Detection of Receptors for Interleukin-6, Interleukin-11, Leukemia Inhibitory Factor, Oncostatin M, and Ciliary Neurotrophic Factor in Bone Marrow Stromal/ Osteoblastic Cells

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Abstract

The functional receptor complexes assembled in response to interleukin-6 and -11 (IL-6 and IL-11), leukemia inhibitory factor (LIF), oncostatin M (OSM), and ciliary neurotrophic factor (CNTF), all involve the signal transducer gp130: IL-6 and IL-11 induce homodimerization of gp130, while the rest heterodimerize gp130 with other gp130-related β subunits. Some of these cytokines (IL-6, IL-11, and CNTF) also require a specificity-determining α subunit not directly involved in signaling. We have searched for functional receptor complexes for these cytokines in cells of the bone marrow stromal/osteoblastic lineage, using tyrosine phosphorylation of the β subunits as a detection assay. Collectively, murine calvaria cells, bone marrow-derived murine cell lines (+/+LDA11 and MBA13.2), as well as murine (MC3T3-E1) and human (MG-63) osteoblast-like cell lines displayed all the previously recognized α and β subunits of this family of receptors. However, individual cell types had different constellations of α and β subunits. In addition and in difference to the other cell types examined, MC3T3-E1 cells expressed a heretofore unrecognized form of gp130; and MG-63 displayed an alternative form (type II) of the OSM receptor. These findings establish that stromal/osteoblastic cells are targets for the actions of all the members of the cytokine subfamily that shares the gp130 signal transducer; and suggest that different receptor repertoires may be expressed at different stages of differentiation of this lineage. (*J. Clin. Invest.* 1996. 97:431–437.) Key words: gp130 • signal transduction • tyrosine phosphorylation • bone biology

Introduction

Members of the subfamily of cytokines that includes interleukin-6 and -11 (IL-6 and -11),[1] leukemia inhibitory factor (LIF), oncostatin M (OSM), and ciliary neurotrophic factor (CNTF) induce the assembly of related multi-component receptors. IL-6, CNTF, and IL-11 initially bind to receptor subunits that recognize the respective cytokine but do not participate directly in the transduction of the signal (α subunits) (1–4). LIF and OSM, on the other hand, bind to receptor components (β subunits) that are also involved in the transduction of the signal. Irrespectively of whether a cytokine binds to its α subunit or directly to a β subunit, binding of the ligand leads to the formation of gp130 homodimers or gp130 heterodimers with an additional β subunit. Ligand-induced dimerization of the β subunits initiates intracellular signaling by activating members of a family of receptor-associated tyrosine kinases, known as the Janus kinases (Jaks) (2, 5). This step induces tyrosine phosphorylation of several proteins including the β components of the receptor complex and the kinases themselves and a series of cytoplasmic proteins termed STATs (signal transducers and activators of transcription) (6, 7). Specifically, binding of IL-6 to the membrane-anchored IL-6 receptor (IL-6R) or a soluble form of this protein (sIL-6R) induces homodimerization and tyrosine phosphorylation of gp130. On the other hand, binding of LIF to its receptor (LIFRβ) induces phosphorylation and heterodimerization of LIFRβ with gp130. LIFRβ/gp130 heterodimers are also formed upon binding of CNTF to soluble or membrane-anchored receptors for CNTF (CNTFRα) (3, 8). OSM can bind directly to gp130 and induce the formation of LIFRβ/gp130 heterodimers (9, 10). Certain cells, however, express receptors that can bind OSM but not LIF and thereby exhibit unique responses to the former cytokine. This alternative OSM receptor has been termed type II, and is believed to consist of a heterodimer of gp130 and a putative OSMRβ (10). A diagrammatic illustration of the ligand–receptor complexes for this cytokine subfamily is provided in Fig. 1.

Demonstration of the fact that gp130 and LIFRβ are shared by these cytokines, the former being an obligatory β subunit for all of them, largely explains the functional pleiotropy and redundancy of the members of this subfamily (2, 5). In spite of such redundancy, however, each cytokine also has unique biologic effects. Such specificity is most probably due to the expression of different cytokine binding subunits by different cell types and/or different signal transducing components. Additionally, unique effects by each cytokine can be due to the presence of different kinases, distinct combinations of Jaks, or substrate choice by the β subunit of the receptor, in different cells (11, 12).

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1. *Abbreviations used in this paper:* CNTF, ciliary neurotrophic factor; ECL, enhanced chemiluminescence; gp130, glycoprotein 130; LIF, leukemia inhibitory factor; IL-6, interleukin-6; IL-11, interleukin-11; Jaks, Janus kinases; LIFRβ, leukemia inhibitory factor receptor β; OSM, oncostatin M; OSMRβ, oncostatin M receptor β; PVDF, polyvinylidene difluoride; sCNTFRα, soluble ciliary neurotrophic factor receptor α; sIL-6R, soluble IL-6 receptor.

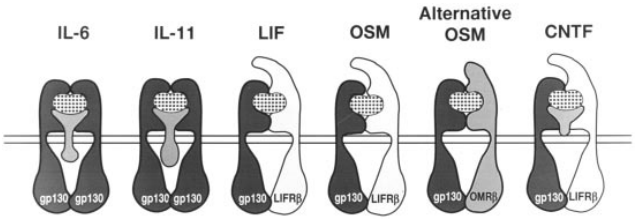

Figure 1. Illustration of the complexes formed between cytokines and components of the cytokine receptors that share the gp130 signal transducing protein. The cytokines are represented by black dotted symbols. The α subunits of the receptors for IL-6, IL-11, and CNTF are depicted in dark grey. The β subunits gp130, LIFRβ, and OSMRβ are indicated in black, white and light grey, respectively.

Earlier work from our group has revealed that IL-6 and IL-11 play a critical role in osteoclast development from its hematopoietic precursors of the bone marrow (13, 14). Moreover, altered production and responsiveness to IL-6, and perhaps to other cytokines that use gp130, appear to be key pathogenetic events in disease states characterized by increased bone resorption, such as osteoporosis (15). Besides IL-6 and IL-11, another member of the cytokine subfamily that uses gp130, namely LIF, has been shown to play a role in bone biology. Indeed, LIF receptors have been detected in stromal/osteoblastic cells and LIF potentiates the expression of alkaline phosphatase and type I collagen synthesis in these cells (16–19). Further, overexpression of LIF causes ectopic bone formation and resorption (20), and targeted disruption of the LIFRβ gene results in decreased bone volume (threefold) and increased osteoclast numbers (sixfold) (21).

Bone marrow stromal cells and osteoblasts are believed to be of the same lineage, as their phenotypic properties overlap. Indeed, bone marrow–derived stromal cell lines express phenotypic markers of osteoblasts, such as alkaline phosphatase and collage type I, and can even form calcified nodules. Conversely, osteoblasts are capable of secreting the same colony-stimulating factors and cytokines that are secreted by stromal cells, including IL-6 and IL-11, and appear to play a central part in myelopoiesis (15). The purpose of the present study was to establish whether cells of the stromal/osteoblastic lineage express some or all of the members of this receptor family, by assessing tyrosine phosphorylation of the β subunits upon stimulation with IL-6, IL-11, LIF, CNTF, and OSM alone or in combination with the soluble forms of their respective receptors. We present evidence that collectively the cells of this lineage express all the known components of this family of receptors; as well as a heretofore unrecognized form of gp130 and an alternative OSM receptor. More interestingly, although these receptors are present in cells of this lineage, individual cell types display a different repertoire of α or β subunits.

Methods

Materials. IL-6, LIF, mouse anti–phosphotyrosine antibody, and rabbit polyclonal IgG anti–human gp130 antibody were obtained from Upstate Biotechnology Inc. (Lake Placid, NY); IL-11 was from Genzyme (Cambridge, MA); OSM and soluble IL-6 receptor were from R&D Systems (Minneapolis, MN). McCoy's 5A medium, FBS, and Brij 96 were from Sigma Chemical Co. (St. Louis, MO). MEM was from GIBCO-BRL (Gaithersburg, MD). CNTF, soluble CNTF receptor, and antisera against human gp130, and LIF receptor β used for immunoprecipitations were obtained as previously described (3, 8, 22, 23). Goat anti–mouse IgG + IgM conjugated with HRP was obtained from Caltag Laboratories (San Francisco, CA). Protein A-Sepharose was purchased from Pharmacia (Piscataway, NJ). Enhanced chemiluminescence (ECL) reactives were purchased from New England Nuclear (Boston, MA). RNAzolB reactive was purchased from Biotecx Laboratories, Inc. (Houston, TX). Oligo-dT-cellulose and glycogen were from Boeringer-Mannheim Biochemicals (Indianapolis, IN).

Cells and culture conditions. Primary bone cell cultures were prepared from neonatal murine calvaria from 3–6-d-old mice, as previously described (24). The +/+LDA11 stromal cell line was established from hematopoietically inactive long-term murine marrow cultures, as previously reported (25). These cells produce a variety of hematopoietic factors including IL-6, LIF, granulocyte–macrophage colony stimulating factor, and stem cell factor (26, 27). They also express receptors for estrogens, androgens, and vitamin D_3 (28, 29). The murine stromal/osteoblastic cell line MBA13.2 expresses type I and IV collagen (30). Both cell lines were cultured in phenol red–free McCoy's 5A medium supplemented with 10% FBS. The murine calvaria-derived osteoblast-like cell line MC3T3-E1 (31), the human osteosarcoma cell line MG-63, and the murine fibroblastic cell line MG-87 were cultured in phenol red–free MEM supplemented with 10% FBS.

Cell stimulation and immunoprecipitations. Cells were cultured until 85–90% of confluence and maintained in serum-free medium for 2 h before stimulation. Cytokines alone (50 ng/ml) or in combination with their soluble receptors (1 μg/ml) were added to cell monolayers and maintained for 5 min at 37°C. Cells were then lysed in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate, 5 μg/ml leupeptin, 0.14 U/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1% Brij 96. Insoluble material was pelleted in a microcentrifuge at 14,000 rpm for 10 min. Aliquots from clear lysates were incubated overnight with specific antisera raised against gp130 or LIFRβ. This was followed by precipitation of the complexes with protein A conjugated with Sepharose.

Immunoblotting. Immunoprecipitated proteins were analyzed as described previously (3, 8). Briefly, immunoprecipitates were dissolved in buffer for protein electrophoresis, separated on SDS–polyacrylamide (7.5%) gels, and electrotransferred to polyvinylidene difluoride (PVDF). Membranes were blocked for 1 h at room temperature in 20 mM Tris, pH 7.5, 150 mM NaCl containing 10% BSA. For the detection of tyrosine phosphorylated proteins, membranes were subjected to immunoblotting using a mouse anti–phosphotyrosine antibody as primary antibody, and a goat anti–mouse IgG + IgM conjugated with HRP as secondary antibody. For the detection of gp130, immunoblotting of the membrane was performed using anti-gp130 antibody (Upstate Biotechnology Inc.) and protein A conjugated with HRP. Blots were developed using ECL according to the manufacturer's recommendations.

RNA extraction and Northern blot analysis. Total cellular RNA was isolated from confluent cell cultures or from freshly isolated murine brain using a commercially available kit (RNAzolB) based on the single step guanidinium-isothiocyanate method (32). Polyadenylated RNA was selected by oligo-dT-cellulose spin columns and precipitated with ethanol in the presence of glycogen. Messenger RNA was separated by electrophoresis in 1% agarose formaldehyde gels, transferred to nylon membranes, and fixed by heating at 80°C under vacuum for 2 h. Blots were probed with radiolabeled cDNAs for the rat CNTFRα (33) or the housekeeping gene Cho-B (25, 34), and analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Results

IL-6 in combination with its soluble receptor (IL-6 + sIL-6R), but not by itself, as well as LIF, CNTF alone or in combination

with its soluble receptor (CNTF + sCNTFRα), IL-11, and OSM induced the phosphorylation of gp130- or LIFRβ-containing complexes in primary cultures of murine calvaria cells (Fig. 2). In this and subsequent experiments with cell lines, tyrosine phosphorylation studies were done using a 5-min time point, based on results of earlier kinetic analysis with hematopoietic and neural cell lines (35) as well as the MBA13.2 cell line (not shown), indicating that this time point is optimal.

The effects of cytokines on the preadipocytic stromal cell line +/+LDA11 are shown in Fig. 3. Following stimulation with IL-6 alone, a faint band of tyrosine phosphorylated gp130 could be seen. The phosphorylation of this protein increased greatly following stimulation of the cells with IL-6 + sIL-6R or IL-11 (Fig. 3, *top*, *arrow*). Unlike IL-6 + sIL-6R and IL-11, stimulation of +/+LDA11 cells with LIF, CNTF, or OSM (each one of which uses LIFRβ for signal transduction) failed to induce phosphorylation of gp130 or LIFRβ. A tyrosine phosphorylated protein precipitated by the antibodies to LIFRβ was detectable in this and repeat experiments with this cell line. Although this protein exhibited a similar migration pattern to that of LIFRβ, it was consistently present in unstimulated as well as stimulated cells. Further, immunoprecipitation with anti-LIFRβ antibody in the presence of an excess of the peptide against the antibody was raised (8) failed to block its precipitation (not shown), indicating that this protein is not LIFRβ.

To investigate whether cells representing phenotypes with more osteoblast-like characteristics than +/+LDA11 cells express different receptors, we examined cytokine responsiveness in two other murine cell lines, MBA13.2 and MC3T3-E1. MBA13.2 cells have several phenotypic characteristics of osteoblastic cells, including expression of type I and type IV collagen, as well as PTH receptors (30). MC3T3-E1 cells represent a differentiated osteoblastic phenotype, as evidenced by the fact that they are able to secrete and mineralize matrix (31). As shown in Fig. 4 *A*, MBA13.2 cells did not respond to

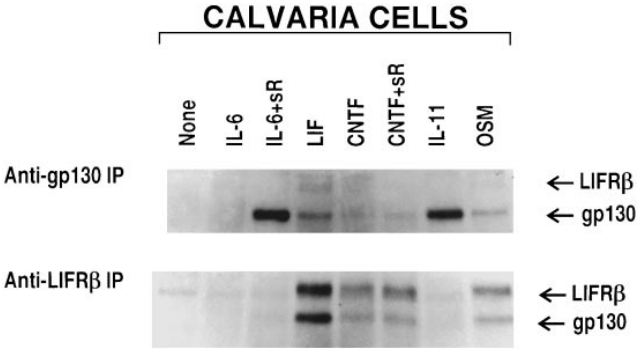

Figure 2. Phosphorylation pattern of primary cultures of bone cells in response to cytokines. Confluent cultures of bone cells isolated from murine calvaria were maintained in serum-free medium for 2 h, and subsequently incubated for 5 min in the absence (*None*) or in the presence of IL-6 alone, IL-6 + sIL-6R (*IL-6 + sR*), LIF, CNTF alone, CNTF + sCNTFR (*CNTF + sR*), IL-11, or OSM, at the concentrations indicated in Methods. Subsequently, cells were lysed with 1% Brij 96 and immunoprecipitated with either an anti-gp130 antibody (*top*) or an anti-LIFRβ antibody (*bottom*). Proteins were separated by SDS-PAGE and transferred to PVDF membranes. Western blotting was performed with anti-phosphotyrosine antibodies. Arrows indicate the positions of LIFRβ (190 kD) and gp130 (145 kD).

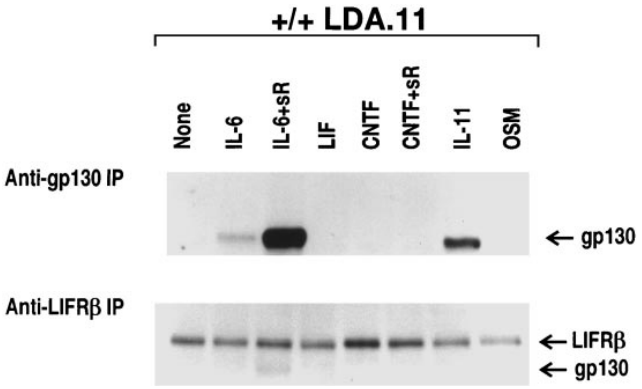

Figure 3. Phosphorylation pattern of +/+LDA11 bone marrow derived stromal cells in response to cytokines. Cultures of murine bone marrow derived stromal +/+LDA11 cells were starved for 2 h, incubated for 5 min in the absence (*None*) or in the presence of the indicated cytokines, and lysed. Cell lysates were immunoprecipitated with either an anti-gp130 antibody or an anti-LIFRβ antibody, separated by SDS-PAGE, and transferred to PVDF membranes. Western blotting was performed with anti-phosphotyrosine antibodies, as detailed in Methods. Arrows indicate the positions of LIFRβ (190 kD) and gp130 (145 kD).

IL-6, but they responded to IL-6 in the presence of sIL-6R. These cells also responded to IL-11, with tyrosine phosphorylation of gp130 homodimers. Unlike +/+LDA.11 cells, MBA13.2 cells responded to LIF, CNTF + sCNTFRα (but not to CNTF alone), and OSM, with the phosphorylation of gp130

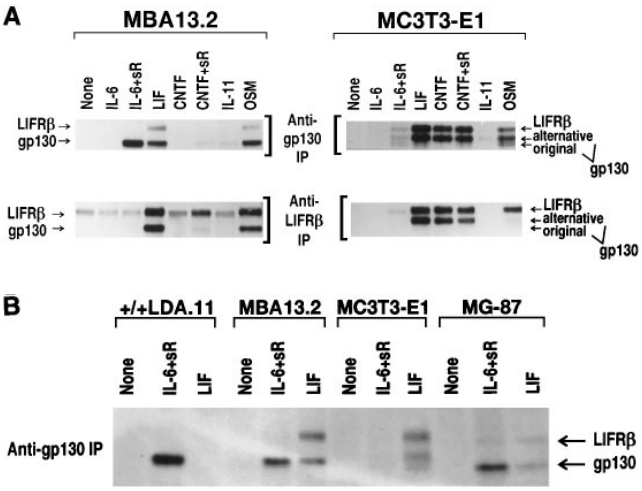

Figure 4. Comparison of gp130 complexes in cells of the stromal/osteoblastic lineage: expression of a distinct form of gp130 in MC3T3-E1 cells. (*A*) Cultures of murine bone marrow derived stromal cells MBA13.2 and murine osteoblast-like MC3T3-E1 cells were stimulated with the indicated cytokines as detailed in Methods. Cell lysates were immunoprecipitated with either an anti-gp130 antibody or an anti-LIFRβ antibody. Proteins were separated by SDS-PAGE and transferred to PVDF membranes and Western blotting were performed with anti-phosphotyrosine antibodies. Arrows indicate the positions of LIFRβ (190 kD), the alternative form of gp130 (165 kD); and the original gp130 (145 kD). (*B*) Cultures of +/+LDA11, MBA13.2, and MC3T3-E1 cells were stimulated with none, or IL-6 + sIL-6R (*IL-6 + sR*), or LIF; and cell lysates were immunoprecipitated with anti-gp130 antibody. Western blotting was performed as in *A*.

and LIFRβ, indicating that in difference to +/+LDA11, MBA13.2 cells express LIFRβ.

MC3T3-E1 cells responded to LIF, CNTF, and OSM with tyrosine phosphorylation of LIFRβ and another protein that, although it was immunoprecipitated by the anti-gp130 antibody, had a different response to cytokines than the classical gp130. Thus, MC3T3-E1 cells exhibited a very weak response to IL-6 + sIL-6R (Fig. 4 *A*). This observation alerted us to the possibility that the protein phosphorylated in response to LIF, CNTF, and OSM in MC3T3-E1 cells is an alternative form of gp130. Consistent with this, the alternative gp130 appeared to migrate slower than the classical gp130. When MC3T3-E1 cells were stimulated with OSM, we detected three distinct phosphorylated proteins: the LIFRβ, the alternative gp130, as well as a weak band that migrated at the expected position of the classical gp130. These findings suggested that MC3T3-E1 cells express both the classical as well as the alternative gp130; and that the latter is the form predominantly phosphorylated in response to cytokine stimulation. MC3T3-E1 cells exhibited minimal response to IL-11, suggesting that these cells either express very low levels of the α subunit of the IL-11 receptor or that the alternative gp130 is as poorly responsive to IL-11 stimulation as it is to IL-6 + sIL-6R stimulation. It should be noted, however, that in other thaws of MC3T3 cells, the alternative gp130 responded better to IL-6 + sIL-6R.

That the alternative gp130 represents a slower migrating form than the classical gp130 was confirmed in a subsequent experiment where we compared the size of the proteins immunoprecipitated by the anti-gp130 antibody in MC3T3-E1 cells with the proteins immunoprecipitated by the same antibody in +/+LDA11, and MBA13.2 cells (Fig. 4 *B*). In this experiment, immunoprecipitated lysates from all three lines were run in the same gel, along with lysates from the murine fibroblastic cell line MG-87. MG-87 cells have been previously shown to express the classical gp130 and were used in this experiment as an additional control. The anti-gp130 precipitated protein of the MC3T3-E1 cells exhibited mobility of a 165-kD protein, whereas the gp130 precipitable protein in all the other three cell lines exhibited the expected (145 kD) mobility of the classical gp130.

These data suggest that the slower migrating protein seen in MC3T3-E1 cells is either an alternative processed form of gp130 or a close gp130 relative that directly binds the gp130 antibodies. These results alone could not exclude the possibility that this is a protein unrelated to gp130 that is tightly associated with gp130. To address this possibility, MC3T3-E1 cells were stimulated with LIF. Cells were then lysed and half of the lysate was heated at 95°C to disrupt protein-protein interactions; the other half was used unheated. Subsequently, both aliquots were immunoprecipitated with anti-gp130 antibody and were immunoblotted using the antiphosphotyrosine antibody (Fig. 5 *A*). Two bands corresponding to the alternative gp130 and LIFRβ were detected in the unheated aliquot (lane *1*). However, only one band, corresponding to the alternative gp130, was detected in the heated aliquot (lane *2*), confirming that disruption of the cytokine receptor complexes by the heating step prevented the coprecipitation of LIFRβ. Heating, however, did not interfere with the recognition of the alternative gp130 by the anti-gp130 antibody. As an alternative experimental approach in establishing that the alternative gp130 is indeed recognized by the anti-gp130 antibody directly and is not a fortuitously coprecipitated protein, lysates from unstimulated (i.e., not treated with cytokines) MC3T3-E1, +/+ LDA11, and MBA13.2 cells were prepared. The lysates were then immunoprecipitated with anti-gp130 antibodies and the proteins were identified by Western blot using the same anti-gp130 antibody. As seen in Fig. 5 *B*, the anti-gp130 antibody recognized two distinct proteins in MC3T3-E1 cells. In contrast, +/+LDA11 and MBA13.2 contained only one protein that migrated exactly as the faster migrating protein of the MC3T3-E1 cells. These results argue strongly against the possibility that the alternative gp130 is an irrelevant protein fortuitously associated with gp130.

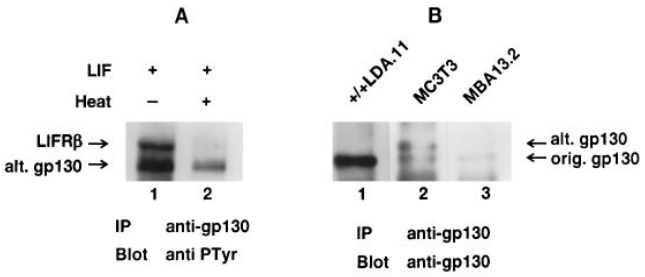

Figure 5. The alternative gp130 expressed in MC3T3-E1 cells is directly recognized by anti- gp130 antibody. (*A*) Confluent cultures of MC3T3-E1 cells were stimulated with LIF for 5 min, and cell lysate was obtained. Half of the lysate was heated at 95°C for 5 min and subsequently both aliquots were immunoprecipitated with anti-gp130 antibody. Immunoprecipitated proteins were separated by SDS-PAGE, electrotransferred, and blotted using anti-phosphotyrosine antibody. Lane *1*, Immunoprecipitates from unheated lysates; lane *2*, immunoprecipitates from heated lysates. (*B*) Lysates of unstimulated +/+ LDA.11 (lane *1*), MC3T3-E1 (lane *2*), and MBA13.2 (lane *3*) cells were immunoprecipitated with anti-gp130 antibody and separated by SDS-PAGE, electrotransferred, and Western blotting was performed with anti-gp130 antibody and HRP-conjugated protein A, as detailed in Methods.

As in the case of calvaria cells (Fig. 2), MC3T3-E1 cells responded to CNTF in the absence of soluble CNTFRα, suggesting that both of these preparations express the membrane-anchored CNTFRα (Fig. 4 *A*). In agreement with the results of the phosphorylation studies indicating that MC3T3-E1 cells express the membrane-anchored CNTFRα (Fig. 4), whereas +/+LDA11 and MBA13.2 cells do not (Figs. 3 and 4, respectively), we detected a single 2.4-kb transcript in MC3T3-E1 cells (Fig. 6, lanes *4* and *5*). This transcript comigrated with CNTFRα expressed in murine brain (lane *1*); but was absent in mRNA preparations from +/+LDA11 cells and MBA13.2 cells (lanes *2* and *3*, respectively).

Finally, the responsiveness of a human osteoblast-like cell to the various cytokines was investigated using the osteosarcoma cell line MG-63 as a model (Fig. 7). MG-63 cells responded very weakly to IL-6 alone. This response was greatly enhanced when IL-6 was used in combination with the sIL-6R. MG-63 cells also responded to IL-11 by forming gp130 homodimers. However, these human cells did not respond to LIF, CNTF alone, or CNTF + sCNTFRα. Further, antibodies to LIFRβ failed to precipitate any tyrosine-phosphorylated protein, suggesting the absence of LIFRβ expression in these cells. On the other hand, stimulation of MG-63 cells with OSM induced tyrosine phosphorylation of gp130 and another β subunit with molecular weight intermediate between gp130 and LIFRβ. This second β subunit was associated with gp130, as

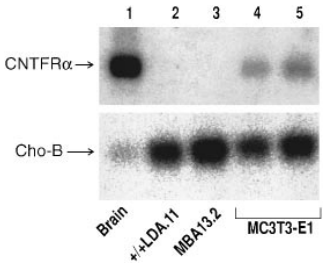

Figure 6. Expression of CNTFRα in osteoblast-like MC3T3-E1 cells. PolyA+ RNA isolated from murine brain (2 μg, lane *1*) or from confluent cultures of the indicated cell lines (5 μg, lanes *2–4*; 10 μg, lane *5*) was separated by electrophoresis in 1% agarose formaldehyde gels, transferred to nylon membranes and fixed. Blot was probed with radiolabeled cDNAs for the rat CNTFRα or the housekeeping gene Cho-B, and analyzed using a PhosphorImager.

evidenced by the fact that it was immunoprecipitated with the anti-gp130 antibody. These results are compatible with the expression of OSMRβ in MG-63 osteosarcoma cells.

Discussion

The results of the experiments presented in this study demonstrate that cells of the stromal/osteoblastic lineage express all the known α and β subunits of the receptors for IL-6, IL-11, LIF, OSM, and CNTF. Indeed, cells of the lineage displayed gp130, as well as LIFRβ; and, as evidenced by tyrosine phosphorylation of these two β subunits upon stimulation with the respective cytokines, express the cytokine-binding (α) subunits for IL-6, IL-11, and CNTF.

In agreement with earlier reports by us and others, IL-6 and CNTF were able to induce signaling in the presence of exogenous sIL-6R α or the soluble CNTF receptor (sCNTFRα), respectively (2, 5, 36). This effect was independent of whether a particular cell expressed the membrane-anchored forms of these receptors. Thus, IL-6 alone had a weak effect on the phosphorylation of gp130 in the preadipocytic stromal cell line +/+LDA11 and the osteoblast-like osteosarcoma cell line MG-63, but no effect in the rest of the lines tested here. However, the effect of IL-6 was greatly enhanced in the weakly responding cells, and appeared in the unresponsive cells, when the cells were stimulated by IL-6 in the presence of the soluble receptor.

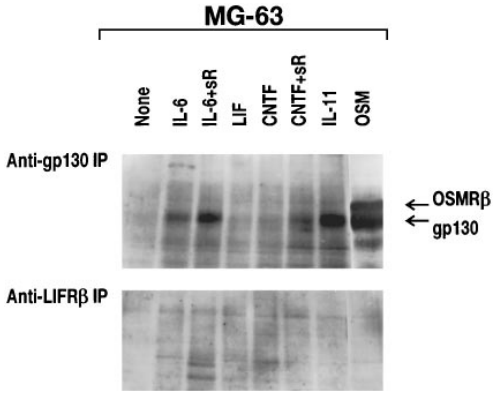

Figure 7. Phosphorylation pattern of human osteoblast-like osteosarcoma MG-63 cells in response to cytokines. Cells were maintained in serum free medium for 2 h and then incubated for 5 min in the absence (*None*) or presence of the indicated cytokines, and lysed. Cell lysates were immunoprecipitated with either an anti-gp130 antibody or an anti-LIFRβ antibody, separated by SDS-PAGE and transferred to PVDF membranes. Western blotting was performed with anti-phosphotyrosine antibodies. Arrows indicate the positions corresponding to gp130 (145 kD) and OSMRβ (160 kD).

IL-6 and IL-11 are produced by stromal/osteoblastic cells and are capable of stimulating the development of osteoclasts from their hematopoietic precursors (13–15). At this stage, however, it remains unclear whether these cytokines exert their osteoclastogenic effects via receptors present on osteoclast precursors, or via receptors present on stromal/osteoblastic cells (which are required for osteoclast development), or both. In any event, whereas the osteoclastogenic effects of IL-11 are readily demonstrated in vitro, the osteoclastogenic effect of IL-6 can only be demonstrated when exogenous IL-6R is provided (37–40), raising the possibility that expression of the α subunit of the IL-6R in bone is a limiting factor for the effects of the latter cytokine. Consistent with this possibility, the α subunit of the IL-6R was weakly expressed in the preadipocytic bone marrow stromal cell line +/+LDA11 and in the human osteosarcoma cells MG-63, but was absent from the calvaria cells and the MBA13.2 and MC3T3-E1 cell lines. The α subunit of the IL-11 receptor, on the other hand, was expressed in all cell types. This apparent difference in IL-6 versus IL-11 receptor expression in stromal/osteoblastic cells may account for the observations that IL-11 is important for osteoclastogenesis in general, whereas IL-6 attains its importance for osteoclastogenesis in certain pathologic states such as osteoporosis. In studies reported elsewhere, we have obtained evidence that both estrogens and androgens regulate the expression of the IL-6 receptor as well as gp130 (41, 42). Moreover, Girasole et al. have reported that the circulating levels of soluble IL-6 receptor increase following the loss of ovarian function in women (43). This evidence adds strength to the contention that the expression of the α subunit of the IL-6 receptor is a limiting factor for the biologic effects of IL-6 in bone metabolism; and suggest that loss of gonadal function, besides its effects on IL-6 production, may also cause an increase in the sensitivity of the osteoclastogenic process to the action of IL-6, due to an upregulation of the membrane-anchored and/or the soluble form of the IL-6 receptor (15, 44).

The binding subunit of the IL-11 receptor has been recently cloned and shown to interact with gp130 but not with LIFRβ (4, 45). In line with this observation, we found that tyrosine phosphorylated β subunits could not be precipitated with the LIFRβ antibody in IL-11–stimulated stromal/osteoblastic cells. In fact, our results strongly suggest that IL-11 induces homodimerization and tyrosine phosphorylation of gp130, since only a single band corresponding to gp130 was immunoprecipitated by the anti-gp130 antibody.

The results reported in this paper also provide evidence that both the type I and the type II receptor for OSM are expressed in bone marrow stromal/osteoblastic cells. Specifically, MBA13.2 and MC3T3-E1 cells (as well as the rat osteosarcoma cell line ROS 17/2.8; data non shown) displayed the type I receptor as evidenced by their ability to respond to both LIF and OSM with phosphorylation of the LIFRβ/gp130 heterodimer. On the other hand, the osteosarcoma cells MG-63 responded to OSM with the tyrosine phosphorylation of a heterodimer of gp130 with a different β subunit; and failed to respond to LIF (or CNTF, even in the presence of CNTFRα). The absence of LIFRβ in MG-63 cells was directly documented by the inability of anti-LIFRβ antibody to precipitate

tyrosine phosphorylated complexes. These observations clearly indicate that MG-63 cells express the OSM type II receptor (10). In studies not shown here, we have demonstrated that the type II OSM receptors in MG-63 cells are functional by showing that OSM inhibits the proliferation of MG-63 cells by arresting them in the G0/G1 phase of the cell cycle and increases IL-6 production by these cells; whereas LIF has no effect on either of these parameters (46).

CNTF also induces the phosphorylation of gp130/LIFRβ heterodimers, however the receptor complex for CNTF is tripartite and includes the ligand-specific subunit CNTFRα. Both the membrane-anchored and the soluble forms of CNTFRα can bind CNTF and trigger signaling (3, 8). The results presented here demonstrate that primary cultures of calvaria cells and osteoblast-like MC3T3-E1 cells (originally derived from murine calvaria) express the ligand-binding subunit of CNTF receptor. Although the abundance of this message in MC3T3 cells was much lower than that in brain, the amount of membrane-anchored receptor expressed by the cells seemed to be sufficient to obtain maximal cell response, since addition of the soluble form of the receptor did not result in an increase in the phosphorylation of the β subunits. Consistent with the evidence for the presence of CNTF receptors in osteoblastic cells, we have recently demonstrated modulatory effects of CNTF on proliferation, alkaline phosphatase activity and IL-6 production in MC3T3-E1 cells (47), indicating that these receptors are indeed functional. These observations reveal one of the first actions for CNTF outside of the central nervous system and skeletal muscle (48).

Besides previously recognized (or suspected, in the case of OSMRβ) α and β subunits for this cytokine receptor family, we have detected a heretofore unrecognized form of gp130, with a very small, if any, response to IL-6 (even in the presence of sIL-6R) or IL-11, but full response to LIF, CNTF, and OSM. The evidence that IL-6, even in the presence of sIL-6R, cannot induce gp130 homodimerization in the MC3T3-E1 cells that express this alternative gp130 has, to our knowledge, no precedent, and supports the contention that these cells do indeed express an alternative form of gp130. At this stage we do not know whether the alternative gp130 is an aberration of the particular cell line or a form of gp130 that occurs in normal cells. We also do not know if it represents an alternatively processed form of gp130 or is instead a closely related protein that is recognized by the anti-gp130 antibody. In any event, the presence of an alternative gp130 with distinct responsiveness to various members of this cytokine family suggests additional means whereby specificity can be imparted in the actions of distinct cytokines using the same signal transduction pathway, as sequences present in the β subunits determine the activation of specific substrates (12).

In conclusion, the evidence presented in this paper indicates that bone marrow stromal/osteoblastic cells are targets for the effects of all the cytokines that act through the gp130 receptor family. Taken together with the evidence that at least some of these cytokines are produced by stromal/osteoblastic cells and that they control osteoclastogenesis, these results indicate further that members of this family have both autocrine as well as paracrine actions on bone. Hence, this family of cytokines may be involved in the development of both osteoblasts and osteoclasts, and perhaps in the coordination of these two processes. Even though collectively the cell models used in this study expressed all the known α and β subunits of these receptors, none of them expressed the entire repertoire. Moreover, different combinations of α and β subunits were expressed in cells with distinct phenotypes. This finding strongly suggests that expression of these receptors in cells of the mesenchymal lineage is a function of the stage of differentiation. A differentiation-dependent gain or loss of responsiveness to a particular cytokine would be consistent with a highly specialized role for each of them in bone metabolism. The evidence that increased production of IL-6 and/or increased expression of the IL-6R (13, 29, 41–43), or loss of LIFRβ (21), has detrimental effects on bone homeostasis, is consistent with such a highly specialized and critical role.

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References

1. Taga, T., M. Hibi, Y. Hirata, K. Yamasaki, K. Yasukawa, T. Matsuda, T. Hirano, and T. Kishimoto. 1989. Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell.* 58:573–581.

2. Kishimoto, T., T. Taga, and S. Akira. 1994. Cytokine signal transduction. *Cell.* 76:253–262.

3. Davis, S., T. H. Aldrich, N. Stahl, L. Pan, T. Taga, T. Kishimoto, N. Y. Ip, and G. D. Yancopoulos. 1993. LIFRβ and gp130 as heterodimerizing signal transducers of the tripartite CNTF receptor. *Science (Wash. DC).* 260:1805–1808.

4. Hilton, D. J., A. A. Hilton, A. Raicevic, S. Rakar, M. Harrison-Smith, N. M. Gough, C. G. Begley, D. Metcalf, N. A. Nicola, and T. A. Willson. 1994. Cloning of a murine IL-11 receptor alpha-chain: requirement for gp130 for high affinity binding and signal transduction. *EMBO J.* 13:4765–4775.

5. Stahl, N., and G. D. Yancopoulos. 1993. The alphas, betas, and kinases of cytokine receptor complexes. *Cell.* 74:1–4.

6. Zhong, Z., S. Wen, and J. E. J. Darnell. 1994. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science (Wash. DC).* 264:95–98.

7. Feldman, G. M., E. F. I. Petricoin, M. David, A. C. Larner, and D. S. Finbloom. 1994. Cytokines that associate with the signal transducer gp130 activate the interferon-induced transcription factor p91 by tyrosine phosphorylation. *J. Biol. Chem.* 269:10747–10752.

8. Stahl, N., S. Davis, V. Wong, T. Taga, T. Kishimoto, N. Y. Ip, and G. D. Yancopoulos. 1993. Cross-linking identifies leukemia inhibitory factor-binding protein as a ciliary neurotrophic factor receptor component. *J. Biol. Chem.* 268: 7628–7631.

9. Gearing, D. P. 1993. The leukemia inhibitory factor and its receptor. *Adv. Immunol.* 53:31–58.

10. Thoma, B., T. A. Bird, D. J. Friend, D. P. Gearing, and S. K. Dower. 1994. Oncostatin M and leukemia inhibitory factor trigger overlapping and different signals through partially shared receptor complexes. *J. Biol. Chem.* 269: 6215–6222.

11. Boulton, T. G., N. Stahl, and G. D. Yancopoulos. 1994. Ciliary neurotrophic factor/leukemia inhibitory factor/interleukin 6/oncostatin M family of cytokines induces tyrosine phosphorylation of a common set of proteins overlapping those induced by other cytokines and growth factors. *J. Biol. Chem.* 269:11648–11655.

12. Stahl, N., T. J. Farruggella, T. G. Boulton, Z. Zhong, J. E. Darnell, Jr., and G. D. Yancopoulos. 1995. Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. *Science (Wash. DC).* 267:1349–1353.

13. Jilka, R. L., G. Hangoc, G. Girasole, G. Passeri, D. C. Williams, J. S. Abrams, B. Boyce, H. Broxmeyer, and S. C. Manolagas. 1992. Increased osteoclast development after estrogen loss: Mediation by interleukin-6. *Science (Wash. DC).* 257:88–91.

14. Girasole, G., G. Passeri, R. L. Jilka, and S. C. Manolagas. 1994. Interleukin-11: a new cytokine critical for osteoclast development. *J. Clin. Invest.* 93: 1516–1524.

15. Manolagas, S. C., and R. L. Jilka. 1995. Bone marrow, cytokines, and bone remodeling. *N. Engl. J. Med.* 332:305–311.

16. Gimble, J. M., F. Wanker, C.-S. Wang, H. Bass, X. Wu, K. Kelly, G. D. Yancopoulos, and M. R. Hill. 1994. Regulation of bone marrow stromal cell differentiation by cytokines whose receptors share the gp130 protein. *J. Cell. Biochem.* 54:122–133.

17. Rodan, S. B., G. Wesolowski, D. J. Hilton, N. A. Nicola, and G. A. Rodan. 1990. Leukemia inhibitory factor binds with high affinity to preosteoblastic RCT-1 cells and potentiates the retinoic acid induction of alkaline phosphatase. *Endocrinology.* 127:1602–1608.

18. Evans, D. B., B. Gerber, and J. H. M. Feyen. 1994. Recombinant human leukemia inhibitory factor is mitogenic for human bone-derived osteoblast-like cells. *Biochem. Biophys. Res. Commun.* 199:220–226.

19. Allan, E. H., D. J. Hilton, M. A. Brown, R. S. Evely, S. Yumita, D. Metcalf, N. M. Gough, K. W. Ng, N. A. Nicola, and T. J. Martin. 1990. Osteoblasts display receptors for and responses to leukemia-inhibitory factor. *J. Cell. Physiol.* 145:110–119.

20. Metcalf, D., and D. P. Gearing. 1989. Fatal syndrome in mice engrafted with cells producing high levels of the leukemia inhibitory factor. *Proc. Natl. Acad. Sci. USA.* 6:5948–5952.

21. Ware, C. B., M. C. Horowitz, B. R. Renshaw, J. S. Hunt, D. Liggitt, S. A. Koblar, B. C. Gliniak, H. J. McKenna, T. Papayannopoulou, B. Thoma, et al. 1995. Targeted disruption of the low-affinity leukemia inhibitory factor receptor gene causes placental, skeletal, neural and metabolic defects and results in perinatal death. *Development.* 121:1283–1299.

22. Masiakowski, P., H. Liu, C. Radziejewski, F. Lottspeich, W. Oberthuer, V. Wong, R. M. Lindsay, M. E. Furth, and N. Panayotatos. 1991. Recombinant human and rat ciliary neurotrophic factors. *J. Neurochem.* 57:1003–1012.

23. Panayotatos, N., D. Everdeen, A. Liten, R. Somogyi, and A. Acheson. 1994. Recombinant human CNTF receptor alpha: production, binding stoichiometry, and characterization of its activity as a diffusible factor. *Biochemistry.* 33:5813–5818.

24. Passeri, G., G. Girasole, R. L. Jilka, and S. C. Manolagas. 1993. Increased interleukin-6 production by murine bone marrow and bone cells after estrogen withdrawal. *Endocrinology.* 133:822–828.

25. Girasole, G., R. L. Jilka, G. Passeri, S. Boswell, G. Boder, D. C. Williams, and S. C. Manolagas. 1992. 17β-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in-vitro: a potential mechanism for the antiosteoporotic effect of estrogens. *J. Clin. Invest.* 89:883–891.

26. Boswell, H. S., D. Y. Mochizuki, G. S. Burgess, S. Gillis, E. B. Walker, D. Anderson, and D. E. Williams. 1990. A novel mast cell growth factor (MCGF-3) produced by marrow-adherent cells that synergizes with interleukin 3 and interleukin 4. *Exp. Hematol.* 18:794–800.

27. Williams, D. E., J. Eisenman, A. Baird, C. Rauch, K. Van Ness, C. J. March, L. S. Park, U. Martin, D. Y. Mochizuki, H. S. Boswell, G. S. Burgess, D. Cosman, and S. D. Lyman. 1990. Identification of a ligand for the c-*kit* proto-oncogene. *Cell.* 63:167–174.

28. Bellido, T., G. Girasole, G. Passeri, X. Yu, H. Mocharla, R. L. Jilka, A. Notides, and S. C. Manolagas. 1993. Demonstration of estrogen and vitamin D receptors in bone marrow derived stromal cells: upregulation of the estrogen receptor by $1,25(OH)_2D_3$. *Endocrinology.* 133:553–562.

29. Bellido, T., R. L. Jilka, B. F. Boyce, G. Girasole, H. Broxmeyer, S. A. Dalrymple, R. Murray, and S. C. Manolagas. 1995. Regulation of interleukin-6, osteoclastogenesis and bone mass by androgens. The role of the androgen receptor. *J. Clin. Invest.* 95:2886–2895.

30. Zipori, D., J. Toledo, and K. Von der Mark. 1985. Phenotypic heterogeneity among stromal cell lines from mouse bone marrow disclosed in their extracellular matrix composition and interactions with normal and leukemic cells. *Blood.* 66:447–455.

31. Sudo, H., H. A. Kodama, Y. Amagai, S. Yamamoto, and S. Kasai. 1983. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. *J. Cell Biol.* 96:191–198.

32. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

33. Davis, S., T. H. Aldrich, D. M. Valenzuela, V. Wong, M. E. Furth, S. P. Squinto, and G. D. Yancopoulos. 1991. The receptor for ciliary neurotrophic factor. *Science (Wash. DC).* 253:59–63.

34. Harpold, M. M., R. M. Evans, M. Salditt-Georgieff, and J. E. Darnell. 1979. Production of mRNA in Chinese hamster cells: relationship of the rate of synthesis to the cytoplasmic concentration of nine specific mRNA sequences. *Cell.* 17:1025–1035.

35. Ip, N. Y., S. H. Nye, T. G. Boulton, S. Davis, T. Taga, Y. Li, S. J. Birren, K. Yasukawa, T. Kishimoto, D. J. Anderson, N. Stahl, and G. D. Yancopoulos. 1992. CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130. *Cell.* 69:1121–1132.

36. Rose-John, S., and P. C. Heinrich. 1994. Soluble receptors for cytokines and growth factors: generation and biological function. *Biochem. J.* 300:281–290.

37. Tamura, T., N. Udagawa, N. Takahashi, C. Miyaura, S. Tanaka, Y. Yamada, Y. Koishihara, Y. Ohsugi, K. Kumaki, T. Taga, T. Kishimoto, and T. Suda. 1993. Soluble interleukin-6 receptor triggers osteoclast formation by interleukin 6. *Proc. Natl. Acad. Sci. USA.* 90:11924–11928.

38. Udagawa, N., T. Katagiri, T. Takahashi, H. Murakami, I. Nakamura, D. Zhang, T. Tamura, T. J. Martin, and T. Suda. 1995. The activity of IL-6 to induce osteoclast differentiation depends on the presence of IL-6 receptors expressed on osteoblastic cells. *Bone.* 16:92S(Abstr.)

39. Suda, T., N. Udagawa, I. Nakamura, C. Miyaura, and N. Takahashi. 1995. Modulation of osteoclast differentiation and function by local factors. *Bone.* 16:97S(Abstr.)

40. Suda, T., N. Takahashi, and T. J. Martin. 1995. Modulation of osteoclast differentiation: update 1995. *Endocr. Rev.* 4:266–270.

41. Bellido, T., G. Girasole, G. Passeri, R. L. Jilka, and S. C. Manolagas. 1994. Sex steroids regulate the expression of the gp130 transduction pathway by bone marrow and bone cells. *Bone Miner.* 25(S1):S39.

42. Lin, S.-C., T. Bellido, V. Z. C. Borba, C. A. O'Brien, and S. C. Manolagas. 1995. Estrogen and androgen downregulate the expression of both the ligand-binding subunit and the gp130 signal transducing subunit of the IL-6 receptor by a sex steriod receptor-mediated mechanism independent of new protein synthesis. *J. Bone Miner. Res.* 10:S170(Abstr.)

43. Girasole, G., M. Pedrazzoni, N. Giuliani, G. Passeri, A. Bacchi, P. Sansoni, and M. Passeri. 1995. Increased levels of human serum soluble interleukin-6 receptors in ovariectomized women: evidence of a relationship with parathyroid hormone and FSH. *Bone.* 16:98S(Abstr.)

44. Manolagas, S. C., T. Bellido, and R. L. Jilka. 1995. New insights into the cellular, biochemical, and molecular basis of postmenopausal and senile osteoporosis: roles of IL-6 and gp130. *Int. J. Immunopharmac.* 17:109–116.

45. Yin, T., T. Taga, M. L.-S. Tsang, K. Yasukawa, T. Kishimoto, and Y.-C. Yang. 1993. Involvement of IL-6 signal transducer gp130 in IL-11-mediated signal transduction. *J. Immunol.* 151:2555–2561.

46. Borba, V. Z. C., T. Bellido, I. Grigorieva, J. Epstein, N. Stahl, G. Yancopoulos, and S. C. Manolagas. 1995. Demonstration of functional type II receptors for oncostatin-M (OSM) in human osteosarcoma cells (MG-63). *J. Bone. Miner. Res.* 10:S318(Abstr.)

47. Bellido, T., V. Z. C. Borba, N. Stahl, G. Yancopoulos, R. L. Jilka, and S. C. Manolagas. 1995. Ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) stimulate cell proliferation and increase alkaline phosphatase and interleukin-6 production by osteoblastic cells. *J. Bone Miner. Res* 10: S319(Abstr.)

48. Stahl, N., and G. D. Yancopoulos. 1994. The tripartite CNTF receptor complex: activation and signaling involves components shared with other cytokines. *J. Neurobiol.* 25:1454–1466.