difluoride membrane (PVD-Plus, Micron Separations Inc.). After blocking, the membrane was incubated with an anti-phospho-STAT-3 polyclonal antibody (New England Biolabs), followed by incubation with a goat anti-rabbit Ig polyclonal antibody conjugated with horseradish peroxidase (Dako, Carpinteria, CA). The phosphorylated STAT-3 protein was visualized by autoradiography using the ECL system (Amersham Pharmacia Biotech). To check the quantity of protein loading, the same membranes were stripped with 0.1 M glycine-HCl, pH 3.0, for 30–60 min and washed three times in PBS, 0.1% Tween 20 before reprobing with a rabbit polyclonal antibody to STAT-3 (R-K, Santa Cruz Biotechnology, Inc.).

**Estimation of Soluble Human LIFRα Concentration in Plasma—**To quantify soluble LIFRα, an aliquot of an outdated normal human plasma sample (obtained from the Royal Melbourne Hospital Blood Bank) was first precleared with protein G-Sepharose beads at a ratio of 1:2 (v/v) for 1 h at 4 °C. The protein G-absorbed plasma was then incubated in the presence or absence of 2 µg of a goat anti-human LIFRα polyclonal antibody for 1 h at 4 °C, followed by the addition of 25 µl of protein G-Sepharose beads and a 2-h incubation at 4 °C. Immunoprecipitation of recombinant shLIFRα at various concentrations was performed in parallel except that the preabsorption step with protein G beads was not included. The immunocomplexes were washed with 3 × 1 ml of PBS containing 0.2% (v/v) Tween 20 and eluted from the protein G beads by boiling in SDS sample buffer under reducing conditions for 5 min before being subjected to 7.5% acrylamide SDS-PAGE. The Western blotting was performed as described above except that the anti-human LIFRα polyclonal antibody and a rabbit anti-goat Ig polyclonal antibody conjugated with horseradish peroxidase (Dako, Denmark) were used as the first and second antibodies, respectively.

**RESULTS**

**Detection of LIF-binding Proteins in Normal Human Urine—**Human urine samples, collected from six healthy individuals (H1–H6) were concentrated and tested for soluble LIF-binding proteins by chemical cross-linking. Analysis of the cross-linking products by SDS-PAGE (Fig. 1) indicated that 125I-hLIF was cross-linked specifically to two species of proteins in all six samples with Mr of approximately 100,000 (here referred to as the “large form”) and 50,000 (here referred to as the “small form”) after subtraction for the Mr of the bound unglycosylated hLIF, respectively. The levels of the two LIF-binding proteins varied in the six samples. This variation was likely to be due to the differences in protein content of these samples (data not shown).
The Two Species of the Urinary hLIF-binding Proteins Are Not Precomplexed—To examine whether the two hLIF-binding proteins were part of a preformed complex in urine, concentrated human urine was fractionated on a Superdex 200 gel filtration column as shown in Fig. 2A, and fractions were then analyzed for $^{125}$I-hLIF binding by chemical cross-linking. Analysis of column fractions 21, 23, 25, 27, 29, and 31 by SDS-PAGE (Fig. 2B) after cross-linking showed that the two hLIF-binding proteins were completely separated from each other according to their sizes, suggesting that they do not exist in a preformed complex in human urine. The $M_r$ estimates of the two proteins by gel filtration were consistent with those obtained above. Also, it can be seen from Fig. 2B that there was a downward trend in $M_r$ for both the large and small forms of the LIF-binding proteins across the fractions being assayed. This may be due to differential glycosylation of the two proteins.

Identification of the Urinary LIF-binding Proteins as Soluble gp130 and LIFRs—In mouse and human serum, the presence of soluble forms of the LIF receptor components, mouse LIFRa and human gp130, respectively, has been described (17–19). It has also been demonstrated that LIF can bind to gp130 directly (22, 23), although the affinity was relatively low (23, 28). To examine whether the detected $^{125}$I-hLIF binding activity in human urine was due to the presence of these reported proteins, we first performed competitive cross-linking experiments, as shown in Fig. 3, in which $^{125}$I-hLIF was mixed with increasing concentrations of unlabeled hLIF prior to cross-linking to a partially purified urinary LIF-binding protein sample (Fig. 3A). This was then compared with the same cross-linking to (a) a recombinant form of soluble human gp130 (shgp130; Fig. 3B), which consists of the Ig-like domain, hemopoietin domain, and three FN III modules with a FLAG™ epitope tag at the N terminus, (b) a recombinant short form of soluble human gp130 (ssshgp130; Fig. 3C) identical to shgp130 except that the construct lacked the FN III modules, or (c) a recombinant form of soluble human LIFRa (shLIFRa; Fig. 3D). Densitometric analyses of these data (not shown) revealed that half-maximal inhibition of $^{125}$I-hLIF cross-linking to both the large and small forms of the urinary binding proteins occurred at approximately 750 ng/ml unlabeled hLIF, similar to the hLIF concentrations required to inhibit 50% of the cross-linking to both shgp130 and sshgp130, whereas an approximately 3-fold smaller amount of hLIF was required to achieve the same inhibition for shLIFRa. These results indicated that the relative binding affinities of $^{125}$I-hLIF to the two forms of urinary binding proteins were similar to those of $^{125}$I-hLIF to recombinant shgp130 and sshgp130, suggesting that the LIF binding activity in human urine might be due to the presence of soluble forms of gp130 with different truncations at the C-terminal ends.

To formally test this possibility, the urinary LIF-binding proteins were first purified by a hLIF affinity chromatography step as described under “Experimental Procedures.” The eluates from the hLIF affinity column were then fractionated on a Superdex 200 gel filtration column to separate the large and small forms of the LIF-binding proteins (data not shown). The appropriate fractions were subsequently analyzed in cross-linking experiments, in which $^{125}$I-hLIF was incubated with the purified large or small form of the urinary LIF-binding protein in the presence of anti-shgp130 mAbs prior to cross-linking. As shown in Fig. 4, cross-linking in the presence of mAb AM64 yielded a higher $M_r$ complex in addition to the $^{125}$I-hLIF-large form complex (Fig. 4A, compare lanes 6 and 8) without a significant effect on $^{125}$I-hLIF cross-linking to the small form (Fig. 4B, compare lanes 6 and 8), whereas the addition of mAb GPZ35 in the cross-linking mixture generated higher $M_r$ complexes in both cases (Fig. 4A and B, lanes 10). In contrast, mAb GPX22 completely inhibited the cross-linking of $^{125}$I-hLIF to the small form (Fig. 4B, compare lanes 6 and 8) but only partially inhibited the $^{125}$I-hLIF cross-linking to the large form (Fig. 4A, compare lanes 6 and 9). For comparison, these mAbs were also added to the cross-linking mixtures of recombinant shgp130, sshgp130, and shLIFRa. As before, mAb AM64 only affected shgp130 (Fig. 4A, lane 3) but not sshgp130 (Fig. 4B, lane 3) cross-linking to $^{125}$I-hLIF. Unexpectedly, mAb GPZ35 did not significantly affect the cross-linking of $^{125}$I-hLIF to either sshgp130 (Fig. 4A, lane 5) or sshgp130 (Fig. 4B, lane 5) despite being able to produce a higher $M_r$ species when added to the cross-linking mixture of the small form (Fig. 4B, lane 10). This may be due to the possibility that mAb GPZ35 could not recognize Pichia-expressed gp130, suggesting that it recog-
FIG. 3. Competitive cross-linking of $^{125}$I-hLIF binding to partially purified urinary LIF-binding proteins (panel A), shgp130 (panel B), sshgp130 (panel C), or shLIFRa (panel D). SDS-PAGE analyses are shown of $^{125}$I-hLIF (200,000 cpm) cross-linked to partially purified urinary LIF-binding proteins, shgp130, sshgp130, or shLIFRa, respectively, in the presence of various concentrations of unlabeled hLIF as indicated. The cross-linked products were analyzed by 10% SDS-PAGE under nonreducing conditions.

FIG. 4. Analysis of the urinary hLIF-binding proteins by anti-human gp130 antibodies. The urinary LIF-binding proteins were first purified by hLIF affinity chromatography as described under “Experimental Procedures.” The eluates from the hLIF affinity column were then fractionated on a Superdex 200 gel filtration column to separate the large and small forms of the LIF-binding proteins (data not shown). Panel A, lanes 1–5, shgp130 (10 µl of 0.5 µg/ml) cross-linked to $^{125}$I-hLIF (200,000 cpm) alone or in the presence of 25 µg/ml unlabeled hLIF or 10 µg/ml mAbs AM64, GPX22, or GPZ35, respectively; lanes 6–10, purified large form LIF-binding fraction (10 µl) cross-linked to $^{125}$I-hLIF (200,000 cpm) alone or in the presence of 25 µg/ml unlabeled hLIF or 10 µg/ml mAb AM64, GPX22, or GPZ35, respectively; lanes 11–15, shLIFRa (10 µl of 0.05 µg/ml) cross-linked to $^{125}$I-hLIF (200,000 cpm) alone or in the presence of 25 µg/ml unlabeled hLIF or 10 µg/ml mAb AM64, GPX22, or GPZ35, respectively. The cross-linked products were analyzed by 10% SDS-PAGE under nonreducing conditions.
predominantly a form of soluble LIFRα with a $M_r$ higher than the soluble LIFRα that co-migrated with the large form of gp130. These were probably different glycosylated or truncated forms of the soluble LIFRα. As a control, $^{125}$I-LIF was also cross-linked to a recombinant shLIFRα or a recombinant sshgp130 in the presence of the same anti-human LIFRα antibody. As expected, the addition of the anti-human LIFRα antibody only yielded extra higher $M_r$ species in the cross-linking mixtures of shLIFRα (Fig. 5, compare lanes 1 and 3 and lanes 5 and 7). Together, these data indicated clearly the presence of a soluble LIFRα in the urinary samples.

These results were further confirmed in the immunoprecipitation experiment in which the resultant cross-linking product of $^{125}$I-LIF to the hLIF affinity column eluate was immunoprecipitated using the same anti-human LIFRα antibody (Fig. 5, lane 18). It was noted that the size of the $^{125}$I-LIF cross-linking proteins immunoprecipitated by the anti-LIFRα antibody were similar to those observed when the antibody was added prior to cross-linking (Fig. 5, compare lanes 1, 3, and 16 and lanes 8, 10, and 18, respectively), suggesting that cross-linking of the antibody to the $^{125}$I-LIF-LIFRα complex continued to occur during immunoprecipitation.

**Ternary Complex Formation of both the Large and Small Forms of gp130 with shLIFRα in the Presence of hLIF**—Both the large and small forms of the urinary gp130 as well as recombinant sshgp130 and sshgp130 were analyzed in cross-linking experiments for their ability to form a ternary complex with shLIFRα in the presence of hLIF. As shown in Fig. 6, cross-linking of $^{125}$I-LIF to the purified large form or recombinant sshgp130 in the presence of shLIFRα generated extra higher $M_r$ species (Fig. 6, lanes 7 and 3, respectively), the $M_r$ of which could be accounted for by summing up the $M_r$ of all three cross-linking components, suggesting the formation of a tripartite complex. The inability of the purified large form LIF-binding fraction to itself (Fig. 6, lane 6) to yield a higher $M_r$ complex corresponding to the ternary complex was probably due to insufficient LIFRα in the sample. When an extra amount of recombinant shLIFRα was added to the cross-linking mixture of this sample, the formation of a ternary complex became detectable (Fig. 6, lane 7). This was consistent with the above observation that the appearance of an extrahigh $M_r$ cross-linking species occurred only in the hLIF affinity column eluate containing the most LIF-binding activity. As above, when the cross-linking was performed with the small form of the urinary gp130 and recombinant sshgp130, both proteins were shown to be capable of forming ternary complexes with shLIFRα in the presence of hLIF (Fig. 6, lanes 9 and 5, respectively).

The affinity of the ternary complex for hLIF was estimated by competing with unlabeled hLIF for the formation of the cross-linked radioactive complex. Fig. 7 shows the results of such an inhibition experiment using a mixture of shLIFRα and sshgp130. In this experiment, cross-linked complexes of hLIF with either receptor alone could not be distinguished, but the ternary complex was clearly seen as a higher $M_r$ band (Fig. 7, indicated by the solid arrow). All cross-linked bands were inhibited by unlabeled hLIF, but the higher $M_r$ complex showed an IC$_{50}$ value 4 times lower than that for the lower $M_r$ complexes. This indicates a higher affinity for hLIF in the ternary complex, although it was not possible to derive accurate affinity constants from this type of data.

**Evidence for a Soluble LIFRα in Human Plasma**—It ap-
peared that the soluble LIFRα in human urine was of low abundance, since the LIF binding activity was hardly detectable in unconcentrated urine (data not shown). This may be the reason that we were unable to detect the soluble LIFRα in human serum in our earlier study, which was based on detecting binding activity for LIF (17). To examine this possibility, a human plasma sample (50 ml) was loaded onto a hLIF affinity column (see “Experimental Procedures” for details), and the column eluates were then analyzed in cross-linking experiments for the presence of LIF binding activity. As shown in Fig. 8A, SDS-PAGE analysis of the cross-linked products of 125I-hLIF to the five column fractions (eluates 1–5) revealed that there were at least three LIF-binding proteins present in human plasma with Mᵋ ranging from 50,000 to 130,000 (after subtraction for the Mᵋ of the bound 125I-hLIF). Judging by the sizes of their cross-linking products with 125I-hLIF, we assumed that two of these proteins, with Mᵋ of approximately 100,000 and 50,000, respectively, corresponded to the large and small forms of gp130 detected in human urine. In two of the eluates (eluates 2 and 3), there was an additional minor band (Fig. 8A, lanes 4 and 7, indicated by the open arrow), which was similar to the extra higher Mᵋ species previously observed in the hLIF affinity column eluate (Fig. 5, lane 8). Upon the addition of the anti-hLIFRα antibody prior to cross-linking, this minor band disappeared and was replaced by an increase in intensity of the band migrating at the top of the separating gel, indicated by the solid arrow (Fig. 8A, compare lanes 4 and 6 and lanes 7 and 9), suggesting that it corresponded to the cross-linked complex between 125I-hLIF and the soluble LIFRα. There was also a similar Mᵋ complex migrating at the top of the separating gel in the affinity column eluates, which predominantly occurred in eluate 1 (Fig. 8A, lane 1). The intensity of this complex was decreased upon the addition of unlabeled hLIF (compare lanes 1 and 2) but unaffected by the addition of the anti-hLIFRα antibody (compare lanes 1 and 3), probably indicating the presence of some aggregated form of gp130 in the sample. It is also worth noting that there was relatively more of the small form of gp130 in urine than in plasma.

Plasma shLIFRα levels were determined by immunoprecipitation with anti-hLIFRα followed by Western blotting with the same antibody (Fig. 8B). A specific band of ~140 kDa was detected in these experiments (lanes 2 and 4) and, by comparison with the intensity obtained for known amounts of the lower Mᵋ recombinant shLIFRα (lanes 6–8), was estimated to be present in human plasma at ~10 ng/ml.

**Inhibitory Action of shLIFRα and shgp130 on LIF Biological Activities**—LIF was originally defined by its ability to induce macrophage differentiation in the M1 myeloid leukemic cell line (1). One of the earliest detectable actions of LIF on M1 cells is the induction of tyrosine phosphorylation and activation of the signal transducer and activator of transcription, STAT-3, which occurs rapidly. Fig. 9 shows that shLIFRα and mixtures of shLIFRα and shgp130 were able to significantly inhibit tyrosine phosphorylation of STAT-3 but that, at the concentrations used, neither form of shgp130 alone nor a mixture of soluble human urinary LIFRα and gp130 could inhibit hLIF-induced STAT-3 phosphorylation.

**DISCUSSION**

There are many examples of receptors in the hemopoietin receptor family that are found in soluble forms in body fluids (29). Because of the small amounts of recombinant receptors available, we decided to test the inhibitory action of the soluble receptors on hLIF-induced STAT-3 activation in M1 cells, which occurs rapidly. Fig. 9 shows that shLIFRα and mixtures of shLIFRα and shgp130 were able to significantly inhibit tyrosine phosphorylation of STAT-3 but that, at the concentrations used, neither form of shgp130 alone nor a mixture of soluble human urinary LIFRα and gp130 could inhibit hLIF-induced STAT-3 phosphorylation.
FIG. 9. Effects of soluble LIFRα and gp130 on hLIF-induced STAT-3 tyrosine phosphorylation in M1 myeloid cells. M1 cells were treated with either saline (−) or 1 ng of hLIF (+), together with 0.05 μg of shLIFRα, 0.45 μg of sshgp130, 0.225 μg of sshgp130, a mixture of 0.05 μg of shLIFRα and 0.45 μg of sshgp130, a mixture of 0.05 μg of shLIFRα and 0.225 μg of sshgp130, or a mixture of soluble human urinary LIFRα and gp130, respectively, in a total volume of 1 ml. The total cellular proteins were analyzed by Western blotting as described under “Experimental Procedures.” The upper panels show the level of phosphorylated STAT-3, and the lower panels show the level of total STAT-3.

(30, 31). In the IL-6 receptor subfamily, these include IL-6 receptor α-chain (IL-6Rα) in human urine (32) and serum (33), LIFRα in mouse serum (17, 18), CNTF receptor α-chain in human cerebrospinal fluid (34), and gp130 in human serum (19). Soluble receptors have been implicated in both enhancing and reducing the biological effects of their cognate ligands. For example, the complex of soluble IL-6Rα and IL-6 is capable of interacting with cell surface gp130 to trigger a variety of biological responses (9), while a naturally occurring soluble form of gp130 in human serum has been implied to serve as a negative regulator in vivo of the signaling mediated by the IL-6-soluble IL-6Rα complex (19), and soluble LIFRα in mouse serum also acts exclusively as an inhibitor of LIF signaling (17, 18).

Here, we describe the presence in normal human urine and plasma of a soluble form of LIFRα and two distinct truncated forms of soluble gp130 (the large and small forms) that can bind hLIF specifically. To our knowledge, this is the first report describing a naturally occurring soluble form of LIFRα in human biological fluids as well as describing a small form of soluble gp130 capable of binding LIF directly and specifically. The large form of the urinary soluble gp130 with a Mr of approximately 100,000 was similar to the previously described soluble form of gp130 in serum with a Mr of 90,000–110,000 (19). The previous detection of the soluble gp130 in serum was facilitated by the anti-human gp130 mAb AM64 (19). We have demonstrated in this study that mAb (AM64) could not recognize the soluble form of gp130 lacking FN III modules. This may be one of the reasons for the earlier inability (19) to detect the small form of gp130, which appeared to be also present in human plasma. Based on the similar sizes of the cross-linked complexes between 125I-hLIF and sshgp130 and between 125I-hLIF and the small form of urinary gp130, as well as the results obtained from the analyses with the anti-gp130 mAbs, we concluded that the small form of the urinary gp130 was likely to contain only the hemopoietin domain and was missing all or almost all of the three FN III modules.

Recently, an alternatively spliced mRNA encoding a soluble form of human gp130 was described from bone mononuclear cells (35). This transcript would encode a form of gp130 truncated within one base pair of the transmembrane domain and would correspond to the long form described here and elsewhere (19). Whether the short form is encoded by a separate transcript or by posttranslational processing of the longer form is currently unclear. It is of interest, however, that three alternative transcripts encoding soluble human LIFRα have been described from liver, placenta, and choriocarcinoma cell line (36). One possibility may be that the FN III domains of the long form allow it to be sequestered at tissue sites and that proteolytic cleavage to generate the short form could serve as an additional control point to regulate circulatory levels of bioactive forms of soluble gp130. Further experiments are required to address this issue.

The recombinant shLIFRα containing the two hemopoietin domains and the intervening Ig-like domain, but lacking all three FN III modules, was shown to be sufficient for hLIF binding, a finding consistent with previous results (28). In the presence of hLIF, this shLIFRα was also capable of forming ternary complexes with both the recombinant gp130 lacking all three FN III modules (sshgp130) and the naturally occurring small form of gp130, probably also lacking all three FN III modules. These findings were in agreement with a previous mutagenesis study of gp130, which demonstrated that only the membrane distal half of gp130, consisting of the Ig-like domain and hemopoietin domain, was responsible for the formation of a ternary complex with IL-6 and the IL-6Rα (37). The exact roles of the three FN III modules in ternary complex formation of these hemopoietin receptors still remain to be determined.

The ternary complexes of shLIFRα with both forms of the recombinant and the urinary gp130 in the presence of hLIF appeared to be heterotrimeric, which agreed with our previous finding that hLIF could form a cross-species heterotrimetric complex with soluble mouse LIFRα and human gp130 in solution (23) but differed from the IL-6-IL-6Rαgp130 and CNTF-CNTF receptor α-chain-gp130-LIFRα complexes, which were hexameric (38–40).

At the concentrations used, the shLIFRα displayed significant inhibition of hLIF-induced STAT-3 phosphorylation in M1 cells, but both forms of soluble gp130 were ineffective. Moreover, no significant increase in inhibition was observed when shLIFRα was mixed with either form of gp130.

These data are in agreement with previous data indicating that shLIFRα acts exclusively as a LIF antagonist (17, 18) but contrast with previous reports that soluble gp130 inhibited the biological actions of IL-6 on Kaposi’s sarcoma cells (41) as well as the proliferative actions of LIF and OSM on TF-1 erythroleukemia cells (22). The reason for these differences is probably the use in the latter study of a dimeric form of soluble gp130 at considerably higher concentrations than those used here (approximately 6 μg/ml compared with 0.45 μg/ml). This is probably also the reason that the soluble human urinary LIFRα and gp130 failed to inhibit STAT-3 phosphorylation in M1 cells stimulated by hLIF (Fig. 9). Nevertheless, taken together, all of these observations suggest that shLIFRα and sshgp130 in serum and urine would serve to act as inhibitors of LIF, OSM, IL-6, CNTF, and IL-11 action. Although the concentrations of these receptors in normal serum appear insufficient to inhibit these cytokines, it is likely that some biological responses may elevate the concentrations of the receptors to a level that is able to suppress the action of these proinflammatory cytokines. For
example, the concentration of shLIFRα detected in normal human plasma (~10 ng/ml) was only 5-fold lower than the dose that gave significant inhibition of LIF-stimulated STAT-3 phosphorylation in M1 cells. Consequently, it will be of some interest to determine stimuli that result in increased secretion and circulatory levels of soluble hLIFRα and gp130.

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