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Functional inhibition of hematopoietic and neurotrophic cytokines by blocking the interleukin 6 signal transducer gp130

(signal transduction/receptor/cytokine receptor family)

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ABSTRACT **Functional pleiotropy and redundancy are characteristic features of cytokines. To understand the signaling mechanisms of such cytokines, we have proposed a two-chain interleukin (IL) 6 receptor model: IL-6 triggers the association of a ligand-binding chain (IL-6 receptor) and a nonbinding signal transducer (gp130) to form a high-affinity receptor complex, resulting in transmission of the signal by the cytoplasmic portion of gp130. This model would explain the functional redundancy of cytokines if we were to assume that gp130 interacts with several different receptor chains. Here we present data indicating that gp130 functions as a common signal transducer for IL-6, oncostatin M, leukemia inhibitory factor, and ciliary neurotrophic factor. We show that anti-gp130 monoclonal antibodies completely block the biological responses induced by all of these factors. Since leukemia inhibitory factor functions as a cholinergic differentiation factor in nerve cells, as does ciliary neurotrophic factor, these results suggest that gp130 may also play a role in the nervous system.**

Most cytokines are characterized by their pleiotropic and redundant functions; i.e., each factor exerts multiple effects in different cells and different factors can act on the same cell to induce similar effects. Some of the biological effects induced by interleukin (IL) 6 (1), a typical example of such cytokines, are also mediated by oncostatin M (OM) and leukemia inhibitory factor (LIF) [e.g., acute-phase protein induction in hepatocytes (2–5) and macrophage differentiation of mouse myeloid leukemia M1 cells (6–10)], suggesting a common signaling mechanism. The two-chain receptor model we have proposed (11, 12), in which a receptor-associated signal transducer generates the cytoplasmic signal, would be one way to explain these overlapping functions, if we assume that a signal transducer is shared by these three cytokines. This idea is supported by the observation that the IL-6-signal transducer gp130 was found to convert the LIF receptor (LIF-R) into a high-affinity binding site for OM and LIF (13, 14). LIF functions as a cholinergic differentiation factor in nerve cells (15) as does ciliary neurotrophic factor (CNTF), another cytokine functioning in the nervous system (16, 17). CNTF-R, which is anchored to the membrane by a glycosyl-phosphatidylinositol linkage, is structurally very similar to IL-6-R except that it possesses no transmembrane and cytoplasmic regions (18). This is reminiscent of the observation that the extracellular soluble(s) form of IL-6-R (sIL-6-R) mediates the IL-6 signal transduction through cell surface gp130 (11, 12, 19). LIF- and CNTF-induced tyrosine phosphorylation of gp130 was observed (20) as had been shown for IL-6 (21).

Thus, it has been hypothesized that gp130 is involved in the biological functions of IL-6, OM, LIF, and CNTF and is not merely involved in mediating cell surface events such as high-affinity receptor complex formation and protein phosphorylation. In the present study, we show that gp130 is essential for transducing the signals of all of these factors, based on the results that anti-gp130 monoclonal antibodies (mAbs) completely block their biological functions.

MATERIALS AND METHODS

Anti-Human gp130 mAbs. Mice were immunized with recombinant human sgp130 (22) and hybridomas were obtained (T.S., K.Y., D.M., T.T., and T.K., unpublished data). Hybridomas producing anti-human gp130 mAbs GPX7, GPX22, and GPZ35 were thus established. Other mAbs (AM64 and AM66) have been described (12). The three anti-human gp130 mAbs GPX7, GPX22, and GPZ35 are likely to recognize different epitopes on gp130 or have different binding properties to gp130 because (*i*) sgp130, captured by GPZ35-precoated microtiter plates, was recognized as well by either GPX7 or GPX22 and (*ii*) GPX22 inhibited the *in vitro* association of sIL-6-R and sgp130 at a severalfold lower concentration compared to GPX7 (T.S., K.Y., D.M., T.T., and T.K., unpublished data).

BAFh130 Cell Proliferation Assay. A mouse pro-B-cell-line-derived transfectant, BAFh130, expressing human gp130 but no endogenous mouse IL-6-R and gp130, was prepared and maintained as described (12, 21). BAFh130 cells were cultured in 96-well microtiter plates (4×10^4 cells per well, 0.1–0.2 ml per well) with test samples for 18–40 hr and pulse-labeled with [^{3}H]thymidine (0.75 μCi per well; 1 Ci = 37 GBq) for 5–6 hr. Cells were harvested on glass filters and cell-associated radioactivity was measured by a scintillation counter.

TF-1 Cell Proliferation Assay. An erythroleukemia-derived cell line, TF-1 (23), was maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum and recombinant human granulocyte–macrophage colony-stimulating factor (3 ng/ml). TF-1 cells were cultured in 96-well microtiter plates (2×10^4 cells per well, 0.1 ml per well) with test samples for 40 hr and pulse-labeled with [^{3}H]thymidine (1 μCi per well) for 3.5–5 hr. Cell-associated radioactivity was measured as described above.

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Abbreviations: IL, interleukin; LIF, leukemia inhibitory factor; OM, oncostatin M; CNTF, ciliary neurotrophic factor; EPO, erythropoietin; R, receptor; s, soluble; mAb, monoclonal antibody.

Fibrinogen Production Assay. Hep3B cells were cultured in 96-well microtiter plates (1×10^4 cells per well, 0.1 ml per well) with test samples for 22 hr as described (24). Culture supernatant was assayed for fibrinogen by the ELISA using a combination of unlabeled and peroxidase-labeled goat anti-human fibrinogen antibody. Absorbance at 450 nm of the enzyme substrate was measured.

Quantitation of the IL-6-Induced Association Between sIL-6-R and sgp130. Microtiter plates were coated with anti-gp130 mAb AM64, blocked with 1% bovine serum albumin in 50 mM Tris Cl, pH 8.1/150 mM NaCl/0.05% Tween 20, and incubated with sgp130 as described elsewhere (22). After the capture of sgp130 on mAb AM64, test samples containing IL-6 and sIL-6-R were added and incubated at room temperature for 2 hr. The bound sIL-6-R was detected by sequential incubation with guinea pig anti-human sIL-6-R polyclonal antibody and alkaline phosphatase-conjugated goat anti-guinea pig IgG antibody (22). Absorbance at 405 nm of the enzyme substrate was measured.

Factors. All the factors were pure recombinant molecules except LIF, which was the concentrated supernatant from CHO cell transfectants with human LIF cDNA (25). Thus, the concentration of LIF was expressed in units/ml (26).

Immunoblot Analysis. Immunoblot analysis of the tyrosine phosphorylation of gp130 was performed as described (21) but with the following modifications. Cells were incubated in factor-free minimal essential medium (MEM) for 7 hr at 37°C and then 10 min in MEM (3×10^7 cells in 1.5 ml) containing bovine serum albumin (1 mg/ml), 20 mM Hepes (pH 7.3), 2 mM sodium vanadate, and stimulants. Cells were then solubilized in lysis buffer (150 mM NaCl/50 mM Tris · HCl, pH 8.0/1% Nonidet P-40/0.1% SDS/3 mM sodium vanadate/2.5 mM phenylmethylsulfonyl fluoride; 2 ml for 3×10^7 cells). Immunoprecipitates obtained with anti-gp130 mAb AM66 were examined by SDS/PAGE under reducing conditions and subsequently by immunoblot analysis with a rabbit anti-phosphotyrosine polyclonal antibody.

RESULTS AND DISCUSSION

Inhibition of IL-6 Functions by Anti-gp130 mAbs. To test the hypothesis that gp130 is involved in the biological functions of IL-6, OM, LIF, and CNTF, we prepared anti-human gp130 mAbs by immunizing mice with sgp130. Each mAb was tested for its ability to block IL-6 functions by using a mouse pro-B-cell-line (BAF-B03; IL-6-R$^-$, gp130$^-$)-derived transfectant, BAFh130, expressing human gp130 (12). The IL-6–sIL-6-R complex has been shown to associate with gp130 in BAFh130 cells, resulting in tyrosine phosphorylation of the gp130 molecule and transmission of a signal to initiate cellular DNA synthesis (21). As shown in Fig. 1*A*, DNA synthesis in BAFh130 cells induced by IL-6 plus sIL-6-R was blocked by the anti-human gp130 mAbs GPX7, GPX22, and GPZ35. We examined whether this effect was due to the inhibition of the IL-6-triggered association between IL-6-R and gp130. The ELISA that can quantitate the association of sIL-6-R with sgp130 (22) was used to assay the effect of these mAbs on this association. As shown in Fig. 1*B*, all three of the mAbs inhibited this association, indicating that these mAbs inhibited the IL-6-mediated DNA synthesis in BAFh130 cells by blocking the interaction between sIL-6-R and cell-surface gp130.

Inhibition of IL-6-, OM-, or LIF-Induced Fibrinogen Production in Hep3B by Anti-gp130 mAbs. IL-6, OM, and LIF are known to induce acute-phase proteins in hepatocytes (2–5). As shown in Fig. 2*A*, Hep3B cells produced one such acute-phase protein, fibrinogen, in response to these cytokines. Fibrinogen induction by LIF, even at a saturating concentration, was much lower than that by IL-6 or OM. This might be due to the smaller number of LIF-R molecules

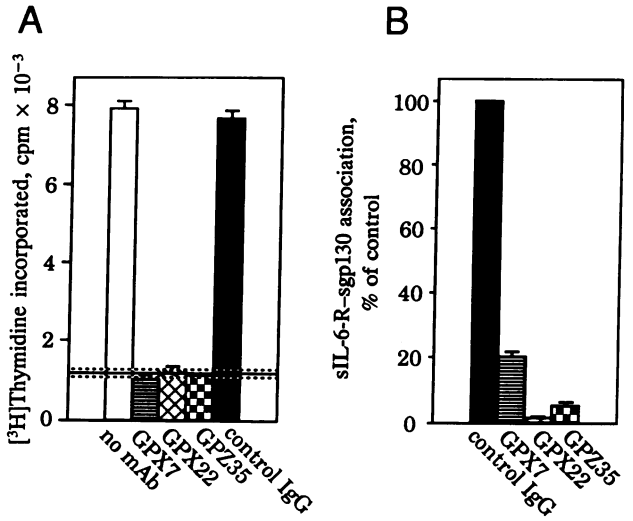

FIG. 1. (*A*) Inhibition of gp130-mediated DNA synthesis in BAFh130 cells. Cells were cultured in triplicate for 40 hr (4×10^4 cells per well, 0.2 ml per well) with recombinant IL-6 (250 ng/ml) and sIL-6-R (250 ng/ml) and the antibodies (1 μg/ml) as indicated and pulse-labeled with [^{3}H]thymidine for 6 hr. Data represent the cell-associated radioactivity (average ± SD; horizontal solid and broken lines indicate the average ± SD, respectively, obtained from the cells cultured in medium alone). (*B*) Inhibition of association between sIL-6-R and sgp130. Microtiter plates were coated with anti-gp130 mAb AM64. After the capture of sgp130 on AM64, IL-6 (5 μg/ml), sIL-6-R (5 μg/ml), and antibodies (2.5 μg/ml) as indicated were added. sIL-6-R bound to sgp130 was detected by the ELISA. Data represent the ratio of the absorbance of the enzyme substrate to the one obtained with control IgG and are expressed as percent of control.

compared to IL-6-R and gp130. In another hepatoblastoma, HepG2, comparable amounts of fibrinogen were induced by IL-6, OM, or LIF (5). To examine the involvement of gp130 in factor-mediated fibrinogen induction, Hep3B cells were cultured with nonsaturating doses of these cytokines plus anti-gp130 mAbs. As shown in Fig. 2*B*, fibrinogen induction was completely inhibited by anti-gp130 mAbs, indicating that the IL-6 signal transducer gp130 plays a key role in fibrinogen production mediated not only by IL-6 but also by OM and LIF.

Involvement of gp130 in the Signaling Processes of IL-6, OM, LIF, and CNTF. To further test our hypothesis, we used human erythroleukemia TF-1 cells expressing gp130, which had been shown to proliferate in response to IL-3, IL-5, IL-6, erythropoietin (EPO), granulocyte–macrophage colony-stimulating factor, and LIF (23). We examined possible responsiveness of TF-1 cells to OM and CNTF. As shown in Fig. 3*A*, TF-1 cells incorporated [^{3}H]thymidine in response to IL-6, OM, and LIF. The IL-6-induced response was enhanced by the addition of sIL-6-R, probably because there may be fewer IL-6-R molecules than gp130 on TF-1 cells and thus exogenous sIL-6-R can stimulate the otherwise unbound gp130. TF-1 cells did not respond to CNTF alone but did initiate DNA synthesis after the simultaneous addition of CNTF and sCNTF-R, indicating that CNTF plus sCNTF-R could cause biological responses in originally CNTF-unresponsive cells through cell surface molecule(s) that could presumably include gp130 (S.D. and G.D.Y., unpublished data). To examine whether all the above factor-induced biological responses in TF-1 cells were mediated through gp130, the cells were incubated with IL-6, OM, LIF, CNTF plus sCNTF-R, or EPO, each at a dose inducing cellular DNA synthesis to a similar extent, in the presence of anti-gp130 mAbs. As shown in Fig. 3*B*, TF-1 responses induced by all of these factors, except for EPO, were inhibited by GPX7 and

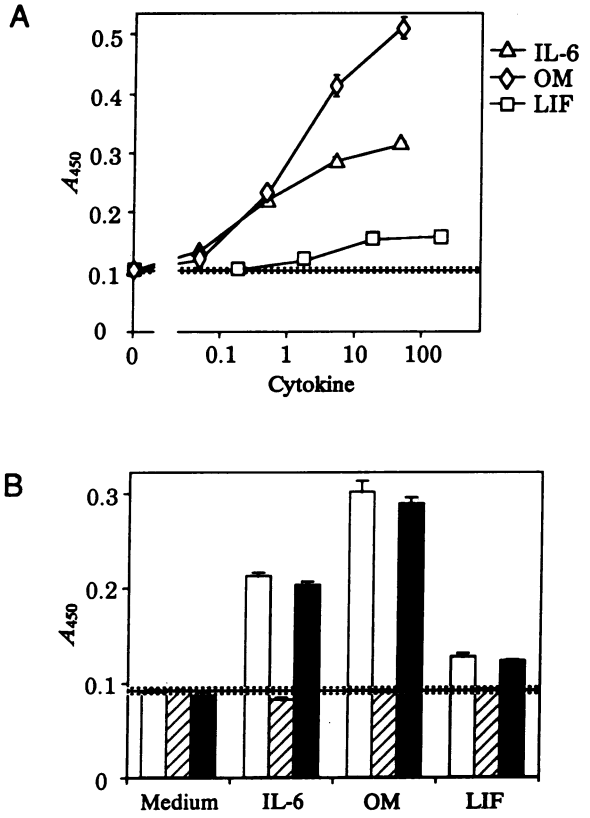

FIG. 2. (*A*) Fibrinogen induction in Hep3B by IL-6, OM, and LIF. Cells were cultured in triplicate in microtiter plates with the three cytokines for 22 hr. IL-6 and OM are expressed as ng/ml; LIF is expressed as units/ml ($\times 10^{-1}$). Absorbance (A_{450}) from the ELISA for fibrinogen is plotted (average ± SD). (*B*) Inhibition of fibrinogen induction by anti-gp130 mAbs. Hep3B cells were cultured for 22 hr in triplicate with medium alone, IL-6 (2 ng/ml), OM (2 ng/ml), or LIF (200 units/ml) with no mAb (open bars) or in the presence of a mixture of GPX7 (1 μg/ml), GPX22 (1 μg/ml), and GPZ35 (1 μg/ml) (hatched bars) or control IgG (3 μg/ml) (solid bars). A_{450} from the ELISA is shown (average ± SD). Horizontal solid and broken lines are as in Fig. 1.

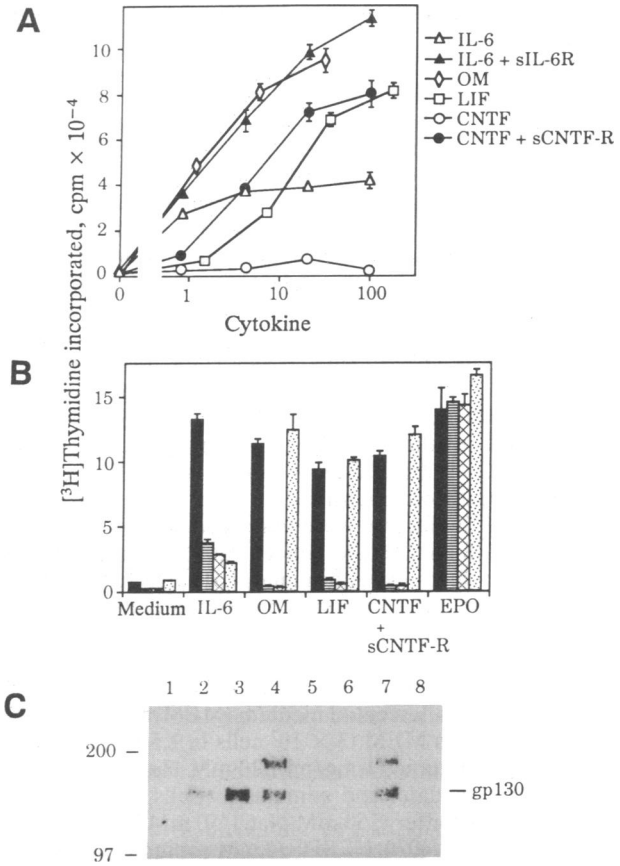

FIG. 3. (*A*) DNA synthesis induced in TF-1 cells by IL-6, OM, LIF, and the CNTF–sCNTF-R complex. Cells were cultured in triplicate as indicated for 40 hr and pulse-labeled with [^{3}H]thymidine for 3.5 hr. Each soluble receptor was used at 1 μg/ml. Cell-associated radioactivity is shown (average ± SD). (*B*) Inhibition of TF-1 responses by anti-gp130 mAbs. Cells were cultured in triplicate with IL-6 (4 ng/ml), OM (1 ng/ml), LIF (60 units/ml), CNTF (4 ng/ml) plus sCNTF-R (1 μg/ml), or EPO (20 ng/ml) in the presence of control IgG (closed bars), anti-gp130 mAb GPX7 (striped bars) or GPX22 (cross-hatched bars), or anti-IL-6-R mAb PM1 (dotted bars), each at 3 μg/ml. Incorporated radioactivity during the 5-hr pulse-labeling with [^{3}H]thymidine is indicated (average ± SD). (*C*) Factor-induced tyrosine phosphorylation of gp130 in TF-1 cells. Lysates were prepared from unstimulated cells (lane 1) or cells stimulated with IL-6 plus sIL-6-R (4 and 20 μg/ml, respectively; lane 2), OM (2 μg/ml; lane 3), LIF (40,000 units/ml; lane 4), CNTF (2 μg/ml; lane 5), sCNTF-R (3 μg/ml; lane 6), CNTF plus sCNTF-R (2 and 3 μg/ml, respectively; lane 7), and EPO (10 μg/ml; lane 8). The immunoprecipitates obtained with anti-gp130 mAb AM66 were separated by SDS/PAGE and the immunoblot was analyzed with anti-phosphotyrosine antibody. Molecular masses in kDa are indicated to the left.

GPX22. Similar results were obtained with another anti-gp130 mAb GPZ35. None of the three anti-gp130 mAbs immunoprecipitated IL-6-R or affected EPO-R-mediated TF-1 responses. Thus, it is unlikely that the inhibition caused by these mAbs is due to recognition of some common epitope present in the gp130, IL-6-R, LIF-R, CNTF-R, and EPO-R molecules that belongs to the cytokine receptor family (27). In contrast, PM1 (28), a mAb specific to IL-6-R, inhibited only the IL-6-induced responses in TF-1 cells. The results indicate that gp130 is required for the signaling of CNTF, IL-6, OM, and LIF. We next examined whether these cytokines could induce tyrosine phosphorylation of gp130 in TF-1 cells, as was shown for IL-6 in BAFh130 cells (21). As shown in Fig. 3*C*, OM, LIF, and the CNTF–sCNTF-R complex, in addition to the IL-6–sIL-6-R complex, induced tyrosine phosphorylation of gp130, supporting the idea that gp130 is involved in transducing the signals elicited by each of these factors. LIF and CNTF also induced the tyrosine phosphorylation of a protein with a molecular mass of 190 kDa. It should be noted that this 190-kDa phosphoprotein was coprecipitated with gp130, indicating a possible physical association. Similar results were recently obtained that LIF and CNTF induced the tyrosine phosphorylation of a set of proteins including gp130 and a 190-kDa protein in Ewing sarcoma EW-1 cells (ref. 20; S.D. and G.D.Y., unpublished data).

Responses of BAFh130 to IL-6, OM, LIF, and CNTF. As mentioned above, the transfectant BAFh130 initiated gp130-mediated DNA synthesis in response to the IL-6–sIL-6-R complex. BAFh130 was tested for its possible responsiveness to OM, LIF, or CNTF. As shown in Fig. 4, LIF did not induce DNA synthesis, presumably due to the absence of LIF-R in BAFh130 cells. OM at 200 ng/ml (6.7 nM) did not induce DNA synthesis in BAFh130 cells, although gp130 was observed to possess a low-affinity OM-binding capability (K_d = 7.7 nM) (13, 14). Thus, gp130 does not appear to be sufficient to transduce the OM signal, and another molecule important for signalings may possibly be missing in BAFh130 cells. This transfectant did not respond to CNTF alone or CNTF plus sCNTF-R (Fig. 4). As shown in Figs. 1 and 3*A*, gp130 in BAFh130 cells did function in response to the IL-6–sIL-6-R complex and the CNTF–sCNTF-R complex was able to

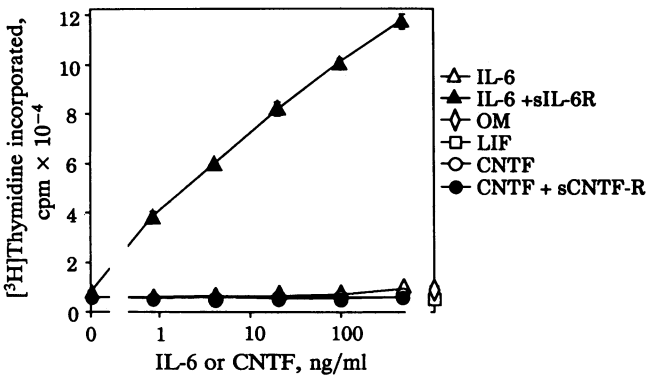

FIG. 4. Responses of BAFh130 cells to IL-6, OM, LIF, and CNTF. Cells (0.1 ml per well) were cultured in triplicate for 18 hr with various concentrations of IL-6 and CNTF either individually or with sIL-6-R (1 μg/ml) and sCNTF-R (1 μg/ml), respectively. OM at 200 ng/ml and LIF at 2000 units/ml were also present. Cell-associated radioactivity during 5-hr pulse-labeling with [3H]thymidine is shown (average ± SD).

induce a biological response in TF-1 cells that could be blocked by anti-gp130 mAbs. Thus, we imagine that gp130 by itself may not be sufficient to transduce the signal of CNTF and that another macromolecule may, therefore, be required. A possible candidate is the 190-kDa protein observed in Fig. 3*C* because in TF-1 cells (*i*) CNTF rapidly induced the tyrosine phosphorylation of both gp130 and the 190-kDa protein, whereas IL-6 induced tyrosine phosphorylation of only the former, and (*ii*) the 190-kDa phosphoprotein was found in anti-gp130 immunoprecipitates. Since the gp130-associated 190-kDa phosphoprotein was also observed in LIF-stimulated TF-1 cells and its molecular mass was comparable to that of LIF-R (29), this molecule could possibly be LIF-R as suggested (20). Although further binding and immunoprecipitation experiments remain to be done to understand the receptor complexes that interact with OM, LIF, and CNTF, the present study clearly shows that the gp130 molecule associated with each of these receptor complexes is essential for transducing their respective cytokine signals. Considering that the hematopoietic cytokine LIF also functions in nerve cells as does CNTF (15–17), this study indicates that gp130 plays a role in the nervous system and that there may be a family of neurotrophic cytokines utilizing gp130 as a signal transducer. This family may function in addition to the family of neurokines such as nerve growth factor, brain-derived neurotrophic factor, and neurotrophin 3 that utilize trk-type receptors (30–32) for their signaling.

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