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Proliferative responses and binding properties of hematopoietic cells transfected with low-affinity receptors for leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor

(hematopoietin receptor family/signal transduction)

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ABSTRACT **Specific low-affinity receptors for leukemia inhibitory factor (LIF), oncostatin M (OSM; gp130), and ciliary neurotrophic factor (CNTF; receptor α, CNTFRα) may be utilized in various combinations to generate high-affinity binding sites and signal transduction. We have tested the ability of combinations of these receptors to transduce a proliferative signal in BAF-B03 cells. Coexpression of the LIF receptor and gp130 in these cells conferred high-affinity LIF and OSM binding and responsiveness to LIF and OSM. These cells also responded to CNTF in the absence of detectable binding. The further addition of CNTFRα conferred high-affinity CNTF binding and enhanced responsiveness to CNTF but did not modify responses to LIF or OSM. Coexpression of LIF receptor and CNTFRα resulted in a nonfunctional high-affinity binding site. These data are consistent with a role for the CNTFRα in enhancing CNTF action but the CNTFRα is not absolutely required for CNTF action and suggest a wider range of targets for CNTF.**

Leukemia inhibitory factor (LIF) and oncostatin M (OSM) are sibling cytokines related by protein structure, genetic arrangement, and chromosomal localization (1, 2) that share a common receptor complex (3, 4). Both cytokines act on a wide variety of embryological and adult cells including cells of hemopoietic, hepatic, and neuronal origin. The varied biological effects encompass induction or suppression of pathways leading to differentiation or proliferation and induction of other cellular target genes. Ciliary neurotrophic factor (CNTF) is more distantly related to LIF and OSM (5) and has been proposed to act through an overlapping receptor complex (6). The effects of CNTF are thought to be restricted to neural cells (7, 8), but CNTF is also active on hepatocytes (9, 10).

The low-affinity LIF receptor (LIFR) is a type I membrane glycoprotein of ≈200 kDa that binds LIF but not OSM (11). Similarly, the low-affinity OSM receptor (OSMR), first identified as a subunit of the related interleukin (IL) 6 receptor (IL-6R) complex (12, 13), is a type I membrane glycoprotein of ≈130 kDa (gp130) that binds OSM but not LIF (4). Expression of the combination of low-affinity LIFRs and OSMRs on COS-7 cells resulted in higher affinity binding for both LIF and OSM (4). OSM also binds to a separate receptor complex that involves gp130 but does not bind LIF (3, 14).

A CNTF-specific receptor (CNTFRα) has been described that is attached to the outer surface of the membrane via a glycosyl-phosphatidylinositol linkage (15) and can function as a CNTF agonist when expressed as a soluble form (16). The signal-transducing CNTFR complex has been proposed to include CNTFRα, gp130, and LIFR, and the CNTFRα has been proposed to be required for CNTF signaling (6, 16–18). Support for LIFR involvement in CNTF-mediated signal transduction came from its role in activation of acute phase reporter genes in hepatic cell lines by CNTF, LIF, and OSM (10) and in phosphorylation of membrane proteins induced by CNTF and LIF (18, 19). To confirm the roles of the individual receptor components in signal transduction mediated by these cytokines, we attempted to reconstitute LIF, OSM, and CNTF responses in the factor-dependent murine hematopoietic cell line BAF-B03, which expresses none of these receptor subunits.

MATERIALS AND METHODS

Expression Constructs. A full-length human LIFR cDNA was subcloned into pTgls(+)HyTK (20) under the control of a cytomegalovirus promoter cassette and selected via the hygromycin-resistance gene. A full-length human gp130 cDNA was subcloned into the expression site of pLXSN (21) and selected via the neomycin-resistance gene. A full-length human CNTFR cDNA was subcloned into the expression site of pLXSHD (22) and selected via the histidinol-resistance gene. The mouse pro-B-cell line BAF-B03 was transfected with 10 μg of the appropriate DNA by electroporation as described (23) and selection of transfected cells was achieved with G418 (0.6 mg/ml), hygromycin (1000 units/ml), and/or L-histidinol (0.5 mg/ml) as appropriate.

Cytokines and Binding Assays. Recombinant human LIF and OSM were produced in yeast and purified as reported (11). Rat CNTF (which displays 85% homology with human CNTF), produced in *Escherichia coli*, was purchased from Peprotech (Rocky Hill, NJ). A cDNA encoding the soluble human CNTFRα (solCNTFRα) protein was incorporated into an expression vector (pDC406) (24) utilizing the unique *Bgl* II restriction site in the coding region and terminated in vector sequences. Three-day supernatants were collected after transient transfection into CV1/EBNA cells as described (24). Radiolabeling with ^{125}I using the Enzymobead reagent (Bio-Rad) and binding assays were performed as described for LIF (11). Adherent SK-N-SH cells were harvested with nonenzymatic cell dissociation solution (Sigma) for binding assays. Binding was carried out for 30–60 min at either 37°C (with radiolabeled LIF and OSM) or 4°C (with radiolabeled CNTF).

Proliferation Assays. Transfected BAF cells were cultured in 96-well microtiter plates (1×10^4 cells per well, 0.2 ml per well) with test samples for 72 h and pulse-labeled with [^3H]thymidine (0.5 μCi per well; 1 Ci = 37 GBq) for 5 h. Cells

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Abbreviations: LIF, leukemia inhibitory factor; OSM, oncostatin M; CNTF, ciliary neurotrophic factor; IL, interleukin; sol, soluble; LIFR, OSMR, etc., LIF receptor, OSM receptor, etc.

were harvested on glass filters and cell-associated radioactivity was measured by a scintillation counter.

RESULTS

Binding Characteristics of CNTF. We have described (4, 11) the binding characteristics of LIFR and gp130 when cDNAs encoding these proteins were transfected into simian COS-7 cells. Such studies suggested that the combination of LIFR and gp130 could explain the shared receptor for LIF and OSM displayed by many responsive cell types, although a second OSM-specific receptor also appeared to exist. Many recent studies have described the biological effects of CNTF although none have reported binding parameters for CNTF to responsive cells. To better analyze CNTF binding to different combinations of receptor subunits, we first defined CNTF binding characteristics to native receptors on the human neuroblastoma cell line SK-N-SH utilizing ^{125}I-labeled rat CNTF. The radiolabeled protein was shown by SDS/PAGE analysis to be a single band of molecular mass 23 kDa. In some preparations a small amount (<5% relative to the primary band) of a higher molecular mass species was also present and may represent CNTF homodimers. Preliminary experiments showed that ^{125}I-labeled CNTF (specific radioactivity, 1–4 × 10^{15} cpm/mmol) bound rapidly to these cells at both 4°C and 37°C, reaching equilibrium within 20 min (data not shown). Fig. 1 illustrates the Scatchard analysis of a typical equilibrium binding experiment performed at 4°C, where two classes of CNTF binding sites were detectable. From an average of 10 binding experiments at 4°C, the high-affinity portion had a calculated apparent K_a of 6.8 ± 5.4 × 10^{10} M^{-1} and 830 ± 360 specific binding sites per cell and the low-affinity portion had a K_a of 1.8 ± 0.9 × 10^8 M^{-1} and 44,000 ± 25,000 sites per cell. Similar values were obtained

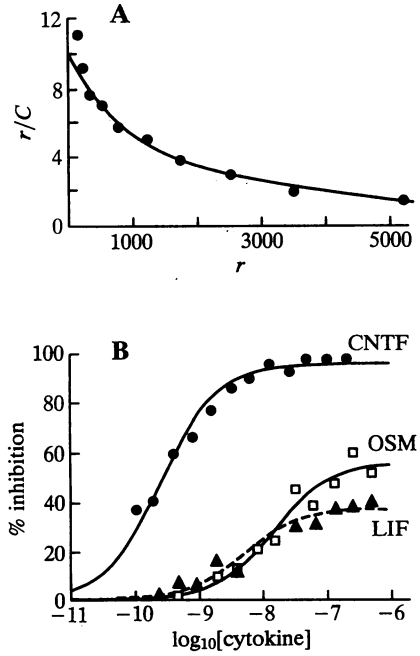

FIG. 1. Equilibrium binding of ^{125}I-labeled CNTF to SK-N-SH cells and inhibition by unlabeled LIF and OSM. (*A*) Scatchard analysis of ^{125}I-labeled CNTF binding (K_{a1} = 8.2 × 10^9 M^{-1} and r_1 = 940; K_{a2} = 2.5 × 10^8 M^{-1} and r_2 = 9600). *r*, No. molecules bound per cell; *C*, concentration in pM. (*B*) Inhibition of binding of ^{125}I-labeled CNTF (1.6 × 10^{-10} M) by various concentrations of unlabeled CNTF (solid circles), LIF (solid triangles), and OSM (open squares). The continuous curves passing through the data were calculated from a one-site competitive inhibition equation using a K_a value for ^{125}I-labeled CNTF of 8.2 × 10^9 M^{-1}.

when binding experiments were performed at 37°C. Fig. 1*B* shows the ability of unlabeled CNTF, LIF, and OSM to compete ^{125}I-labeled CNTF binding at 4°C. These data suggest that the high-affinity CNTFR complex shares common subunits with the receptors for LIF and OSM. The inability of LIF and OSM to completely inhibit CNTF binding to the SK-N-SH cells probably reflects the ability of CNTF to bind with low affinity to CNTFRα in the absence of gp130 or LIFR. The data in Fig. 1*B* also show that the affinity of unlabeled CNTF (K_i = 9.2 × 10^9 M^{-1}) was very similar to the affinity of ^{125}I-labeled CNTF determined in the same experiment (K_a = 8.3 × 10^9 M^{-1}), verifying that radiolabeled CNTF retains full receptor binding ability.

Reconstitution of LIF, OSM, and CNTF Proliferative Responses in BAF-B03 Cells. To assess the signaling capacity of the various receptor subunits, we turned to stable expression of the LIFR, gp130, and CNTFRα in the murine IL-3-dependent cell line, BAF-B03 (25). These cells have been reported to express no endogenous gp130 (13). Preliminary experiments demonstrated that BAF-B03 cells did not bind ^{125}I-labeled LIF, OSM, or CNTF, nor did they respond to LIF, OSM, or CNTF by proliferation (data not shown). Constructs encoding the various subunits were electroporated into BAF-B03 cells along with unique drug-selectable marker genes. BAF-B03 cells expressing LIFR (BAF-LR), gp130 (BAF-gp), CNTFRα (BAF-CR), LIFR–gp130 (BAF-LRgp), LIFR–CNTFRα (BAF-LRCR), gp130–CNTFRα (BAF-gpCR), and LIFR–gp130–CNTFRα (BAF-LRgpCR) were selected. The various cell lines were then assessed for their capacity to bind ^{125}I-labeled LIF, OSM, and CNTF and to proliferate in response to various concentrations of each ligand.

The binding parameters of each cytokine to the various BAF-B03 cell lines are summarized in Table 1. Briefly, ^{125}I-labeled LIF bound to BAF-LR and BAF-LRCR cells with low affinity, to BAF-LRgp and BAF-LRgpCR cells with both high and low affinity, and undetectably to the other cell lines. ^{125}I-labeled OSM bound to BAF-LRgp and BAF-LRgpCR cells with high affinity and did not bind detectably to the other lines. ^{125}I-labeled CNTF bound to BAF-CR and BAF-gpCR cells with low affinity, to BAF-LRCR and BAF-LRgpCR cells with both high and low affinity, and undetectably to the other cell lines.

The proliferative response of the various transfected BAF cells to LIF, OSM, and CNTF was also measured. Proliferation was observed in response to LIF, OSM, and/or CNTF in only two of the cell lines, BAF-LRgp and BAF-LRgpCR. Both lines responded to all three cytokines (Fig. 2). The responsiveness to LIF and OSM was similar in both lines (LIF, 50% maximal stimulation at 1–2 ng/ml; OSM, 50% maximal stimulation at 3–6 ng/ml). In contrast, BAF-LRgpCR cells were ≈200 times more sensitive to CNTF (50% maximal stimulation at 0.06 ng/ml) than BAF-LRgp cells (50% maximal stimulation at 20 ng/ml). To confirm the expression of gp130 on transfected cells, we assessed the response of each of the cell lines to the combination of IL-6 and soluble IL-6 receptor (solIL-6R), as this complex is sufficient to initiate a response in gp130-bearing cells (12). All cell lines transfected with gp130 were responsive to the combination of solIL-6R and IL-6 [recombinant solIL-6R (R&D Systems) at 300 ng/ml and IL-6 at 1 ng/ml], whereas cell lines not transfected with gp130 had no response (data not shown). By analogy to the IL-6–solIL-6R complex, the solCNTFRα subunit has been shown to augment the action of CNTF (18, 26). To confirm the association of the solCNTFRα complex with LIFR–gp130, we incubated BAF-LRgp cells with solCNTFRα-containing supernatants and CNTF. As shown in Fig. 3, the solCNTFRα enhanced the responsiveness of BAF-LRgp cells to CNTF, although it was

Table 1. Binding of ^{125}I-labeled LIF, OSM, and CNTF to transfected BAF-B03 cells

Cell line	^{125}I-labeled ligand	K_{a1}, M^{-1}	K_{a1} sites, no. per cell	K_{a2}, M^{-1}	K_{a2} sites, no. per cell	*n*
BAF-LR	LIF*†	ND	ND	$5.0 \pm 2.8 \times 10^8$	120–1970 ± 700	5
BAF-CR	CNTF*‡	ND	ND	$1.0 \pm 0.6 \times 10^8$	4500–30,000 ± 14,500	3
BAF-LRCR	LIF‡	ND	ND	$4.0 \pm 1.4 \times 10^8$	320–360 ± 30	2
	CNTF	$1.2 \pm 0.4 \times 10^{10}$	260–430 ± 90	$6.0 \pm 5.0 \times 10^7$	14,500–85,000 ± 36,000	3
BAF-gpCR	CNTF*‡	ND	ND	$8.3 \pm 4.6 \times 10^7$	7000–28,000 ± 8800	5
BAF-LRgp	LIF	$9.3 \pm 4.3 \times 10^9$	70–1420 ± 670	$2.8 \pm 1.0 \times 10^8$	740–6850 ± 3000	5
	OSM	$2.5 \pm 1.4 \times 10^9$	100–1150 ± 500	ND	ND	4
	CNTF	ND	ND	ND	ND	5
BAF-LRgpCR	LIF	$5.4 \pm 1.4 \times 10^9$	130–350 ± 100	$2.3 \pm 0.7 \times 10^8$	850–1580 ± 300	4
	OSM	$1.2 \pm 0.3 \times 10^9$	150–240 ± 50	ND	ND	3
	CNTF	$2.4 \pm 1.3 \times 10^{10}$	160–670 ± 200	$1.2 \pm 0.5 \times 10^8$	2800–31,500 ± 11,000	5

Binding to parental BAF-B03 cells and BAF-gp cells was not detected for all three ligands. *K* values are average ± SD. Sites per cell are the range ± SD. ND, not detected; *n*, number of observations.
*No LIF binding observed.
†No CNTF binding observed.
‡No OSM binding observed.

not able to cause enhanced activity of CNTF on any other BAF-B03 transfected cell line (data not shown).

In summary, the presence of high-affinity LIFRs and OSMRs correlated with biological response to all three ligands, but high-affinity CNTFRs did not. The CNTFRα, in membrane-associated or soluble form, altered the LIFR–gp130 complex from being primarily LIF and OSM responsive to being particularly responsive to CNTF with no change in responsiveness to LIF and OSM but was not essential for CNTF signaling.

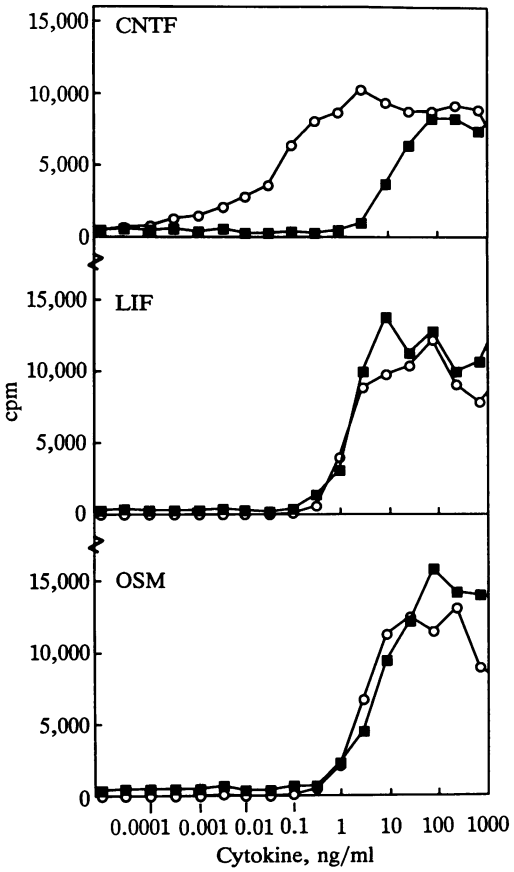

FIG. 2. Response of BAF cells transfected with LIFR and gp130 (BAF-LRgp cells; solid squares) or LIFR, gp130, and CNTFRα (BAF-LRgpCR cells; open circles) to CNTF, LIF, and OSM. Cells (0.2 ml per well) were cultured in duplicate for 72 h with various concentrations of CNTF, LIF, and OSM. Cell-associated radioactivity after a 5-h pulse labeling with [^{3}H]thymidine is shown. The concentrations of cytokine achieving 50% maximal stimulation (mean ± SD, determined from at least three experiments) were as follows. BAF-LRgp cells: LIF, 2.0 ± 0.15 ng/ml; OSM, 3.0 ± 1.9 ng/ml; CNTF, 20 ± 14 ng/ml. BAF-LRgpCR cells: LIF, 0.40 ± 0.09 ng/ml; OSM, 5.6 ± 6.2 ng/ml; CNTF, 0.06 ± 0.03 ng/ml.

DISCUSSION

We have attempted to clarify the roles of the subunits involved in LIFR, OSMR, and CNTFR function. We have reported (4) that the combination of LIFR and gp130, when transfected into COS-7 cells, forms a cross-competable LIF–OSM binding complex similar to that on responsive cell lines. We therefore expressed LIFR and gp130 in the IL-3-dependent cell line BAF-B03 to test whether this complex correlated with signaling. Our results showed that expression of the LIFR–gp130 complex, but not LIFR or gp130 alone, correlated with LIF- and OSM-induced proliferation. The binding properties and half-maximal stimulation of LIF and OSM to the LIFR–gp130 complex expressed on BAF-B03 cells satisfy known criteria for the native receptor for LIF and OSM (e.g., M1 cell differentiation) (1, 3).

Analysis of the response of neural cells to LIF and CNTF has prompted the suggestion that the two receptors might be structurally similar (6, 7). Specifically, a trimeric complex of CNTFR, gp130, and LIFR has been proposed for the functional CNTFR (6). In this study, we have attempted to resolve this issue (*i*) by analysis of the binding properties of LIF, OSM, and CNTF to the neuroblastoma cell line SK-N-SH and (*ii*) by reconstitution of various subunit combinations in BAF-B03 cells.

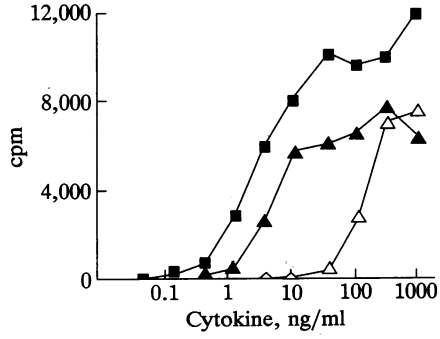

FIG. 3. SolCNTFRα is an agonist of CNTF action on BAF-LRgp cells. BAF-LRgp cells were incubated with LIF (solid squares) or with CNTF in the presence of supernatants from cells transfected with either vector (open triangles) or solCNTFRα (solid triangles). Cell-associated radioactivity (average of duplicate measurements) after a 5-h pulse labeling with [^{3}H]thymidine is shown.

The observation of biphasic CNTF binding to SK-N-SH cells (Fig. 1) suggested that CNTF was binding to two types of receptors, of relatively low affinity ($K_a = 1.8 \times 10^8\ M^{-1}$) and high affinity ($K_a = 6.8 \times 10^{10}\ M^{-1}$). Such binding may be explained by the presence of excess low-affinity binding subunits in the presence of limiting high-affinity converter subunits (4). Competition studies with LIF and OSM showed that the CNTF binding was partially competed by both cytokines, suggesting the presence of a common high-affinity binding site for all three cytokines and CNTF-specific low-affinity binding sites on SK-N-SH cells.

Binding analyses of LIF, OSM, and CNTF to transfected BAF-B03 cells correlated well with previously determined binding characteristics although they produced some surprising results. The CNTFRα chain alone, as expected, formed a low-affinity CNTFR ($K_a = 1.0 \times 10^8\ M^{-1}$) that probably accounts for the low-affinity binding site for CNTF observed on SK-N-SH cells. Unexpectedly, the combination of CNTFRα and LIFR formed a high-affinity CNTF binding site ($K_a = 1.2 \times 10^{10}\ M^{-1}$), but was nonfunctional. The combination of LIFR and gp130 was CNTF-responsive, albeit less responsive than to LIF and OSM, yet definitive CNTF binding could not be measured. Given that the BAF-LRgp cells clearly respond to CNTF, these results indicate that the interaction of CNTF with the LIFR–gp130 complex is of very low affinity, where the low number of binding sites expected on these cells (inferred from the LIF and OSM binding to be 70–1400 sites per cell) likely contributes to the inability to measure CNTF binding. Similarly, low expression of gp130 (OSM $K_a \approx 10^8\ M^{-1}$; ref. 4) likely contributes to the inability to measure OSM binding on BAF-gp cells. Expression of the CNTFRα in BAF-LRgp cells resulted in increased responsiveness to CNTF and high-affinity CNTF binding ($K_a = 2.4 \times 10^{10}\ M^{-1}$) but no change in binding affinity or responsiveness to LIF or OSM. Thus the CNTFRα acts as a receptor modifier subunit, enabling cells that normally respond to LIF and OSM and weakly respond to CNTF to become more responsive to CNTF. The amount of CNTF required to stimulate the LIFR–gp130 complex suggests that this interaction is less productive than the interaction to stimulate the LIFR–gp130–CNTFRα complex. Nevertheless, when the CNTF concentration is high, cells bearing the LIFR–gp130 complex become targets for CNTF action. The high-affinity CNTF site on SK-N-SH cells ($K_a = 6.8 \times 10^{10}\ M^{-1}$) is probably explained by the LIFR–gp130–CNTFRα complex since both LIF and OSM were able to compete for CNTF binding. Experiments with BAF-LRCR cells suggested that OSM would not be expected to compete if the high-affinity site was a complex of LIFR and CNTFRα (Table 1).

The purpose of the nonfunctional high-affinity CNTF binding site formed by the LIFR and CNTFRα is unclear. Perhaps this complex can act as a sink for excess CNTF and plays a part in down-regulating a CNTF response. Alternatively, binding of CNTF to the LIFR–CNTFRα complex might be an intermediate in the formation of the signaling LIFR–CNTFRα–gp130 complex. However, since gp130 expression is widespread, it is possible that LIFR–CNTFRα complexes may not be prevalent *in vivo*. The inability of gp130 to convert the affinity of the CNTFRα in BAF-gpCR cells and the similar high-affinity CNTF binding displayed by BAF-LRCR and BAF-LRgpCR cells suggest that gp130 plays a minimal role, if any, in generating high-affinity binding by CNTF. When expressed in COS cells, gp130 can associate with the CNTFRα in the presence of CNTF but not LIF (18), suggesting that such a low-affinity interaction can occur. The role of gp130, therefore, appears to be primarily as a signaling component. Clearly, binding of CNTF to various cell types will not be an adequate indicator of CNTF-responsive targets, since high-affinity binding can occur in the absence of signaling (via the LIFR–CNTFRα complex) and vice versa, signaling can occur in the absence of detectable CNTF binding (via the LIFR–gp130 complex).

The role of the CNTFRα is similar to that of the homologous IL-6R (15) since both are found in association with other receptor subunits on the cell membrane and both act as agonists in soluble form (12, 13, 16). In these respects the soluble forms of CNTFRα and IL-6R are also similar to the homologous p40 subunit of IL-12 (27) except that p40 is synthesized as a disulfide-linked heterodimer with p35, a cytokine-like subunit. The expression of the soluble forms of CNTFRα and IL-6R has not been proven to be linked to the expression of their respective ligands but CNTFRα mRNA is expressed close to the sites of CNTF synthesis (17). The existence of soluble receptor components suggests that wider activity spectra might exist for some cytokines than would be predicted from binding experiments or from experiments *in vitro* using purified materials. Therefore, in this branch of the cytokine receptor family, there appears to be tremendous diversity: multimeric signaling complexes (e.g., the LIFR–gp130 heterodimers and gp130 homodimers) (28) interact with affinity-modifying subunits (CNTFRα and IL-6R) to form a series of overlapping target sites. These experiments define some of the principles underlying the tremendous redundancy of action of LIF, OSM, CNTF, and IL-6 and suggest that delicate control mechanisms exist for their control *in vivo*.

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