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Multiple Regions within the Cytoplasmic Domains of the Leukemia Inhibitory Factor Receptor and gp130 Cooperate in Signal Transduction in Hepatic and Neuronal Cells

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The receptor for leukemia inhibitory factor (LIFR), in combination with the signal-transducing subunit for interleukin-6-type cytokine receptors, gp130, and LIF, activates transcription of acute-phase plasma protein genes in human and rat hepatoma cells and the vasoactive intestinal peptide gene in a human neuroblastoma cell line. To identify the regions within the cytoplasmic domain of LIFR that initiate signal transduction independently of gp130, we constructed a chimeric receptor by linking the extracellular domain of the granulocyte colony-stimulating factor receptor (G-CSFR) to the transmembrane and cytoplasmic domain of human LIFR. The function of the chimeric receptor protein in transcriptional activation was assessed by G-CSF-mediated stimulation of cotransfected cytokine-responsive reporter gene constructs in hepatoma and neuroblastoma cells. By using the full-length cytoplasmic domain and mutants with progressive carboxy-terminal deletions, internal deletions, or point mutations, we identified the first 150 amino acid residues of LIFR as the minimal region necessary for signaling. The signaling reaction appears to involve a cooperativity between the first 70-amino-acid region containing the two sequence motifs conserved among hematopoietin receptors (box 1 and box 2) and a critical sequence between residues 141 and 150 (box 3). Analogous analyses of the cytoplasmic domains of G-CSFR and gp130 indicated similar arrangements of functional domains in these receptor subunits and the requirement of a box 3-related motif for signaling.

A group of cytokines related to interleukin-6 (IL-6), including IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), and ciliary neurotrophic factor (CNTF), appear to play critical regulatory roles during development and homeostasis (1, 35). These cytokines elicit pleiotropic responses in multiple cell types, including stimulation of proliferation and transcriptional activation of tissue-specific genes (25). In a given cell type, the responses to the individual members of this cytokine group appear to be similar. The redundant action of IL-6-type cytokines is particularly evident in the coordinate activation of acute-phase plasma protein (APP) genes in hepatic cells or neuropeptide genes in neuroblastoma cells (4–6, 14, 28, 40, 43, 46).

Although the IL-6-type cytokines are structurally distinct, their receptors share the extracellular elements that define the hematopoietin receptor family (7, 10). The receptors for the IL-6-type cytokines are multimeric and share a common subunit termed gp130 (24, 47). LIF, OSM, and CNTF receptor complexes share an additional subunit, the low-affinity LIF receptor (LIFR) (11, 18, 19, 27). Within the family, three members, LIFR, gp130, and the granulocyte colony-stimulating factor receptor (G-CSFR), show a high degree of similarity that also extends to their cytoplasmic domains (17, 19, 29). This similarity suggested that the cytoplasmic domain of LIFR might participate in the signal transduction process as previously shown for gp130 and G-CSFR. G-CSFR is thought to form a homodimer when G-CSF is bound (15, 16, 50), whereas gp130 forms a homodimer in the presence of IL-6 and the IL-6 receptor (37), and LIFR and gp130 form a heterodimer when LIF or OSM is bound (18) or in the presence of CNTF and the CNTF receptor (2, 13, 27).

In hepatic cells, the action of IL-6-type cytokine receptors is primarily characterized by the regulation of APP genes, which is regarded to be independent of proliferation control (14, 25, 28), whereas in the nervous system, LIF and CNTF mediate a more complex response that involves neuronal survival and differentiation (30, 39, 40, 44). The components of an active LIFR signaling complex, gp130 and LIFR, are distributed widely in the organism, including the nervous system (26). The ability of cells to respond to CNTF appears to depend on the presence of the CNTFα receptor subunit, which is restricted largely to the nervous system (26). CNTF, LIF, and OSM coordinately activate neuropeptide genes in the human neuroblastoma cell line NBFL (43, 47). These cytokines activate transcription of the vasoactive intestinal peptide (VIP) gene in NBFL cells through a 180-bp cytokine-responsive element (43, 45, 47). The structural requirements of the LIFR and gp130 for these effects, and whether they are similar to those observed in hepatic cells, are not known.

In a previous report (6), we documented that the cloned human LIFR is functional when introduced into the human hepatoma cell line Hep3B. The hepatoma cells acquire the ability to bind LIF and to activate cytokine-responsive APP genes. The functional significance of the LIFR cytoplasmic

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domain became apparent by the loss of response to exogenously added LIF in cells expressing a mutant LIFR that lacked the cytoplasmic domain. In complementary experiments, we used chimeric molecules based on the homodimeric G-CSFR extracellular domain to study the function of the cytoplasmic domains of the LIFR and gp130. Both G-CSFR-LIFR and G-CSFR-gp130 chimeras conferred G-CSF responsiveness upon hepatic cells (6, 50), suggesting that each cytoplasmic domain is capable of signal transduction when induced to form a homodimer. Since the G-CSFR-gp130 chimera mimics the natural gp130 homodimer found in the presence of IL-6 and IL-6 receptor, the chimera permits functional analysis of the gp130 cytoplasmic domain in cells already bearing gp130. Similarly, G-CSF binding to the G-CSFR-LIFR chimera promotes the formation of dimers of the LIFR cytoplasmic domain. Such complexes do not mimic the native state of the heterodimeric LIFR-gp130 complex, but because they signal in hepatic cells, they permit analysis of the functional domains of the LIFR cytoplasmic domain in the absence of gp130.

Mutational analyses of gp130 and G-CSFR have shown that the first 60 residues of the cytoplasmic domains of both receptors proved sufficient to mediate a proliferative signal (15, 38). These regions contain two conserved motifs that are critical for function (termed box 1 and box 2) and are necessary and sufficient for delivering proliferation signal by several members of the hematopoietin receptor family (9, 11, 23, 42). When testing G-CSFR in hepatic cells, we recognized that additional sequences of the cytoplasmic domain were needed for achieving activation of IL-6-responsive APP genes (50). In light of the sequence similarity between G-CSFR and LIFR, we asked whether cytokine-mediated transcriptional activation by the LIFR utilizes the analogous region of the cytoplasmic domain. In this report, we show that at least the first 150 amino acid residues of the LIFR cytoplasmic domain are required for signaling to IL-6-responsive gene elements and that a functionally critical sequence between residues 141 and 150 is also represented in both gp130 and G-CSFR. Furthermore, we show that the same sequences are required for signaling in a neuroblastoma cell line, suggesting that similar signal transduction mechanisms may exist in these distinct cell types.

MATERIALS AND METHODS

Expression constructs. To facilitate discussion in this report, the description of each receptor utilizes a numbering system of the amino acid residues of the cytoplasmic domain, starting with the first residue after transmembrane domain as number 1. Previous publications describe the expression vectors for full-length human LIFR(238) (pH-LIFR-FL) (6) and the carboxy-terminally truncated forms LIFR(131) (= pHLIFR-65 [10]) and LIFR(Δcyto) (6); human G-CSFR (phGR clone D7 [29]) and truncated forms G-CSFR(96), G-CSFR(56), and G-CSFR(Δcyto) (50); the chimeric G-CSFR-LIFR(238) containing the extracellular domain of G-CSFR (residues 1 to 601) and the transmembrane and complete cytoplasmic domains of LIFR (residues 823 to 1097) (6); and human CNTFR (6). Truncated versions of the cytoplasmic domains of LIFR and gp130 were produced by using PCR and 3′ oligonucleotides containing 17 nucleotides of target sequence, an in-frame termination codon, and the recognition sequence for *Not*I [e.g., the 3′ oligonucleotide used to construct G-CSFR-LIFR(40) was 5′-AAGCGGCCGCTTATTCCAATGTTTTAAGAG-3′, where the termination codon is underlined]. PCR primers used to construct LIFR(238; Y142A), G-CSFR-LIFR(150; Y142A), and G-CSFR-LIFR(150; Y142F) incorporated the Y142 codon mutation into the 3′ oligonucleotide sequence. The 5′ oligonucleotide for G-CSFR-LIF chimeras was 5′-CCGGAATTCAGTATGTATGTGGTGACAAAG-3′. In the LIFR-based constructs, this resulted in the formation of an *Eco*RI restriction site corresponding to the external face of the transmembrane domain (the gp130-based chimeras used a 5′ PCR primer corresponding to the *Eco*RI site already present in the sequence). PCR products were digested with *Eco*RI and *Not*I and ligated to a PCR-derived *Asp* 718-*Eco*RI-digested fragment encoding the extracellular domain of the G-CSFR and *Asp* 718-*Not*I-digested expression vector pDC302. LIFR(140) and LIFR(150) cytoplasmic domain mutants were constructed from *Asp* 718-*Cel*II-digested pH-LIFR clone 65 (19) and the insert from *Cel*II-*Not*I-digested G-CSFR-LIFR(140) or G-CSFR-LIFR(150). LIFR(131) is identical to pHLIFR clone 65 (19). LIFR(65) was constructed by insertion of linkers encoding an in-frame termination signal at the unique *Cel*II restriction site of pHLIFR clone 65. Internal deletion constructs G-CSFR-LIFR(Δ4-70) and G-CSFR-LIFR(Δ141-150) were constructed by oligonucleotide-directed site-specific mutagenesis on double-stranded DNA templates, using a commercial kit (Stratagene). The inserts of all constructs were sequenced in their entirety. The receptor activities at the cell surface and binding affinities for LIF, OSM, and G-CSF (where appropriate) were determined for many of the receptor forms by transiently transfecting the receptor constructs into CV-1/EBNA cells and measuring the ligand isotherms as described previously (33). The number of ligand binding sites per cell was calculated for the average cell within transfected cell cultures.

Cell lines and cytokines. Rat H-35 hepatoma cells (clone T-7-18) (5), human hepatoma cells Hep3B cells (12), and human neuroblastoma NBFL cells (46) were cultured as described previously. Treatment of hepatoma cells with cytokines occurred in serum-free minimal essential medium. The following cytokines were used at 100 ng/ml: human LIF, OSM, and G-CSF (Immunex), human IL-6 (Genetics Institute), and rat CNTF (Peprotech). Unless indicated otherwise, all treatments of hepatoma cells included 1 μM dexamethasone. To suppress autocrine stimulation of transfected LIFR in Hep3B cells, the cells were treated in the presence of neutralizing antibodies against human LIF (6).

Cell transfection and analysis. DNA was transfected into Hep3B and NBFL cells as a calcium phosphate precipitate (21) and into H-35 cells as a DEAE-dextran complex (31). The following reporter gene constructs were used to obtain highest possible response in the indicated cell lines: pHP(190)OCT in Hep3B cells (4), pβFB(350)CAT in H-35 cells (3), and VH3-LUC in NBFL cells (45, 48). VH3-LUC contains a region (−1330 to −1151) of the human VIP 5′-flanking sequences upstream of 90 bp of the Rous sarcoma virus promoter linked to the luciferase gene (45). On the basis of dose-response analyses, maximal cytokine regulation was achieved by using in the transfection mixture the following concentrations of the expression vector for any of the various receptor types (see Fig. 2): 5 μg/ml for H-35 cells, 0.5 μg/ml for Hep3B cells, and 2.5 μg/ml for NBFL cells. Higher concentrations of receptor expression vector did not detectably enhance the cytokine response; in fact, high-level expression of receptors bearing the extracellular domain of G-CSFR resulted in hepatoma cells in a nonspecific inhibition of the action of the endogenous IL-6-type cytokine receptors, similar to the finding reported earlier

(50). Internal markers for transfection efficiency were pIE-MUP in hepatoma cells (41) and pRSVcat in NBFL cells (46). Following overnight recovery, hepatoma cell cultures were subdivided, and 24 h later, the subcultures were treated with cytokines. NBFL cells were treated without subdividing. After 24 h (hepatoma cells) or 40 h (NBFL cells), the activities of the reporter gene products were determined. The values were normalized to the level of the internal transfection markers and expressed relative to the basal activity of the reporter gene in control cells (defined as 1.0). The cytokine response of the endogenous APP genes in H-35 cells was visualized by immunoelectrophoretic analysis of medium aliquots for the amounts of secreted fibrinogen.

RESULTS

Function of human LIFR in H-35 cells. Recently we have described the reconstitution of response to LIF and OSM in Hep3B cells following expression of a full-length human LIFR cDNA (6). Because the cells displayed a strong autocrine stimulation, an analysis of the functionally relevant receptor domains was complicated. Therefore, we decided to characterize LIFR in H-35 cells, which do not express detectable LIF (2, 36). Although H-35 cells have endogenous LIFR and gp130 and respond to LIF (Fig. 1A), the function of transfected human LIFR in the presence of the endogenous LIFR was recognized by the following prominent effects (Fig. 1): the stimulation by LIF of cotransfected reporter gene constructs containing IL-6-responsive elements was increased 10-fold and the chloramphenicol acetyltransferase (CAT) gene expression was above the level achieved by IL-6; and transfection of human LIFR into rat H-35 cells improved the response not only to LIF but also to CNTF and particularly to OSM. The comparison of control and LIFR-transfected H-35 cells showed a higher magnitude of OSM response than of LIF response, partly because human OSM was less effective than human LIF in stimulating the reporter gene constructs via the endogenous rat LIFR (Fig. 1).

Role of the cytoplasmic domain in LIFR function. To define the contribution of the cytoplasmic domain of LIFR in signal transduction, mutant LIFR forms were generated and assayed in H-35 cells. Stimulation of the cotransfected IL-6-responsive CAT reporter gene construct by LIF, OSM, and CNTF above the level mediated by the endogenous LIFR in control cultures was used as an indicator for the activity of the transfected LIFR forms. Truncation of the LIFR cytoplasmic domain to 150 or 140 amino acids yielded stepwise reduction in the reporter gene regulation (Fig. 1B). Further deletion of the LIFR cytoplasmic domain to 131 or 65 amino acids did not appreciably lower receptor function. Deletion of the entire domain resulted in complete loss of signal above the basal level. From these data, we concluded that at least two cytoplasmic domain regions are necessary for full LIFR signaling function in cells expressing endogenous gp130; one region resides in the segment from residues 140 to 238, and the other residues in the segment from residues 1 to 65.

Identification of the signaling region within the cytoplasmic domain of the LIFR in hepatoma cells. To assess the function of the LIFR cytoplasmic domain independently of gp130, we made a chimeric receptor consisting of the extracellular part of the human G-CSFR linked to the transmembrane and cytoplasmic domain of LIFR (Fig. 2). The chimera with the entire 238-residue LIFR cytoplasmic domain elicited a G-CSF response that was roughly one-half of the response through the LIFR in H-35 cells and one-quarter of that in

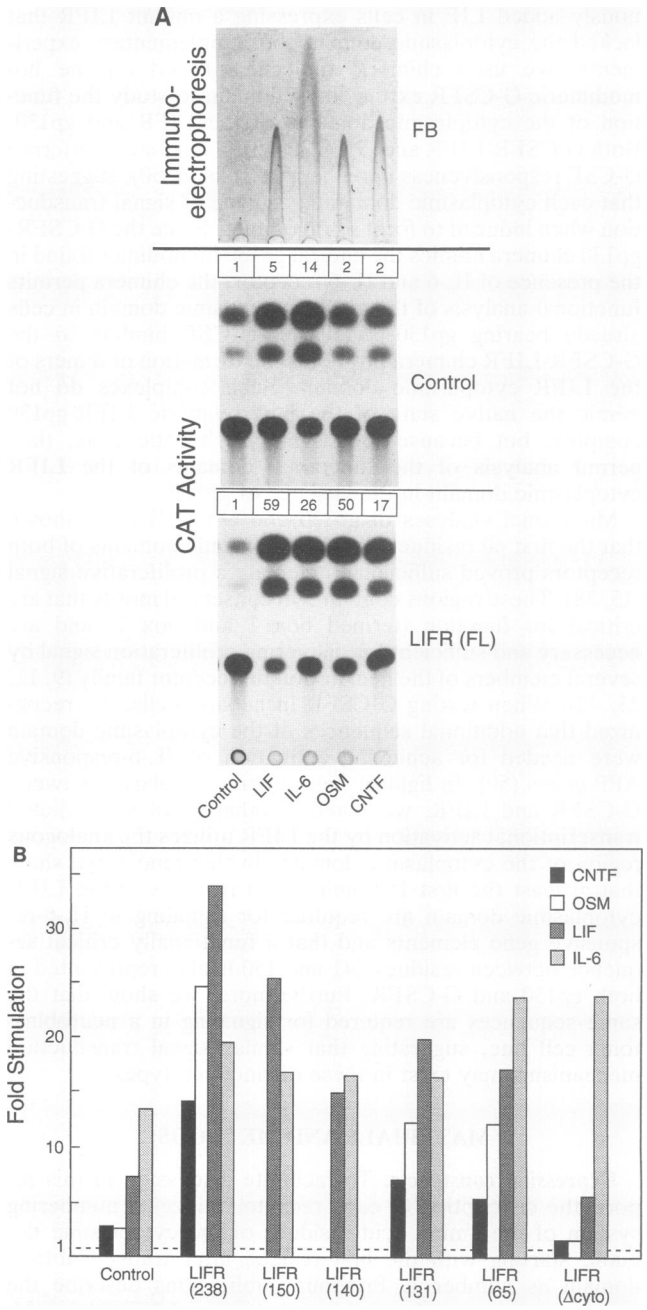

FIG. 1. Activity of human LIFR in H-35 cells. (A) H-35 cells were transfected with plasmid DNA mixtures consisting of pβFB(350)CAT (10 μg/ml), pIE-MUP (2 μg/ml), and either pIE (5 μg/ml) (control) or pHLIFR-FL (5 μg/ml). The subcultures of the transfected cells were treated with the indicated factors. The effect of the treatment on synthesis and secretion of fibrinogen (FB) was quantitated by immunoelectrophoresis of 25 μl of medium. The CAT activity in the cell extract was determined, and the normalized values were calculated relative to the level for untreated control (numbers above autoradiograms). (B) H-35 cells were transfected with pβFB(350)CAT and the expression vectors for the indicated LIFR forms. Subcultures were treated with the listed cytokines. [Cells transfected with LIFR(150) and LIFR(140) were not assayed for CNTF and OSM response.] The specific CAT activities were calculated relative to the level for the control culture. Mean values of two separate experiments are shown.

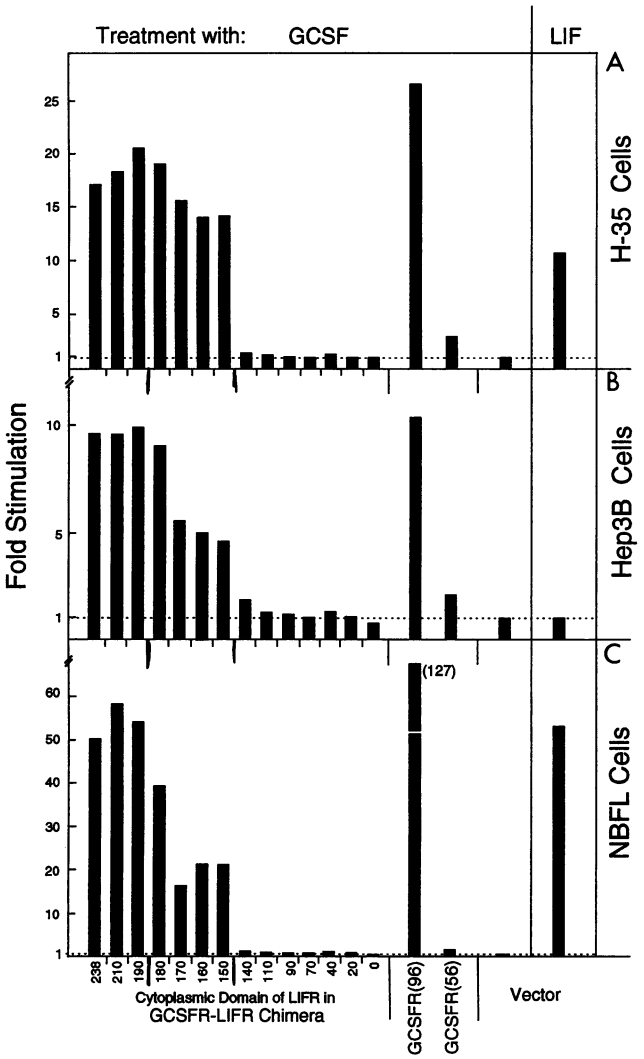

FIG. 2. Functional characterization of the cytoplasmic domain of LIFR. Reporter gene constructs together with expression vectors for the indicated receptor forms (5 μg/ml for H-35 cells, 0.5 μg/ml for Hep3B cells, and 2.5 μg/ml for NBFL cells) were transfected in the cells. Expression of the reporter gene was calculated relative to the level for untreated controls. The results of one representative series for each cell line are shown. Quantitative values are given in Table 1.

Hep3B cells (Table 1). Results for receptors with progressive carboxy-terminal truncations indicated that the sequence beyond residue 190 was not required for maximal G-CSFR function (Fig. 2A and B). Further truncation of the cytoplasmic domain to between residues 180 and 150 resulted in slightly lower activity in H-35 cells and more prominent reduction of activity in Hep3B cells (Table 1). Deletion of the cytoplasmic domain to residue 140 resulted in almost complete loss of the G-CSFR response in both hepatoma cell lines.

To determine whether differences in activity of the various truncation and deletion constructs were attributable to differences in receptor expression at the cell surface or in ligand affinity, we measured binding of LIF, OSM, and G-CSF to a variety of the constructs transiently expressed in CV-1/EBNA cells by Scatchard analyses of binding isotherms (Table 2). All receptor constructs yielded similar levels of receptor expression and ligand binding activities. Equivalent receptor activity measurements in transfected H-35 cells were not feasible because of the much lower transfection efficiency and less prominent activity of the cytomegalovirus promoter in this cell line. Comparable expression of receptor constructs in H-35 cells, however, could be confirmed at the level of mRNA by Northern (RNA) blot analysis (data not shown). Since the various receptor forms appeared to be appropriately process in CV-1 cells, it was reasonable to assume that an equally efficient processing of the receptor proteins existed in H-35 cells.

By comparing the results from Fig. 1 and 2, we concluded that residues 141 to 238 of the LIFR cytoplasmic domain are necessary for signaling in a context that is independent of both gp130 and the extracellular domain of LIFR. The removal of the same region probably accounts for the reduced activity of the LIFR(140) construct (Fig. 1B). As the region from residues 1 to 140 fails to signal in the G-CSFR-LIFR context but does signal in the LIFR context, residues 1 to 140 appear to cooperate with gp130.

The region between 141 to 150 in the LIFR cytoplasmic domain important to G-CSF signaling (Fig. 2) contains a tyrosine residue in position 142 (Fig. 3). The potential involvement of tyrosine 142 in signaling by G-CSFR-LIFR(150) was determined by mutating it to phenylalanine (Y142F) or alanine (Y142A). Both mutant receptor forms were substantially less active than the wild-type form in either hepatoma cell line (Table 1). These results suggested an essential function for the sequence from residues 141 to 150, in particular tyrosine Y142. We therefore deleted residues 141 to 150 from G-CSFR-LIFR(238), but this mutation did not significantly impair the function of the receptor in hepatoma cells (Table 1). Moreover, the mutation Y142A in full-length LIFR did not significantly affect the function of the receptor (Table 1). These data suggest that additional independently active elements exist outside the segment from residues 141 to 150 of the cytoplasmic domain region. The locations and identities of these elements remain to be determined.

The relevance of the membrane-proximal region containing the box 1 and box 2 motifs for the signaling reaction was assessed by deleting residues 4 to 70 from G-CSFR-LIFR(238). The mutant receptor form G-CSFR-LIFR(Δ4-70) was unable to elicit a G-CSF response in transfected cells (Table 1), suggesting that the functional domain that we have defined carboxy terminal to residue 140 acts in concert with boxes 1 and 2.

The LIFR cytoplasmic domain is active in neuronal cells. The structure-function analysis of the LIFR cytoplasmic domain indicated similar signaling activities of the different chimeric constructs in two hepatic cell lines, using compatible APP gene elements as target of the signal transduction pathway (Fig. 2; Table 1). We wished to know whether the signaling mechanism activated by LIFR in nonhepatic cells involves the same elements within the cytoplasmic domain. Considering that LIFR is a critical component in neuronal cells as a mediator of LIF and CNTF signals (13, 27), we predicted that the chimeric receptor constructs should also function in neuronal cells. We therefore assayed the G-CSFR-LIFR variants in the neuroblastoma cell line NBFL along with an LIF- and CNTF-responsive VIP-luciferase reporter gene construct (VH3-LUC) (45) (Fig. 2C; Table 1). The relative activities of the receptor forms in NBFL cells were strikingly similar to the activities observed in hepatic cells. LIFR cytoplasmic regions beyond residues 190 were

TABLE 1. Activities of LIFR cytoplasmic domain mutants

Receptor expression vector[a]	Cytokine response (fold stimulation)[a]					
	H-35 cells		Hep3B cells		NBFL cells	
	n	Mean ± SD	*n*	Mean ± SD	*n*	Mean ± SD
G-CSFR-LIFR(238)	6	20.0 ± 7.2	3	8.4 ± 2.2	8	51.8 ± 26.0
G-CSFR-LIFR(190)	4	24.0 ± 6.1	6	11.1 ± 4.1	4	50.2 ± 21.6
G-CSFR-LIFR(180)	4	17.5 ± 4.4	3	7.5 ± 1.8	4	29.0 ± 13.3
G-CSFR-LIFR(170)	3	15.7 ± 2.9	3	5.1 ± 1.1	3	14.2 ± 5.8
G-CSFR-LIFR(150)	4	15.4 ± 2.5	3	4.4 ± 0.9	6	23.3 ± 13.5
G-CSFR-LIFR(140)	3	1.8 ± 0.6	3	1.5 ± 0.5	3	2.0 ± 0.9
G-CSFR-LIFR(150; Y142F)	3	6.5 ± 0.8	3	1.7 ± 0.7	3	4.8 ± 1.7
G-CSFR-LIFR(150; Y142A)	3	5.1 ± 1.3	1	1.8	3	4.0 ± 2.4
G-CSFR-LIFR(Δ141-150)	3	20.4 ± 7.0	3	5.8 ± 4.2	3	49.3 ± 21.2
G-CSFR-LIFR(Δ4-70)	2	0.9 ± 0.1		ND[b]	3	1.0 ± 0.1
LIFR(238)(LIF)	8	40.1 ± 17.8	3	36.3 ± 2.7		ND
LIFR(238; Y142A)(LIF)	4	32.6 ± 8.5	2	30.3 ± 4.0		ND
Controls						
LIF	7	6.4 ± 4.6	3	1.0 ± 0.1	6	65.1 ± 29.0
IL-6	25	26.5 ± 8.9	10	16.9 ± 7.8	4	1.2 ± 0.6

[a] stimulated with G-CSF except for LIFR(238)(LIF), LIFR(238; Y142A)(LIF), and the two controls.
[b] ND, not determined.

not required for G-CSF-mediated activation of VH3-LUC in NBFL cells (Fig. 2C). Deletion between residues 190 and 170 reduced G-CSF-mediated gene activation, and deletion beyond residue 150 inactivated the chimeric receptor (Table 1). As in hepatic cells, mutation of tyrosine residue 142 in G-CSFR-LIFR(150), but not a deletion of residues 141 to 150 from G-CSFR-LIFR(238), resulted in a lower G-CSF-dependent reporter activation (Table 1). One noteworthy difference in the relative activities of the various G-CSFR-LIFR chimeras between NBFL cells and hepatoma cells was observed for the constructs with deletion between residues 190 and 180, which showed a more prominent reduction in G-CSF-mediated gene activation in NBFL cells than in hepatic cells (Table 1). Taken together, these results suggest that the intracellular signal transduction machinery recognizes common receptor structures in neuronal cells and in hepatic cells.

The functional sequences in LIFR are similar to those in G-CSFR and gp130. Comparison of the amino acid sequences of the cytoplasmic domains of LIFR, gp130, and G-CSFR (19) indicated that the functionally important region from residues 141 to 150 in LIFR is not found at similar positions in gp130 and G-CSFR. The cytoplasmic domain of G-CSFR (isoform D7) consists only of 130 amino acids (13), and previous analyses in human hepatoma cells indicated that signaling is accomplished with a 96-amino-acid segment (50). Transfection of G-CSFR(96) into H-35, Hep3B, and NBFL cells revealed that this receptor form mediated a prominent G-CSF response in all three cell types (Fig. 2). Truncation of the G-CSFR cytoplasmic domain to 56 amino acids was accompanied by the loss of most of the signaling activity, suggesting that the signal transduction mechanism in hepatic and neuronal cells requires structural information provided by the region from residues 57 to 96 and that this region performs a function similar to that of residues distal to 141 of LIFR.

To identify the equivalent functional regions of gp130, we constructed a series of cytoplasmic deletions in the chimeric

TABLE 2. Expression and activities of the cloned receptor forms in CV-1/EBNA cells

Receptor construct	No. of receptors/cell (10^3)	Binding affinity (M)		
		G-CSF	LIF	OSM
G-CSFR-LIFR(238)	64	9.6×10^8		
G-CSFR-LIFR(Δ141-150)	42	1.1×10^9		
G-CSFR-LIFR(150)	69	1.4×10^9		
G-CSFR-LIFR(150; Y142F)	70	1.5×10^9		
G-CSFR-LIFR(150; Y142A)	115	1.3×10^9		
G-CSFR-LIFR(140)	203	1.2×10^9		
G-CSFR-LIFR(70)	300	4.5×10^9		
G-CSFR-LIFR(40)	120	1.0×10^9		
LIFR(238)	17		5.4×10^8	ND[a]
LIFR(238) + gp130	27		3.8×10^9	9.7×10^9
LIFR(150)	13		5.2×10^8	ND
LIFR(150) + gp130	16		6.6×10^9	1.3×10^{10}
LIFR(140)	13		5.0×10^8	ND
LIFR(140) + gp130	8		6.5×10^9	7.3×10^9
LIFR(Y142A)	11		5.0×10^8	ND
LIFR(Y142A) + gp130	12		5.0×10^9	7.6×10^9

[a] ND, not determined.

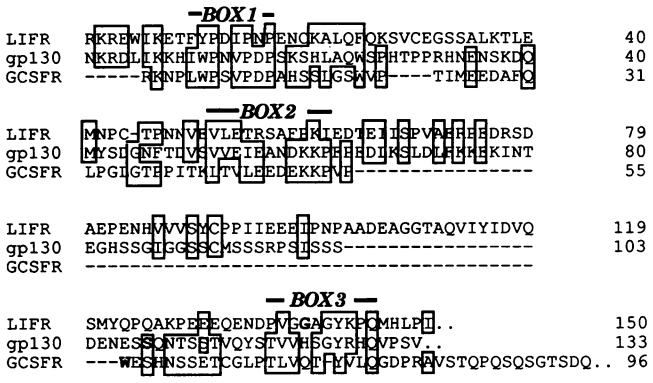

FIG. 3. Comparison of the functional regions in LIFR, gp130, and G-CSFR. The cytoplasmic domains of LIFR, gp130, and G-CSFR minimally necessary for signaling are aligned to demonstrate sequence motifs similarities between boxes 1 to 3.

receptor of G-CSFR-gp130 similarly as done for G-CSFR-LIFR described above. These constructs were assayed in H-35 cells and NBFL cells for the ability to confer G-CSF responsiveness (Table 3). We observed that G-CSFR-gp130(277), among all of the receptor constructs used in this study, was the most active in both cell types. The large magnitude of stimulation achieved in NBFL cells probably contributed to the substantial variation in the measured reporter gene expression. Progressive truncation of the cytoplasmic domain to position 133 lowered the signaling activity of the G-CSFR-gp130 chimera. The reduction was more prominent in NBFL cells than in H-35 cells. Unexpectedly, the relative activity of G-CSFR-gp130(133) was much higher in H-35 cells than in NBFL cells. The cause for this difference is unknown. With additional deletion to residue 109, the chimeric receptor lost essentially all signaling activity in both cell types. We verified in transiently transfected CV-1/EBNA cells that the G-CSFR-gp130 constructs used in Table 3 yielded similar surface expression of G-CSF binding activity (data not shown) (49).

Comparison of the functionally relevant region from residues 109 to 133 of the gp130 cytoplasmic domain with those of the LIFR and G-CSFR (Fig. 3) suggested that the carboxy-terminal sequences of the minimal constructs that retain activity contain a domain of weak homology (termed box 3). This domain consists of a central tyrosine residue in a context that is shared between LIFR and gp130 (Val-X-X-X-Gly-Tyr-Lys/Arg-X-Gln, where X is any amino acid) but in which the conservation of other residues is not strong. The homologous region of G-CSFR includes a tyrosine but is, in general, more similar to the corresponding region in gp130 than to that in LIFR. The functional relevance of the tyrosine residues in box 3 of gp130 and G-CSFR has yet to be established.

TABLE 3. Activities of G-CSFR-gp130 chimeric receptors with cytoplasmic domain deletions

Receptor expression vector	Cytokine response (fold stimulation)			
	H-35 cells		NBFL cells	
	n	Mean ± SD	*n*	Mean ± SD
G-CSFR-gp130(277)	8	66.6 ± 25.9	3	156 ± 64
G-CSFR-gp130(230)	3	40.1 ± 12.5	3	115 ± 45
G-CSFR-gp130(165)	3	15.7 ± 4.2	3	45 ± 21
G-CSFR-gp130(133)	4	49.7 ± 8.7	3	29 ± 18
G-CSFR-gp130(109)	3	1.4 ± 0.1	3	1.6 ± 0.7
G-CSFR-gp130(91)	3	1.0 ± 0.1	3	1.8 ± 0.7
G-CSFR-gp130(65)	2	0.6 ± 0.1	3	1.9 ± 0.1
G-CSFR-gp130(40)	3	1.0 ± 0.2	3	1.2 ± 0.1

DISCUSSION

In this paper, we have reported the following findings. (i) LIFR requires discrete cytoplasmic regions for signaling in hepatic cells. The extracellular and transmembrane regions and the first 65 amino acid residues of the cytoplasmic domain, in combination with gp130, mediate weak signaling. The cytoplasmic domain from residues 141 to 238 is needed for full signaling by the LIFR-gp130 complex or for signaling activity in the context of a G-CSFR-LIFR chimera. (ii) The amino acid architecture of the functionally relevant sequences within the cytoplasmic domain of LIFR is similar to that of gp130 and G-CSFR. (iii) The same structural elements in the cytoplasmic domains of LIFR, gp130, and G-CSFR appear to mediate signaling in hepatic and neuronal cells.

The results have been incorporated into a refinement of a preexisting model of the functional LIF-OSM receptor complex (Fig. 4). In experiments described elsewhere, proliferative response to LIF and OSM in transfected BAF-B03 cells requires expression of both LIFR and gp130 (20). gp130 is expressed in the hepatoma and neuroblastoma cell lines used in the present study. The minimal receptor sequence requirement for gaining transcriptional activation of IL-6-responsive target genes appears to be either a pairing of the first 65 amino acid residues of the cytoplasmic LIFR domain with gp130 or a homodimer of the first 150 amino acid residues as a G-CSFR-LIFR chimera. The response generated by any of the G-CSFR-LIFR chimeras is substantially less than that achieved by 96-residue cytoplasmic domains of G-CSFR (Fig. 2) or the 277-residue domain of gp130 in hepatoma cells (Table 3). The difference in the magnitude of the response may reflect the affinity of each cytoplasmic domain for the molecules of the signal transduction machinery.

Cooperativity of the cytoplasmic domains of gp130 and G-CSFR does not occur since, as described previously (6), the chimeric receptor consisting of the LIFR extracellular domain and G-CSFR cytoplasmic domain is inactive. This negative result also appears to indicate that formation of homodimers by the chimeric receptor LIFR-G-CSFR does not occur. Moreover, from the observation that G-CSFR constructs expressed in transfected cells did not enhance the cell response to either IL-6 (hepatoma cells) or LIF, we conclude that there is no productive interaction between the introduced G-CSFR forms and the endogenous ligand-activated gp130 or LIFR. In contrast, as noted previously (50), high-level expression of any construct carrying the extracellular domain of G-CSFR leads to a nonspecific G-CSF-independent attenuation of the cell responsiveness to all IL-6-type cytokines. The molecular basis for that inhibitory action is yet to be defined.

The cytoplasmic domain sequence from residues 141 to 150 is critical for the activity of the G-CSFR-LIFR(150) chimera (Fig. 2). The mutation of tyrosine 142 detectably affects signaling of this construct in hepatoma and neuroblastoma cells (Table 1), suggesting a functional role for this residue in cytokine-mediated gene activation. However, the sequence context surrounding tyrosine 142 is not a recognized target of a known protein tyrosine kinase, nor has it

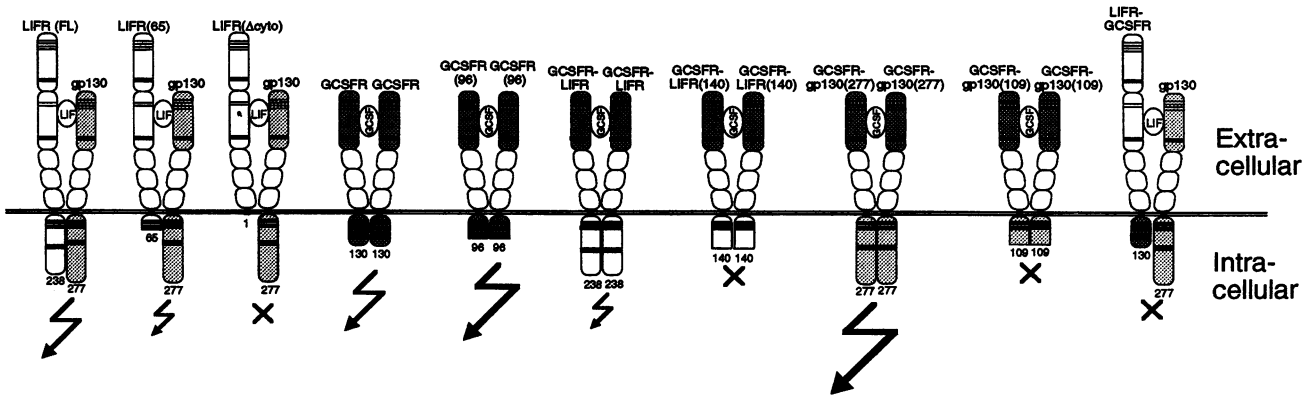

FIG. 4. Signaling action of receptor subunits and chimeras.

been established whether tyrosine 142 is phosphorylated. The decapeptide from residues 141 to 150 or tyrosine 142 is not absolutely critical for the function of the entire cytoplasmic domain, since in its absence, both the LIFR and G-CSFR-LIFR constructs are still active. Other sequences carboxy terminal to residue 150 may contain additional structural information important to the signaling machinery. These presumed functional regions are not recognizable as repeated sequence motifs of the 141-to-150 sequence. The gradual loss of receptor activity by sequential deletions of two decapeptide segments from residues 190 to 170 (Table 1) might indicate the loss of contributing functional sequences. A similar multiplicity of functional sequences is predicted for the cytoplasmic domain for gp130. The truncation to residue 133 has reduced substantially the signaling activity of the G-CSFR-gp130 chimera, particularly in NBFL cells, suggesting that full receptor action requires a cooperativity of receptor elements that exceeds that provided by boxes 1 to 3. Moreover, the recognition of these sequences seems to be subject to cell-type-specific differences. Further structural-functional dissection of the LIFR and gp130 cytoplasmic domains will likely provide definitive information about the identify and complexity of the predicted regulatory sequences.

The present results highlight an important difference between the receptor-mediated control of differentiated cell functions and proliferation. The signaling event measured in hepatoma and neuroblastoma cells as transcriptional activation requires more receptor sequences than does the event for activating proliferation of pre-B-cell lines by the same receptor subunits. A proliferative signal is accomplished by gp130 containing only a 61-amino-acid cytoplasmic domain (38) and by G-CSFR containing 56 residues (15, 50). The activity of these minimal receptor forms has been ascribed in part to the involvement of the two regions with shared sequence motifs, box 1 and box 2. The notion that the two boxes are relevant for achieving a mitogenic signal has been supported by the finding of similar activities for the analogous regions of the related growth hormone receptor (9), IL-2 receptor β (23), and erythropoietin receptor (11, 34, 42). On the basis of sequence comparison, Fukunaga et al. (15) suggested a third sequence motif shared among the cytoplasmic domains of hematopoietin receptors. No functional relevance has yet been demonstrated for this additional box. Moreover, this box is positioned carboxy terminal to box 3 indicated in Fig. 3 and is not contained in the G-CSFR D7 isoform (29).

The sequence information of the functional receptor domains available thus far does not indicate a similarity to interaction sites defined for known signal-transducing proteins such as kinases, G proteins, or SH-2/SH-3-containing proteins. However, the similar structural requirements for cytokine receptor function in hepatic and neuronal cells suggest that components of the signaling machinery which are required for cytokine-mediated cellular differentiation are similar, if not identical, in hepatic and neuronal cells. By extension, we envision that the same mechanism might be operative in other cell types upon which LIF is active, such as monocytic cells (22, 32), or other neuronal cell types in which CNTF is active. The identities of the signaling molecules generated at the receptor and targeted to the APP and VIP genes are unknown. From the observations that β-fibrinogen and haptoglobin gene elements are not regulated by IL-6-type cytokines in nonhepatic cells (36) and cytokine-responsive elements in the VIP gene are not responsive to IL-6 or LIF in HepG2 cells (45), we assume that cell-type-specific signal molecules and/or signal-targeted transcription factors exist. Although the treatment of a variety of cell lines with IL-6-type cytokines is accompanied by immediate changes in the phosphorylation of cellular proteins, including gp130 (1, 25, 32, 35, 37, 38), the activation of a specific kinase or change in phosphorylation state of a transcription factor has yet to be causally linked to the regulation of APP or neuropeptide genes (8, 35).

Our finding of a cooperativity of multiple receptor elements to signal the activation of differentiated cell functions by IL-6-type cytokines directs our future studies to identify (i) the molecules from different cell types recognizing the functional cytoplasmic domain structure that includes boxes 1 to 3 of LIFR, G-CSFR, and gp130, (ii) the intracellular signal transduction machinery, and (iii) the transcription factors mediating gene activation. The current knowledge of cytokine action derives primarily from findings made in systems involving proliferation control. The data described here suggest that the signaling mechanisms of cytokine-mediated cell differentiation and cell proliferation are different. Defining the underlying mechanistic difference is the present challenge.

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