INTERLEUKIN-6 (IL-6) is a pleiotropic cytokine that acts on a wide variety of cells, exerting growth promotion, growth inhibition, and specific gene expression sometimes accompanied by cellular differentiation. IL-6 exerts multiple functions through a specific receptor system. It consists of two membrane proteins, a ligand-binding receptor (IL-6R) and a nonbinding signal transducer (gp130), both of which belong to the cytokine receptor family. Binding of IL-6 to IL-6R triggers the association of IL-6R and gp130, forming a high-affinity IL-6-binding site, and gp130, in turn, transduces the signal.

Genetically engineered recombinant soluble IL-6R (rsIL-6R) lacking transmembrane and cytoplasmic regions can bind IL-6 and mediate the IL-6 signal by associating with membrane-anchored gp130 in vitro. In addition to the recombinant study, naturally produced sIL-6R was reported to be present in human urine and serum. sIL-6R purified from human urine was shown to enhance the IL-6-induced DNA synthesis in mouse plasmacytoma cells, and an enhanced level of sIL-6R was detected in sera of human immunodeficiency virus (HIV)-positive patients. As for the other component of the IL-6R complex, gp130, presence of its soluble form (sgp130) in serum or urine has not been reported yet. At least in vitro, recombinant soluble gp130 (rgp130) lacking transmembrane and cytoplasmic regions has been prepared and shown to associate with the IL-6/sgp130 complex. Thus, rgp130 may have a potential to inhibit the function of IL-6 by competing with membrane-anchored gp130 for the IL-6R association. If sgp130 is present in the biologic fluid such as serum, it is possible that such a molecule has a physiologic role in regulating IL-6 functions.

gp130 has recently been shown to be involved in the signaling processes of oncostatin M (OSM), leukemia inhibitory factor (LIF), and ciliary neurotrophic factor (CNTF), in addition to those of IL-6. This is based on the results that cellular responses initiated by either of these four cytokines are inhibited completely by the addition of anti-gp130 monoclonal antibodies (MoAbs), and gp130 is rapidly tyrosine-phosphorylated after stimulation by these cytokines. Thus, it would also be of interest to examine the effect of sgp130 on the functions of these cytokines.

In this study, we show that naturally produced sgp130 is present in human serum and that this serum sgp130 becomes associated with serum sIL-6R when rIL-6 is added. We further indicate that natural sgp130 contained in human serum inhibits the IL-6 action mediated by serum sIL-6R. In addition, we show that the inhibitory nature of sgp130 can be extended to the function of OSM, LIF, and CNTF that share gp130 as a signal transducing receptor component. Our present study implies that serum soluble forms of gp130 may have a potential to inhibit signals through gp130 in vivo.

MATERIALS AND METHODS

Antibodies. Antihuman gp130 MoAb (AM64) and antihuman IL-6R MoAb (MT18) were obtained as previously described.

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Polyclonal rat anti-human gp130 Ab and polyclonal rabbit anti-human IL-6R were prepared by immunizing recombinant human sgp130 or recombinant human sIL-6R, respectively. Alkaline phosphatase-conjugated antirat IgG Ab and antirabbit IgG were purchased from Tago (Burlingame, CA). Antihamster gp130 MoAb (AM64) was digested by pepsin-coupled beads (Pierce Chemical Co, Rockford, IL) and passed through a protein-A-sepharose column to obtain F(ab)Ь. Its purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Cell proliferation assay. A mouse pro-B-cell line transfected with human gp130 cDNA (BAF-130 cells) was prepared and maintained as previously described.13 BAF-130 was incubated in culture medium (RPMI 1640, 10% fetal calf serum [FCS], 0.1 mg/mL streptomycin, 100 U/mL penicillin G) for 18 hours in 96-well microplates (5 × 10³ cells/mL, 0.1 mL/well) with varying concentrations of rIL-6 in the presence of human serum (25% vol/vol) that was preabsorbed with anti-IL-6R MoAb (MT18), anti-gp130 MoAb (AM64), or control MoAb-conjugated beads. In some experiments, BAF-130 cells were incubated in culture medium with 250 ng/mL of rIL-6, 75 ng/mL of sIL-6R, and various concentrations of rsgp130. The cells were pulse-labeled with [³H]-thymidine (1.25 μCi/well) for 6 hours and harvested on glass filters by an automatic cell harvester. Incorporated radioactivities were measured with a scintillation counter.

A human erythroleukemic cell line TF1 was kindly provided by Dr T. Kitamura (DNAX, Palo Alto, CA) and maintained as previously described.10 TF1 cells were incubated in culture medium (RPMI 1640, 10% FCS, 0.1 mg/mL streptomycin, 100 U/mL penicillin G) for 48 hours in 96-well microplates (2 × 10³ cells/mL, 0.1 mL/well) with varying concentrations of rsgp130 in the presence of 4 ng/mL of IL-6 and 75 ng/mL of sIL-6R, 1 ng/mL of OSM, 60 U/mL of LIF, 4 ng/mL of CNTF, and 1 ng/mL of recombinant sCNTF receptor (sCNTFR), or 20 ng/mL of erythropoietin (EPO). The cells were pulse-labeled with [³H]-thymidine (1 μCi/well) for 6 hours and harvested on glass filters by an automatic cell harvester. Incorporated radioactivities were measured with a scintillation counter.

Factors. All the factors were pure recombinant molecules as previously described except LIF, which was purchased from Amrad Corp (Victoria, Australia) and was expressed in units per milliliter.14

Immunoprecipitation and Western blotting analysis. Human serum was immunoprecipitated with Sepharose 4B beads (Pharmacia LKB Biotechnology, Uppsala, Sweden) coupled with anti-human gp130 MoAb (AM64), antihamster IL-6R MoAb (MT18), or control MoAb. After washing the beads four times with phosphate-buffered saline (PBS) containing 0.05% Tween-20, the immunoprecipitates were analyzed by SDS-PAGE under a reducing condition on 4% to 20% gradient polyacrylamide gel and were electroblotted to nitrocellulose membranes. Membranes were incubated with polyclonal rat anti-gp130 Ab. Bound Ab was detected by alkaline phosphatase-conjugated antirat IgG (100 µL/mL) and subsequent development with phosphatase substrate (Sigma-104; Sigma Chemical Co, St Louis, MO). Absorbance values at 405 nm obtained with the rsgp130 standards were used to construct a standard curve for the calculation of human sgp130 concentration.

ELISA for sgp130 capable of associating with the IL-6/sIL-6R complex was performed as follows. Wells coated with F(ab)Ь fragments of AM64 (100 µL of 2.5 µg/mL in 0.1 mol/L NaHCO₃, pH 9) at 4°C for 8 to 15 hours. The wells were incubated with blocking solution (200 µL of 0.05 mol/L Tris-Cl, pH 8.1, 1 mmol/L MgCl₂, 0.15 mol/L NaCl, 1% bovine serum albumin [BSA], 0.05% Tween-20) at room temperature for 3 hours. Test samples or the standards (rsgp130) diluted in blocking solution were added to the wells (100 µL/well) and incubated for 1.5 hours at room temperature. The wells were washed three times with PBS containing 0.05% Tween-20 and incubated with polyclonal rat anti-human gp130 Ab (100 µL of 2.5 µg/mL) for 1.5 hours at room temperature. The bound Ab was detected by alkaline phosphatase-conjugated antirat IgG (100 µL/mL) and subsequent development with phosphatase substrate (Sigma-104; Sigma Chemical Co, St Louis, MO). Absorbance values at 405 nm obtained with the rsgp130 standards were used to construct a standard curve for the calculation of human sgp130 concentration.

Measurement of the rIL-6/sIL-6R/rgsp130 ternary complex using radiolabeled IL-6. ¹²⁵I-labeled rIL-6 with a specific binding activity of 1.8 × 10⁶ cpm/ng was prepared by using Bolton-Hunter reagent (NEN, Boston, MA) as previously described.13 ¹²⁵I-labeled rIL-6 (100 ng/mL), sIL-6R (75 ng/mL), and increasing concentrations of rsgp130 were incubated in binding solution (100 µL of RPMI 1640 containing 25 mmol/L HEPES, pH 7.4, 10 mg/mL BSA, and 0.05% NaN₃) for 1 hour at room temperature. The reaction mixtures were subjected to immunoprecipitation with anti-IL-6R MoAb (MT18)-conjugated or anti-gp130 MoAb (AM64)-conjugated Sepharose beads. After washing the beads four times with PBS containing 0.05% Tween-20, radioactivities of immunoprecipitates were measured with a gamma counter.

RESULTS

Detection of serum sgp130 that can associate with the IL-6/sIL-6R complex. A presence of soluble IL-6R in human serum and urine that can bind IL-6 and enhance IL-6-induced plasmacytoma cell proliferation has been described.6,7 We examined whether the IL-6 signal-transducing receptor component, gp130, is also present in a soluble form in human serum. Serum was immunoprecipitated with anti-gp130 MoAb-conjugated beads, and the precipitates were analyzed by SDS-PAGE and subsequent immunoblotting with polyclonal anti-gp130 Ab. As shown in Fig 1, two species of sgp130 with molecular weights (Mr) of around 90 and 110 Kd were observed (lane 2). To see a role of naturally produced serum sgp130, it has to be examined whether such proteins are capable of associating with IL-6R. For this purpose, we used genetically engineered sIL-6R (sIL-6R), which had been shown to associate with gp130 in the presence of IL-6. Human serum was mixed with 1 µg/mL each of rIL-6 and rsIL-6R, incubated for 1 hour at room temperature, and immunoprecipitated with anti-IL-6R MoAb-conjugated beads. The immunoprecipitates were subjected to SDS-PAGE, followed by immunoblotting with polyclonal anti-gp130 Ab. As observed in lane 4, both of the above-mentioned two species of sgp130 were found in the anti-IL-6R immunoprecipitates, but they were not found in the absence of rIL-6 (lane 3). The results showed that these serum sgp130 molecules were associated with the rIL-6/sIL-6R complex. We then examined whether naturally produced sIL-6R and sgp130 in serum could actually associate with each other under a condition in which the serum included a substantial level of IL-6. Human serum usually
contains undetectable amount of IL-6. The serum was mixed with 0 or 1 μg/mL of rIL-6 and incubated for 1 hour at room temperature. Immunoprecipitates with anti–IL-6R MoAb-conjugated beads were analyzed by immunoblotting with polyclonal anti-gp130 Ab. Both Mr species of sgpl30 were detected in the presence of rIL-6 (lane 6), but not in its absence (lane 5), suggesting that serum sIL-6R and sgpl30 were present uncomplexed in healthy human serum because of the lack of IL-6, and that they, if not all, would become associated when the serum IL-6 level was upregulated under a certain condition such as inflammation.

**Measurement of natural sgpl30 in human serum.** We then determined the concentrations of sgpl30 in human serum by the two ELISA systems. The first one was the sandwich ELISA established with the use of F(ab)2 fragments of anti-gp130 MoAb for catching and polyclonal anti-gp130 Ab for detection. The standard curve was obtained by using serially diluted rsgp130 (Fig 2A). As shown in Fig 2B (column a), 390 ± 72 ng/mL of sgpl30 was present in sera of healthy volunteers (n = 10). These values were sgpl30 specific, because they became almost negligible when the detecting polyclonal anti-gp130 Ab was substituted by the control Ab (column b). To see how much of serum sgpl30 can associate with the IL-6/sIL-6R complex, serum sgpl30 was assayed by another ELISA that can measure the association of sgpl30 with sIL-6R (see Materials and Methods for details). Figure 2C shows the standard curve obtained by using serially diluted rsgp130. As shown in Fig 2D, sgpl30 possessing a capability to associate with the rIL-6/sIL-6R complex was detected at 330 ± 95 ng/mL (column a) in the same set of sera used in the above experiments. To see the specificity of this ELISA system, rIL-6 (column b) or rsIL-6R (column c) was omitted from the ELISA process, or the detecting polyclonal anti–IL-6R Ab was substituted by control Ab (column d) in this ELISA. In the experiment in column e, the test sera were preabsorbed with anti-gp130 MoAb-conjugated beads. All of these measurements showed that the values were almost negligible, whereas preabsorption of the sera with control MoAb-conjugated beads did not influence the measurements of sgpl30 (column f). Comparing this value with the one obtained by the sandwich ELISA, it was suggested that 86% of sgpl30 in human serum had a capacity to form a ternary complex with sIL-6R together with IL-6. As for the concentrations of sIL-6R in human serum, the value of 75.5 ng/mL (n = 5) has been reported by Honda et al., and our recent measurements by the sandwich ELISA with the use of F(ab)2 fragments of anti–IL-6R MoAb for catching and polyclonal anti–IL-6R Ab for detection showed that comparable concentrations (79 ± 15 ng/mL, n = 10) of sIL-6R existed in the sera used for measuring sgpl30 in this section. Based on these observations, the function of sgpl30 was examined by using 75 ng/mL of rsIL-6R in the present work.

**Inhibition of the sIL-6R–mediated IL-6 signal by serum sgpl30.** BAF-130 is a mouse pro–B-cell line-derived transfectant that expresses human gp130 but no endogenous mouse IL-6R and gp130. To examine the function of serum sgpl30, BAF-130 cells were cultured under various conditions indicated in Fig 3 and DNA synthesis was measured. As shown in Fig 3A, although rIL-6 alone (column b) or serum alone (column c) showed no effect on BAF-130 cells, the addition of rIL-6 into the serum induced DNA synthesis in BAF-130 cells (column d). Deprivation of sIL-6R from the serum by anti–IL-6R MoAb-conjugated beads (column e) completely abolished this effect, indicating that sIL-6R in serum could mediate the IL-6 signal. In contrast, the DNA synthesis induced in BAF-130 cells by the rIL-6-supplemented human serum was increased by deprivation of sgpl30 from the serum by anti-gp130 MoAb-conjugated beads (compare columns d and f), suggesting that sgpl30 in the serum possessed the ability to negatively regulate the sIL-6R–mediated IL-6 signal. As shown in Fig 3B, the serum sIL-6R–mediated DNA synthesis in BAF-130 cells increased in accordance with the concentration of exogenously added rIL-6, and the inhibitory effect of serum sgpl30 was indicated at each dose of rIL-6 because the depletion of sgpl30 from the serum always made the [3H]–thymidine uptake higher. To further confirm the negative regulatory effect of sgpl30 on the DNA synthesis in BAF-130, various concentrations of genetically engineered rsgp130 were added to the cells together with rIL-6 and rsIL-6R. As shown in Fig 3C, DNA synthesis in BAF-130 cells was reduced in accordance with the concentration of rsgp130.

One-half of the IL-6–occupied rsIL-6R molecules became complexed with rsgp130 at its concentration of 10^{-8} mol/L. Because the inhibitory effect of naturally produced or genetically engineered sgpl30 appeared to be insufficient to completely block the sIL-6R–mediated IL-6 signal even at a concentration of 2 μg/mL (Fig 3C), the efficiency of sgpl30 to associate with the IL-6/sIL-6R complex was likely to be low. Thus, it was examined as to how efficiently the rsgp130 molecule could be associated with
the IL-6/sIL-6R complex to form a ternary complex. In the experiment shown in Fig 4, rsIL-6R at a concentration of 75 ng/mL, which is similar to the sIL-6R level in human serum, was mixed with various concentrations of rsgp130 and a fixed amount of $^{125}$I-labeled rIL-6. Each reaction mixture was divided into two and immunoprecipitated with either anti-IL-6R MoAb-conjugated beads or anti-gp130 MoAb-conjugated beads. Radioactivity of $^{125}$I-labeled rIL-6

Fig 3. Biologic functions of serum sIL-6R and sgp130. (A) BAF-130 cells were cultured with medium alone (column a) or medium supplemented with rIL-6 (column b), human serum (column c), rIL-6 plus human serum (column d), rIL-6 plus human serum deprived of sgp130 by anti-gp130 MoAb-conjugated beads (column e), or rIL-6 plus human serum deprived of sIL-6R by anti-IL-6R MoAb-bearing beads (column f). After 18 hours of culture in 96-well microplates, cells were pulse-labeled with $[^3]$H]-thymidine. rIL-6 and test serum was used at a concentration of 250 ng/mL and 25% vol/vol, respectively. Averages of triplicate measurements of incorporated radioactivity are shown with SD (vertical bars). (B) BAF-130 cells were cultured with rIL-6 (0 to 500 ng/mL) in the presence of 25% vol/vol human serum that was preabsorbed by beads conjugated with anti-gp130 MoAb (a), control MoAb (b), or anti-IL-6R MoAb (c). DNA synthesis was measured as above. (C) BAF-130 cells were cultured with rIL-6 (250 ng/mL), rIL-6R (75 ng/mL), and various concentrations of rsgp130.
in the anti-IL-6R or anti-gp130 precipitates was measured. The radioactivity in the former immunoprecipitates represents total sum of the amount of the rIL-6/sIL-6R binary complex and the rIL-6/sIL-6R/rsgp130 ternary complex, whereas the radioactivity in the latter immunoprecipitates represents the amount of the rIL-6/sIL-6R/rsgp130 ternary complex. Figure 4 shows that in the presence of rsgp130 at a concentration of 0.33 μg/mL, which is almost the same concentration as observed in human serum, approximately 25% of the totally formed rIL-6/sIL-6R complex was bound to rsgp130. Nearly one-half of the IL-6-occupied sIL-6R was bound to rsgp130 when 1 μg/mL (10^-8 mol/L) of rsgp130 was added to the reaction mixture.

**Effect of sgp130 on biologic functions of OSM, LIF, and CNTF.** Human erythroleukemic cell line TF1 is known to initiate DNA synthesis in response to several cytokines, including EPO, IL-6, OSM, LIF, and CNTF, together with sCNTFR. To examine the effect of sgp130 on this biologic response induced by these cytokines, various concentrations of rsgp130 were added to TF1 cells together with the cytokines. As shown in Fig 5, DNA synthesis induced by OSM or the CNTF/srsCNTFR complex was significantly inhibited by rsgp130 in accordance with its concentration. The inhibitory effect of rsgp130 on the DNA synthesis induced by LIF or the IL-6/sIL-6R complex was not so prominent.

**DISCUSSION**

In this study, we have shown the existence of naturally produced sgp130 as well as sIL-6R in human serum. The previous studies have shown that natural sIL-6R potentiates the IL-6 actions on cells already responsive to IL-6, whose surface phenotype is IL-6R^+/gp130^+. In addition to this, the present study indicates that serum sIL-6R, in concert with IL-6, can also act on cells not normally responsive to IL-6; sIL-6R in the serum supplemented with rIL-6 initiates DNA synthesis in BAF-130 cells (IL-6R^+/gp130^+) by forming an IL-6/sIL-6R complex and interacting with membrane-anchored gp130. In relation to the function of sIL-6R, natural killer cell stimulatory factor (NKSF or IL-12) should be noted. NKSF is a disulfide-bonded heterodimeric cytokine consisting of two subunits, p35 and p40. These two subunits share amino acid sequence homology to the IL-6 molecule or the extracellular domain of IL-6R, respectively. Thus, NKSF is structurally similar to the complex of IL-6 and sIL-6R molecules, but the latter complex is formed noncovalently. The heterodimeric cytokine NKSF is inactive when each subunit is present individually. Although specific receptor for NKSF is unidentified, it is postulated to be structurally similar to gp130, from the analogy to the observation with the IL-6/sIL-6R complex, which mediates its signal by associating with membrane-anchored gp130.

Some of soluble cytokine receptors were reported to competitively inhibit the functions of their specific ligands, eg, IL-1, IL-4, platelet-derived growth factor, and fibroblast growth factor, as opposed to the observation with sIL-6R, which enhances the IL-6 actions. The functional differ-
ence between these two types of soluble receptors may be due to the nature of these receptors; IL-6R associates extra-cellularly, in the presence of IL-6, with the signal-transducing receptor component gp130 to mediate the IL-6 signal, and thus its cytoplasmic region is dispensable. In contrast, most other receptors appear to require their own cytoplasmic regions for signals.

As for serum sgp130, this protein is indicated to have capability to associate with the ligand-binding subunit of the IL-6R system. Indeed, serum sgp130 can be complexed with serum sIL-6R in the presence of IL-6 (lane 6 in Fig 1). Serum sgp130 shows an inhibitory effect (~22% inhibition) on the DNA synthesis of BAF-130 cells induced by the IL-6/sIL-6R complex (compare columns d and f in Fig 3A). By using rsgp130, its inhibitory effect on the DNA synthesis induced in BAF-130 cells by rIL-6 plus nsIL-6R (at physiologic concentrations) was also confirmed (Fig 3C). From these observations, serum sgp130 is implied to serve as a negative regulator in vivo of the signal mediated by the IL-6/sIL-6R complex on the cells or tissues with a surface phenotypic of IL-6R^+^/gp130^+^, like BAF-130 cells. Considering the relatively high level of sgp130 (390 ng/mL, Fig 2) compared with the sIL-6R level (75.5 ng/mL) in serum, it is suggested that sIL-6R levels, and not sgp130 levels, might be limiting with regard to maximal effectiveness. Concerning the effect of sgp130 on the IL-6R^+^/gp130^+^ cells normally responsive to IL-6, IL-6-induced DNA synthesis in human erythroleukemic TF1 cells was inhibited only a little (12% inhibition; Fig 5) even by the addition of 4.5 μg/mL of rsgp130 (12-fold higher concentration of natural sgp130 in serum). Based on these results, the inhibitory effect of sgp130 on the IL-6-induced biologic response of the IL-6R^+^/gp130^+^ cells was weaker than that on the IL-6R^+^/gp130^+^ cells. A possible explanation of this difference could be that the association affinity of the membrane-anchored gp130 to the IL-6/sIL-6R complex might be weaker than that to the IL-6/membrane-anchored IL-6R complex. Thus, sgp130 might interfere with the former association more efficiently. In this relation, it will be interesting to examine whether soluble forms of the receptor components (IL-6R and gp130) reconstitute a high- or low-affinity binding site and to determine these affinities. This would help us to explain the above difference in the effectiveness of sgp130.

Recently, gp130 is demonstrated to be involved in the signaling processes of OSM, LIF, andCNTF, in addition to those of IL-6. By using TF1 cells, sgp130 was shown to have inhibitory effect on the signals of OSM and the CNTF/rsCNTFR complex. However, the inhibitory effect of sgp130 on the TF1 response induced by IL-6 and LIF was not so prominent. One possible explanation of these results is that there might be a difference in the association affinity of gp130 protein to the other component of the receptor complex for each of the above four cytokines. These affinity values remain to be tested, and such values would explain the different effectiveness of sgp130 on different cytokines.

Two possible mechanisms have been suggested for the production of soluble cytokine receptors: (1) formation of alternatively spliced mRNA species encoding soluble form of receptor, as reported for IL-4R, IL-5R, IL-7R, IL-9R, and granulocyte colony-stimulating factor (G-CSF)-R; and (2) proteolytic shedding from membrane-anchored form, as suggested for tumor necrosis factor (TNF)-R. In the case of sIL-6R, the mRNA species encoding a soluble form was recently detected in some cell lines. The polymerase chain reaction with oligonucleotide primers acrossing the transmembrane domain of IL-6R produced two different fragments: one possesses the transmembrane domain as expected from the intact IL-6R cDNA structure and the other lacks this domain. The mechanism of the production of sIL-6R found in serum and the tissues releasing sIL-6R remain to be clarified. Two species of sgp130 with a Mr of around 90 and 110 Kd were found in human serum. There might be two different proteolytic cleavage sites, or two different alternatively spliced transcripts encoding transmembrane domainless gp130 protein might exist. In relation to the regulating mechanism of sgp130 production, although the elevated level of sIL-6R (170 ng/mL, n = 11) was found in HIV-infected patients' sera, serum sgp130 levels under disease conditions are not known at this moment.

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REFERENCES

inhibition of hematopoietic and neurotrophic cytokines by blocking the interleukin 6 signal transducer gp130. Proc Natl Acad Sci USA 89:10998, 1992


Soluble forms of the interleukin-6 signal-transducing receptor component gp130 in human serum possessing a potential to inhibit signals through membrane-anchored gp130

M Narazaki, K Yasukawa, T Saito, Y Ohsugi, H Fukui, Y Koishihara, GD Yancopoulos, T Taga and T Kishimoto