Communication

Reconstitution of the Response to Leukemia Inhibitory Factor, Oncostatin M, and Ciliary Neurotrophic Factor in Hepatoma Cells*

(Received for publication, November 16, 1992, and in revised form, January 14, 1993)

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Ciliary neurotrophic factor (CNTF) has been described as a neuro-active cytokine that shares functional similarities with the leukemia inhibitory factor (LIF). We demonstrate here that, like LIF, CNTF stimulates expression of acute phase plasma proteins in rat H-35 hepatoma cells. Transfection of the LIF receptor into Hep3B hepatoma cells reconstituted LIF and oncostatin M regulation of acute phase plasma protein genes. Co-expression of the LIF receptor and the CNTF receptor, but not expression of either subunit alone, generated CNTF responsiveness in Hep3B cells, suggesting cooperativity of these receptor subunits. Evidence is presented for direct interaction of the LIF receptor with the intracellular signal transduction machinery.

The coordinated synthesis of a set of plasma proteins in the liver is one of the manifestations of the systemic response to inflammation (1). Several distinct cytokines, including interleukin-6 (IL-6), LIF, IL-11, and OSM are able to elicit a qualitatively similar response in liver cells (2–7). The usage of common receptors (LIF and OSM) (8) or a common signal transducing subunit (gp130) (9–11) has been proposed to be the cause for the redundancy of action of these cytokines. Characterization of different hepatic cell lines indicated the existence of different spectra of cytokine responses. H-35 and Fao rat hepatoma cells respond to IL-6, LIF, OSM, and IL-11; HepG2 human hepatoma cells to IL-6, LIF, and OSM; and Hep3B cells to IL-6 alone. A common affinity convertor, gp130, has recently been described for the IL-6 and LIF/OSM receptors (9), but, conversely, differences do exist in the response to these cytokines. For example, IL-6 stimulation of thiostatin gene expression in H-35 cells is strongly enhanced by dexamethasone, but LIF stimulation is inhibited (12). Therefore, the presence of the common gp130 subunit in each receptor does not necessarily trigger identical signaling pathways.

Subunits of the receptors for IL-6 (10, 13, 14), LIF (15), and OSM (9, 16) are related in structure to a large family that includes subunits of the GCSFR (17) and CNTFR (18). The intracellular domains of the LIFR, gp130, and GCSFR display extensive homology (15). GCSFR is thought to act as a high-affinity homodimer (19), whereas the LIFR and gp130 combine to form a heterodimeric high affinity LIF/OSM receptor (9). A three-subunit model for the CNTF receptor complex has been suggested involving the low affinity CNTF receptor, gp130, and the LIF receptor (11). In order to define the nature of the functional LIF/OSM receptor, we attempted to reconstitute the cloned LIF receptor into non-LIF/OSM-responsive Hep3B cells. Similarly, transfection of the LIF and CNTF receptor cDNAs into Hep3B and HepG2 cells was used to reconstitute functional CNTF receptors in hepatoma cells.

EXPERIMENTAL PROCEDURES

Expression Constructs—For all expression constructs, the cyto-megalovirus promoter-based plasmid vector pDC302 was used (15). The full-length human LIFR construct (pHLIFR-FL) was made by ligation of a genomic clone encoding the cytoplasmic domain to the truncated cytoplasmic domain of pHLIFR-65 via a common Cell restriction site (15). A 2.4-kilobase pair Asp718-Xmml fragment of pHLIFR-65 was ligated to Asp718-Smal-digested pDC302 and resulted in a 746-amino acid soluble form of the hLIFR, terminating immediately in vector sequences (LIFR-soluble). A DNA fragment encoding amino acid residues 1–862 of the hLIFR was produced using the polymerase chain reaction and resulted in a clone encoding the extracellular domain of the GCSFR (residues 1–601) (17) and the transmembrane and complete cytoplasmic domain of the hLIFR (residues 823–1097) (15) was conducted by ligation of two polymerase chain reaction fragments. The clone encoding the human CNTFR was isolated from a human brain cDNA library using oligonucleotides based on the published sequence (18). The inserts of all constructions were sequenced. Binding affinities for LIF, CNTF, or GCSF, (where appropriate) corresponded to those previously described (15, 17, 18) when the cDNAs were expressed in COS-7 cells.

Cell Lines, Cytokines, and Antibody Preparations—Rat H-35 hepatoma (clone T-7–18) (20), HepG2 (21), and Hep3B (22) cells were cultured as described. Treatments with cytokines occurred in serum-free minimal essential medium. The following cytokines were used at 100 ng/ml: human LIF, OSM, and GCSF (Immunex), human IL-6 (Genetics Institute), and rat CNTF (Peprotech). Unless indicated, all treatments included 1 μM dexamethasone. To inhibit endogenous LIF, neutralizing antibodies to human LIF, either rabbit polyclonal (5) or monoclonal D25.1.4 and D62.3.2 (generously provided by Dr. K. Jin Kim, Genentech), were included in the culture medium.

Transfections and Plasma Protein Gene Expression Assays—DNA was transfected into HepG2 and Hep3B cells as a calcium phosphate precipitate (26) and into H-35 cells as a DEAE-Dextran complex (27). The following reporter gene constructs were used: pIRR(F2xIL-6RE)ECT, containing two tandem copies of the 34-base pair IL-6RE of the rat β-fibrinogen gene in pC7 (28), and pHIL130-OCt, containing the rat thiostatin gene promoter from —165 to +25 in pC7 (24). pE-MUP served as an internal marker for transfection efficiency in all experiments (25). Following overnight recovery, the cell cultures were subdivided, and 24 h later, the subcultures were treated with cytokines and/or dexamethasone. After another 24 h, the me-
To obtain stable LIFR transfectants of Hep3B cells, pHILFR-FL, together with pSV2neo, was transfected and cells selected using 1 mg/ml G418. Subclones were chosen on the basis of LIFR mRNA expression, LIF and OSM surface binding, and LIF-induced stimulation of haptoglobin and fibrinogen synthesis. Polyadenylated RNA was subjected to Northern blot analysis according to standard procedures.

RESULTS AND DISCUSSION

Transient transfection of a LIFR expression construct with the cytokine-responsive CAT gene reporter constructs pHp(19O)OCT or pPFG(2xIL-GRE)CT into Hep3B cells, resulted in two effects. 1) The basal activity of the CAT gene construct was elevated 8-fold (Fig. 1A); 2) LIF and OSM stimulated CAT expression above this basal level and were equally effective (Table I). The IL-6 response was unaffected.

The elevated basal stimulation was caused, in part, by endogenous LIF production. Northern blot analysis revealed the presence of LIF mRNA, and incubation of Hep3B cells with neutralizing LIF antibodies decreased basal activity of the reporter gene by as much as 60% (Fig. 1C). Antibodies against OSM were not effective (data not shown). LIFR appeared to function as well as the endogenous IL-6 receptor, since stimulation by OSM (in the presence of anti-LIF) was comparable to IL-6; no additive action was observed with any combination of LIF, OSM, and IL-6. Endogenous acute phase protein genes were similarly affected by transfection of LIFR. A stable cell line, Hep3B-LIFR, was derived which expressed LIFR mRNA and displayed small numbers of high affinity cell surface LIF and OSM binding sites (LIF: site number per cell (R) = 760, equilibrium dissociation constant (Kd) = 1.1 x 10^{-10}, OSM: R = 129, Kd = 1 x 10^{-10}), and responded to LIF treatment by an increased synthesis of fibrinogen (Fig. 1B), and haptoglobin (data not shown). Hep3B-LIFR cells showed a 2-3-fold increase in basal fibrinogen and haptoglobin expression that was partially reduced with anti-LIF, suggesting that these cells also experience autocrine stimulation.

Expression of truncated LIFR forms revealed two interesting features (Table I). 1) With deletion of the cytoplasmic domain, the response to exogenously added LIF/OSM was lost; 2) the 6-8-fold elevated basal expression of the reporter gene construct was maintained in all cells receiving the extracellular domain of LIFR, regardless of whether this was part of a membrane-associated or a soluble form. Since this basal activation was virtually unaffected by anti-LIF, we hypothesize that the truncated LIFR protein exerts either a ligand-independent action possibly via binding to signal transducing subunits, or autocrine stimulation occurs intracellularly and,
TABLE I
Activity of LIFR in transiently transfected Hep3B cells
Hep3B cells were transfected with a plasmid DNA mixture composed of 15 µg/ml pSP64(190)OCT, 2 µg/ml pIE-MUP, and 5 µg of the indicated receptor expression vectors. All treatments except with LIF were carried out in the presence of neutralizing LIF antibodies. The values represent normalized CAT activities (means of 2 separate experiments are shown). ND, not determined.

| Treatment | Receptor expression vector |
|-----------|---------------------------|
|           | Control | LIFR (FL) | LIFR (Δcyto) | LIFR (soluble) | Mouse LIFR (soluble) |
| No addition | 1.0 | 6.6 | 8.0 | 6.6 | 1.0 |
| IL-6       | 20.4 | 25.2 | 22.3 | 23.7 | 15.2 |
| LIF        | 1.0  | 35.0 | 10.0 | 8.1 | 1.0 |
| OSM        | 1.0  | 33.0 | 11.0 | ND   | ND  |

TABLE II
Activity of chimeric receptors in Hep3B cells
Hep3B cells were transfected with a plasmid DNA mixture composed of 15 µg/ml pSP64(190)OCT, 2 µg/ml pIE-MUP, and 5 µg of the indicated receptor expression vectors. The values represent normalized CAT activities (means of at least 2 experiments are shown). ND, not done.

| Treatment | Receptor expression vector |
|-----------|---------------------------|
|           | Control | LIFR | GCSFR | LIFR-GCSFR | GCSFR-LIFR | GCSFR-gp130 |
| No addition | 1.0  | 7.6 | 0.7 | 3.9 | 1.1 | 1.0 |
| IL-6       | 18.4 | 14.7 | 6.0 | 17.0 | 7.1 | 5.0 |
| GCSF       | 0.9  | 6.9 | 17.1 | 3.6 | 9.2 | 30.7 |
| LIF        | 1.4  | 13.3 | 0.8 | 4.3 | 0.7 | ND |

Therefore, is inaccessible to anti LIF inhibition. Surprisingly, expression of soluble mouse LIFR (Table I), which is capable of binding human LIF (15), or addition of purified soluble mouse LIFR and LIF (data not shown) was unable to elicit a basal level stimulation of the reporter gene construct, suggesting that interaction of the extracellular domain of LIFR with any signal transducing receptor subunit is dependent upon species-specific structural features.

Since the extracellular domain of LIFR exerted such a prominent effect on the signaling event, the precise contribution of the cytoplasmic domain, if any, was not clearly apparent. gp130 has been described as a common signal transducing subunit for IL-6-type cytokine receptors (11, 18, 28). LIFR has a 238-amino acid cytoplasmic domain, which is highly conserved between man and mouse (87% identity) and which is homologous to gp130 (9, 15). This extensive homology between LIFR and gp130 suggests that the cytoplasmic domain of LIFR may act as a signal transducer and may contribute to the LIF/OSM response. Since there are no hepatic cell lines that lack gp130, we decided to assess the function of the cytoplasmic domain of LIFR as part of a GCSFR extracellular domain-LIFR transmembrane and cytoplasmic domain (GCSFR-LIFR) chimera (Table II). The functional form of the GCSFR is a homodimer that does not require gp130 for function (19, 29). We have previously observed that transfected GCSFR is functional in hepatic cells and that substitution of the cytoplasmic domain of gp130 onto the GCSFR extracellular domain confers GCSF responsiveness to Hep3B cells (Table II and Ref. 30). A GCSFR-LIFR chimera expression construct was able to confer GCSF responsiveness on Hep3B cells (Table II), HepG2 cells, and Hep-3 cells (data not shown). The chimera receptor did not yield a basal level activation of the reporter gene construct as seen for LIFR but was consistently less effective than the GCSF-

**Fig. 2. CNTF response in H-35 cells.** Confluent monolayers of H-35 cells were treated in duplicate for 24 h with serum-free medium containing the indicated serially diluted factors. The amount of fibrinogen and thioatin secreted was measured by rocket immunoelectrophoresis. Mean values of the duplicate cultures are shown.

**TABLE III**
Reconstitution of CNTFR function in Hep3B cells
The indicated receptor expression vectors were tested in Hep3B cells using pHp(190)OCT as the reporter gene construct as described in Fig. 1. All treatments were carried out in the presence of neutralizing CNTF antibodies. Means ± S.D. of 3 separate experiments are shown; last column represents mean of two experiments.

| Treatment | Receptor expression vector |
|-----------|---------------------------|
|           | Control | LIFR | CNTFR | LIFR + CNTFR | LIFR (aceto) + CNTFR |
| No addition | 1.0  | 6.0 | 2.6 | 1.1 | 0.7 | 5.4 | 1.4 | 9.1 |
| IL-6       | 24.4 | 7.1 | 31.1 | 12.9 | 30.4 | 7.8 | 26.9 | 4.3 | 21.6 |
| CNTF       | 0.9  | 0.1 | 8.8 | 3.4 | 1.6 | 0.6 | 18.6 | 6.4 | 7.6 |
| OSM        | 1.0  | 0.2 | 27.2 | 10.2 | 1.3 | 0.2 | 29.8 | 8.0 | 8.8 |

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of endogenous LIF, the cytokine treatments were carried out in the presence of anti-LIF antibodies. While LIFR-transfected cells responded to OSM, CNTF was only minimally active. An equally low CNTF response was recorded in Hep3B cells that had been transfected with CNTFR. Only co-expression of both receptors yielded a 3-fold enhanced CNTF response, although this was only half of the OSM response. Using LIFR with a deleted cytoplasmic domain, no CNTF response was achieved. These results support the proposal (11) that CNTFR cooperates with gp130 and LIFR in order to generate CNTF signal transduction. However, the data also indicate that the subunit combination in Hep3B cells was less effective in CNTF signal transduction than it was for OSM signal transduction. To achieve both CNTF and LIF/OSM response, intact LIFR was required.

HepG2 cells responded to CNTF in a fashion similar to LIF. CNTF and LIF were not additive in action, and OSM caused a much greater stimulation (Table IV). HepG2 cells express both the LIF/OSM receptor and the OSM-specific receptor. CNTFR-transfected HepG2 cells displayed a higher response to CNTF than control transfectants but no elevated LIF or OSM response (Table IV). These data suggest that either CNTFR does not enhance LIF or OSM signaling by the LIFR-gp130 complex, or that CNTFR can interact with other receptors on the surface of HepG2 cells. One candidate on HepG2 cells is a second receptor that binds OSM but not LIF (8).

These data, together with the previous findings (9, 11), suggest an expansion of the model for the function of the IL-6 receptor-related subgroup of the hematopoietin receptor family. In order to transduce a signal, dimers of intracellular domains may be required. Such active dimers have been documented for LIFR (Table II), gp130 (30), and LIFR-gp130. A variety of subunit combinations between ligand-binding subunits (IL-6R, CNTFR and probably IL-11R) and signaling subunit dimers is likely. Involvement of heterotrimERIC combinations cannot yet be ruled out, as previously suggested for the high affinity receptor for LIF, OSM, and IL-6 (9). Heterotrimeric complexes seem to be necessary for CNTF responsiveness and would include CNTFR-gp130-LIFR (Table III) and possibly CNTFR-gp130-OSMR (Table IV). We have been unable to detect CNTFR mRNA by Northern blot analysis in HepG2 or H-35 cells despite their ability to respond to CNTF. We concluded that either CNTFR might be able to interact weakly with the LIFR-gp130 dimer and that expression of CNTFR enhances this interaction (Tables III and IV), or CNTFR is acting in these cells via a yet to be defined second form of CNTFR. Additional work will be required to understand which combinations of subunits direct cellular responses and to determine how these subunits are linked to the intracellular signal transduction apparatus.

Acknowledgments—We thank Mike Comeau and Della Friend for excellent technical assistance; Jackie McGourty for LIF and OSM; Susana P. Campos, Linda Park, Bethina Thoma, David Cosman, Steven Gillis, and Steven Dower for continued support and comments on the manuscript; Marcia Held for secretarial assistance; and Dr. K. Jin Kim for the neutralizing anti-LIF antibodies.

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