Receptor Recognition Sites of Cytokines Are Organized as Exchangeable Modules

TRANSFER OF THE LEUKEMIA INHIBITORY FACTOR RECEPTOR-BINDING SITE FROM CILIARY NEUROTROPHIC FACTOR TO INTERLEUKIN-6*

Karl-Josef Kallen, Joachim Grötzinger†, Eric Lelièvre§, Petra Vollmer, Dorthe Aasland, Christoph Renné, Jürgen Müllerberg, Karl-Hermann Meyer zum Büschenfelde, Hugues Gascan§, and Stefan Rose-John†

From the I. Medizinische Klinik, Abteilung Pathophysiologie, Johannes Gutenberg Universität Mainz, Obere Zahlbacher Str. 63, D-55101 Mainz, Germany, and INSERM, C.J.F. 97-08, 4 rue Larrey, CHU Angers, 49033 Angers Cedex, France

(Received for publication, October 2, 1998, and in revised form, January 28, 1999)

Interleukin-6 (IL-6) and ciliary neurotrophic factor (CNTF) are “4-helical bundle” cytokines of the IL-6 type family of neuropoietic and hematopoietic cytokines. IL-6 signals by induction of a gp130 homodimer (e.g. IL-6), whereas CNTF and leukemia inhibitory factor (LIF) signal via a heterodimer of gp130 and LIF receptor (LIFR). Despite binding to the same receptor component (gp130) and a similar protein structure, IL-6 and CNTF share only 6% sequence identity. Using molecular modeling we defined a putative LIFR binding epitope on CNTF that consists of three distinct regions (C-terminal A-helix/N-terminal AB loop, BC loop, C-terminal CD-loop/N-terminal D-helix). A corresponding gp130-binding site on IL-6 was exchanged with this epitope. The resulting IL-6/CNTF chimera lost the capacity to signal via gp130 on cells without LIFR, but acquired the ability to signal via the gp130/LIFR heterodimer and STAT3 on responsive cells. Besides identifying a specific LIFR binding epitope on CNTF, our results suggest that receptor recognition sites of cytokines are organized as modules that are exchangeable even between cytokines with limited sequence homology.

Ciliary neurotrophic factor (CNTF) is a survival and differentiation factor for a variety of neuronal and glial cells (1). Several groups have demonstrated its ability to prevent or slow down neuronal degeneration in animal models of neurodegenerative diseases (2–4). Non-neuronal effects of CNTF include maintenance of embryonic stem cells in an undifferentiated state (5), induction of an acute-phase response in liver cells (6), and a myotrophic effect on denervated skeletal muscles of mice (7).

CNTF belongs to the IL-6 type family of hematopoietic and neurotrophic cytokines that also encompasses interleukin 6 (IL-6), leukemia inhibitory factor (LIF), oncostatin M, cardiotrophin-1, and interleukin 11 (IL-11). All IL-6 type cytokines use a membrane spanning 130-kDa glycoprotein, gp130, as a signal transducing subunit (8, 9). Some IL-6 type cytokines also use the LIF receptor (LIFR) and the oncostatin M receptor for signaling. Despite very limited sequence homology, the IL-6 type cytokines were predicted to share a common structure consisting of four anti-parallel α-helices (A, B, C, and D) connected by two long cross-over loops (AB, CD) and one short loop (BC) (10, 11). This so-called “four-helix bundle” structure represents a fundamental protein fold characteristic of most cytokines (11). Crystallographic and NMR studies have confirmed this structure for IL-6, CNTF, and LIF (12–14).

The biological response to CNTF is elicited by formation of a multiunit receptor complex (15). CNTF first binds in a 1:1 stoichiometry to a glycosylphosphatidylinositol-anchored ligand binding α-unit, CNTF receptor (CNTFRα), which is not involved in signal transduction (16). This is followed by the recruitment of gp130 and LIFR as membrane spanning signal transducing β-units (15), which in turn form a disulfide-linked heterodimer that activates the JAK/STAT and the Ras/MAP kinase pathways (8). Signaling of cardiotrophin-1, LIF, and oncostatin M also occurs via a gp130/LIFR heterodimer. Similar to CNTF, cardiotrophin-1 first binds to non-signaling receptor α-unit (8), whereas LIF and oncostatin M directly bind to LIFR and gp130, respectively (17). In contrast, binding of IL-6 to a non-signaling α-unit, gp80 (IL-6Rα), induces gp130 homodimerization and subsequent activation of Jak/Tyk kinases (8).

Immunoprecipitation experiments in solution as well as biochemical evidence suggested that the IL-6 and CNTF receptor complexes are hexamers consisting of IL-6, IL-6Rα, and gp130 in a 2:2:2 stoichiometry (18, 19) or CNTF, CNTFRα, gp130, and LIFR in a 2:2:1:1 ratio (20). However, recent analyses of crystallographic and mutagenesis data of CNTF suggested a tetrameric complex as the simplest model of the CNTF complex (13, 21). Furthermore, an arrangement of cytokine and cytokine receptors as in the hexameric models of Paonessa and de Serio (18, 20) has been considered to be inconsistent with the known dimensions of IL-6 and CNTF (22). Other authors argued that structural constraints of the above hexameric models would lead to contradictions with the hitherto observed general conservation of the structural arrangement of cytokine receptors (23). A new hexameric model for the IL-6 receptor complex could alleviate these structural objections (23). Adapted to the CNTFR receptor complex it nevertheless implies, as does the model suggested by de Serio et al. (20) that the same site of.
CNTF is able to contact either gp130 or LIFR which rules out the existence of a genuine and specific LIFR-binding site on CNTF.

Mutagenesis studies of CNTF, IL-6, and LIF have identified contact sites of these cytokines with the subunits of their respective receptor complexes. For IL-6 and CNTF the contact site with the receptor α-unit could be mapped to a site that includes residues of the C-terminal AB loop and the C-terminal D-helix (13, 21, 22, 24–28). This site corresponds to site I of growth hormone in its receptor complex (29). Residues of the A- and C-helices of CNTF, LIF, and IL-6 constitute a gp130-binding site which is equivalent to site II of growth hormone in its receptor complex (21, 30–32). In IL-6, a second gp130-binding site consists of amino acids residues of the N-terminal AB loop, the C-terminal CD loop, and the N-terminal D-helix (27, 33). This site is now termed site III in continuation of the growth hormone terminology. Crystallographic and mutagenesis studies of CNTF and LIF indicated that residues of the C-terminal B-helix, possibly the BC loop, CD loop, and the N-terminal D-helix constitute site III in these cytokines (13, 21, 30, 34, 35). These experiments also suggested site III as a potential LIFR binding epitope in LIF and CNTF.

Considering the conserved four-helical bundle structure of most cytokines we reasoned that receptor recognition sites of cytokines might have evolved as discontinuous modules which should principally be exchangeable between different cytokines. A comparison of the homology based IL-6 model and the x-ray structure of CNTF (13, 27) prompted us to define boundaries of the potential LIFR binding epitope of CNTF which encompasses residues of the C-terminal A-helix, the N-terminal AB loop, the BC loop, the C-terminal CD-loop, and the N-terminal D-helix. The transfer of this putative “LIFR binding module” from CNTF to IL-6 resulted in a chimeric IL-6/CNTF molecule that binds to IL-6Rα and signals via a heterodimer of gp130 and LIFR. Effectively, this “module swap” created a new cytokine with LIF-like, but IL-6R dependent activity on cells expressing gp130, IL-6Rα, and LIFR. On a more general basis, our results indicate that cytokines are organized as a set of modules, making specific contacts to different receptors.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Human SK-N-MC neuroblastoma, HepG2 and Hep3B hepatoma and COS-7 cells (bought from ATCC (Manassas, VA)) were routinely grown in RPMI or Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. BAF/3 cells transfected with human gp130 were a kind gift from Immunex (Seattle, WA). BAF/3-(gp130) cells were stably transfected with cDNAs coding for human IL-6R, human CNTFR, or human LIFR as described elsewhere (17). Thus four different cell lines were generated: BAF/3-(gp130,IL-6R) cells, BAF/3-(gp130,LIFR) cells, BAF/3-(gp130,LIFR,IL-6R) cells, and
BAF/3-(gp130,LIFR,CNTFR) cells. The relative expression of the receptors of the IL-6 family on the different BAF/3 cell lines as well as the SK-N-MC cells was analyzed by fluorescence-activated cell sorter analysis using the murine monoclonal antibodies B-S12 (anti-gp130), B-R6 (anti-IL-6R), AN-E1 (anti-LIFR), and AN-D3 (anti-CNTFR) and is shown in Table I. B-S12 and B-R6 have been described in detail before (36), AN-E1 and AN-D3 are newly developed murine monoclonal anti-LIFR and anti-CNTFR antibodies. Anti-STAT3 mAb was obtained from Transduction Laboratories (Lexington, KY), anti-phosphotyrosine mAb 4G10 was bought from UBI (Lake Placid, NY), and the anti-mouse peroxidase-coupled mAb was from BIOSOURCE (Calmarillo, CA). The restriction enzymes NcoI, HindIII, and XbaI were obtained from AGS (Heidelberg, Germany); calf intestinal phosphatase was bought from Boehringer Mannheim (Mannheim, Germany). The restriction enzyme AccI, Vent DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs (Schwalbach, Germany), the T7 sequencing kit was from Pharmacia (Freiburg, Germany). [a-32P]dATP, [thio-a-35S]dATP, and [3H]thymidine were obtained from Amersham International. Oligonucleotides were bought from Eurogentec (Seraing, Belgium), Brij-96 and Nonidet P-40 from Sigma (Munich, Germany).

Construction of Chimeras of Human IL-6 and CNTF—The discrete regions of human IL-6 and CNTF used to construct the chimeras are shown in Fig. 1. Construction of chimeras IC1, IC2, and IC3 relied heavily on PCR-ligation-PCR (37). cDNAs of human IL-6 and CNTF cloned into the pRSET5d bacterial expression vector via NcoI and HindIII restriction sites served as PCR templates (27, 28). To construct chimeras IC1, an IL-6 cDNA fragment encoding the N-terminal part of the molecule to Arg40 and a C-terminal CNTF cDNA fragment starting at the codon for Asp36 were amplified by standard PCR. The PCR products were ligated (37) and the ligation product subsequently amplified by PCR to produce the fragment IL-6(Pro1-Arg40):CNTF(Glu36-Met56). This fragment was purified from a 1% agarose gel and ligated to the amplified C-terminal IL-6 cDNA fragment starting at the codon for Asn60. The ligation product, IC1, was amplified by PCR and subsequently cloned into the bacterial pRSET5d expression vector after digestion with NcoI and HindIII. Chimeras IC2 and IC3 were constructed analogously. The sequences of all primers used are available on request. Chimeras IC4 to IC6 were produced from chimeras IC1 to IC3 using NcoI, AccI, XbaI, and HindIII restriction enzymes. IC7 was produced from IC1 and IC6. The integrity of all constructs was verified by restriction fragment analysis and DNA sequencing according to standard methods (38).

Molecular Modeling—The boundaries of the IL-6 and CNTF regions exchanged were derived from a molecular model of IL-6 (27) and the x-ray structure of CNTF as taken from the Brookhaven data bank (accession code 1cnt). Recently, the x-ray as well as the NMR structure of human IL-6 were solved (12, 39), the regions interchanged are color coded on the ribbon models of the IL-6 NMR and CNTF x-ray structures. Structure comparisons and all computer graphic work were performed with the WHATIF program package running on an SGI-Indigo2 (40). For graphical representation the program Grasp was used (41).

Preparation and Quantification of Mutant Proteins—Escherichia coli bacteria (strains BL21-DE3 and BL21 pLysS) were transformed with the expression vector pRSET5d containing human IL-6, human CNTF, and chimeric cDNAs. Transformed bacteria were grown to an A600 of approximately 0.5-0.7 and induced to produce recombinant protein by addition of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside. After 2 h, purification of inclusion bodies and denaturation with 6 M guanidinium chloride was performed as described before (42). Refolding of proteins was achieved by dialysis against refolding buffer (1 M guanidinium chloride, 3 mM oxidized glutathione, 0.6 mM reduced glutathione, 12 h) and 20 mM Tris-Cl, pH 6.8 (12 h). The purity of the recombinant

---

2 H. Gascan, manuscript in preparation.
proteins was ascertained by 12.5% SDS-PAGE, stained with Coomassie Blue or silver. In addition, protein concentrations were determined by hydrolysis of the proteins in 6 M HCl and subsequent amino acid analysis. LPS concentrations in the purified protein preparations were ascertained with the Limulus amoebocyte lysate assay (Biowhittaker, Walkersville, MD).

**CD Spectra**—CD spectra of all chimeras were taken on an AVIV CD spectrometer 62DS and on a Jasco J-600 spectropolarimeter. Both instruments were calibrated with an aqueous solution of 10-camphosulfonic acid at 25 °C. The spectral band width was 1.5 nm. Protein samples were dissolved in water, the pH was adjusted to 3.5.

**Bioassays**—Proliferation of at least two different clones of the transfected BAF/3-(gp130) cell lines in response to human IL-6, human CNTF, human LIF, and the chimeras IC1 to IC7 was measured in 96-well microtiter plates. The cells were exposed to test samples for 72 h and subsequently pulse-labeled with [3H]thymidine for 4 h. Proliferation rates were measured by harvesting the cells on glass filters and determination of the incorporated radioactivity by scintillation counting. For each mutant and each cell line the proliferation assay was performed at least three times in triplicate. Haptoglobin production by HepG2 and Hep3B cells in response to stimulation with the above cytokines and chimeras was measured by a sandwich enzyme-linked immunosorbent assay as described recently (43).

cDNAs and COS-7 Cell Transfection—COS-7 cells were simultaneously transfected with cDNAs for human gp130, LIFR, IL-6R, and STAT3 with the DEAE-dextran method as described (44). gp130 and LIFR were subcloned into the p409 expression vector (a kind gift from Immunoex). The human IL-6R was in the pCMV8 expression vector, STAT3 in pSVL. After transfection, COS-7 cells were cultured for 2 days in RPMI plus 10% fetal calf serum before starvation in serum-free medium.

**Tyrosine Phosphorylation Analysis**—SK-N-MC, HepG2, and transfected COS-7 cells were starved overnight in serum-free medium before stimulation with cytokines. After stimulation, cells were lysed in 50 mM Tris, pH 7.5, 100 mM NaCl, 50 mM sodium fluoride, and 3 mM sodium orthovanadate containing 1% Brij-96. Insoluble material was pelleted and the supernatants subjected to a second immunoprecipitation with anti-phosphotyrosine mAb 4G10 (5 μg/ml) after addition of 1% Nonidet P-40. The precipitates were subjected to SDS-PAGE and transferred to an Immobilon membrane (Millipore, Bedford, MA). The membranes were incubated with an appropriate primary antibody before being labeled with a secondary antibody coupled to peroxidase. Subsequently, the membranes were developed using the Amersham ECL kit.

**RESULTS**

**Structural Comparison of IL-6 and CNTF**—The four-helix bundle fold (Fig. 1A) is the characteristic structure of most cytokines (11). A schematic representation of the backbone atoms of the IL-6 NMR and the CNTF x-ray structure is shown in Fig. 1B. Both structures were superimposed onto each other using the C-, atoms of the helices to identify putative components of the site III receptor binding epitope. The segments of IL-6 and CNTF which participated in the “epitope shuffle” of site III are color coded and designated as IIA, IIB, and IIC, respectively (Fig. 1A and B).

A structural analysis of CNTF suggested that amino acid residues situated in the C-terminal A-helix (Gln148-Gln152) and N-terminal AB loop (Gly143-Met149) form site IIA, while the BC loop (His109-Arg104) with adjacent residues of the E- (Leu91-Val96) and C-helix (Phe105-Ile109) represents site IIB. Together with site IIC which closely corresponds to Bazan’s D1 motif (10) and consists of the C-terminal CD loop (Gly147-Leu151), and the N-terminal D-helix (Phe152-Leu156), they constitute the putative LIFR-binding epitope on CNTF (site III). Potential site III residues of IL-6 were substituted by these CNTF amino acids. The precise amino acid sequences of the resulting chimeric molecules are given in Fig. 1C. Considering the extensions of helices and loops in IL-6 and CNTF (12, 13), site IIB of CNTF extends the B-helix of the resulting chimeras by five amino acids, whereas the BC loop is lengthened by two amino acids. Site IIA and IIC differ in two and one amino acid residues, respectively. The transferred part of the CNTF AB loop (Gly143-Met149) includes the CNTF mini-helix (Lue50-Ala53) (13), whereas the putative E-helix of IL-6 (Pro141-Gln145) (12) is not affected by the exchange of site IIC.

The chimeras were expressed in E. coli, refolded, and purified to near homogeneity as shown in Fig. 1D. Correct folding of each chimera was checked by CD spectroscopy (data not shown). Despite small differences, the CD spectra of all molecules exhibited band shapes typical of four-helix bundle proteins (28, 45) indicating successful refolding of the chimeric proteins. Chimera IC6 could not be expressed in any of our bacterial expression systems. Protein concentrations were estimated from Coomassie Blue staining, silver staining (data not shown), and amino acid analysis of the individual proteins.

**Chimeras IC5 and IC7 Induce Phosphorylation of the gp130/LIFR Heterodimer**—To verify the successful transfer of a LIFR binding epitope from CNTF to IL-6 we assessed the ability of the chimeras carrying the most extended amino acid stretches from CNTF, IC5, and IC7, to induce heterodimerization and phosphorylation gp130 and LIFR in SK-N-MC neuroblastoma cells (Fig. 2). In SK-N-MC cells express gp130, LIFR, and CNTFR, but no IL-6R on the cell surface (Table I) and display...
particularlty strong tyrosine phosphorylation after stimulation with IL-6-type cytokines (9, 46). Phosphorylation of SK-N-MC gp130 was observed after stimulation with CNTF and also with IL-6, IC5, and IC7 in the presence, but not the absence of the soluble IL-6R (Fig. 2A). IC5, IC7, and CNTF also induced phosphorylation of a protein of around 190-kDa molecular mass coprecipitating with gp130, most likely the LIFR. Immunoprecipitation of SK-N-MC with the anti-LIFR mAb AN-E1 confirmed phosphorylation of LIFR after treatment with IC5, IC7, and CNTF, but not IL-6 (Fig. 2B). The slight band apparent after stimulation with IL-6 in the presence of the soluble IL-6R was due to background phosphorylation, in two further experiments a similar band was seen in the control lane (data not shown). Chimeras IC5 and IC7 and CNTF also induced coprecipitation of phosphorylated gp130. In the presence of the soluble IL-6R, IC5 and IC7 triggered STAT3 phosphorylation as did CNTF (Fig. 2C), thus demonstrating activation of the signaling cascade downstream of gp130 and LIFR. Phosphorylation of gp130, LIFR, and STAT3 after stimulation with IC7 equaled that observed after stimulation with CNTF, whereas we consistently observed weaker phosphorylation of these proteins after application of IC5, although 10-fold higher concentrations of IC5 were used (Fig. 2). Together, these experiments strongly suggest that upon binding to the IL-6R, IC5 and IC7 cause heterodimerization and phosphorylation of gp130 and LIFR as well as activation of STAT3.

The phosphorylation pattern of HepG2 cells after stimulation with IL-6, IC5, IC7, and LIF confirmed the results on SK-N-MC cells (Fig. 3 A-C). gp130 phosphorylation was clearly weaker after treatment with IC5 and IC7 than after IL-6, but stronger than LIF induced gp130 phosphorylation (Fig. 3A). Coprecipitation of an activated LIFR was clearly discernible after stimulation with IC7 and LIF, whereas much longer exposures of the x-ray film were necessary to detect the phosphorylated LIFR after stimulation with IC5 (data not shown). Immunoprecipitation of HepG2 cells with anti-LIFR mAb AN-E1 demonstrated LIFR phosphorylation only after treatment with LIF, IC7, and IC5, but not IL-6 (Fig. 3B). With the former cytokines, phosphorylated gp130 was also coprecipitated, which in the case of IC5 became visible only after longer exposure of the x-ray film. Although IC5 induced weaker phosphorylation of gp130 and LIFR than LIF or IC7 this difference was not observed with regard to the phosphorylation of STAT3 suggesting amplification of the activation signal (Fig. 3C).

Furthermore, we reconstituted the cellular response to IC7 in COS-7 cells by transfecting these cells with cDNAs coding for IL-6R, LIFR, gp130, and STAT3. Stimulation of the transfected COS-7 cells with LIF and IC7 resulted in appearance of the double band typical of the phosphorylated gp130/LIFR heterodimer after immunoprecipitation, while IL-6 only induced phosphorylation of gp130 (Fig. 4A). All three cytokines were able to trigger phosphorylation of STAT3 (Fig. 4B).

**Biological Activity of the IL-6/CNTF Chimeras—To assess the biological activity of the IL-6/CNTF chimeras we first measured secretion of the acute-phase protein haptoglobin by stimulated HepG2 and Hep3B human hepatoma cells. In contrast to HepG2 cells, Hep3B cells express gp130 and IL-6R, but not the LIFR (6). Chimeras IC2, IC5, and IC7 achieved virtually the same activity as IL-6 on HepG2 cells (Fig. 5A) which agrees well with the almost equal phosphorylation of STAT3 induced by IL-6, IC5, and IC7 in these cells (Fig. 3C). IC2 was clearly active on Hep3B cells, although around 100-fold higher concentrations were needed to exert the same half-maximal activity as IL-6. In contrast, IC5 and IC7 were completely inactive on Hep3B cells (Fig. 5B). Thus chimeras IC5 and IC7 have lost the ability to elicit cellular responses via gp130 alone. Biological activity of IC1 and IC3 on HepG2 cells was only
observed at very high concentrations that even exceeded those needed for unspecific stimulation by CNTF (Fig. 5A). These two chimeras also showed some minor activity on Hep3B cells.

In a second bioassay we investigated the proliferative response of transfected BAF/3 cells to the chimeras. Murine BAF/3 cells display a strong proliferative response to IL-3, but do not respond to human IL-6 type cytokines, since they neither express gp130 nor any other receptor of this family (47). However, BAF/3 cell lines transfected with human gp130 and human IL-6R proliferate upon treatment with human IL-6 (Fig. 6A) and after additional transfection of the human LIFR also upon human LIF (Fig. 6B). BAF/3-(gp130,IL-6R) cells cannot be stimulated to proliferate by human LIF (Fig. 6A). The response of the above cell lines to our IL-6/CNTF chimeras is more diverse. Hardly any proliferation was observed in response to IC3 and IC4, whereas IC2 was almost as active as IL-6 on both cell lines. IC1 was also active on both cell lines, but 300–1000-fold higher concentrations than those of IL-6 were needed to achieve half-maximal activity. The most significant findings, however, concern chimeras IC7 and IC5: on BAF/3-(gp130,IL-6R,LIFR) cells IC7 had virtually the same activity as LIF, while roughly 100-fold higher concentrations of IC5 were needed to achieve the same half-maximal activity as IC7. Absence of the LIFR as in BAF/3-(gp130,IL-6R) cells (Fig. 6A) prevented a proliferative activity of IC5 and IC7 on these cells. Chimeras do not possess the ability to elicit LIF-like responses on cells without the IL-6R (Fig. 6C), nor can the CNTFRα substitute for the IL-6R (Fig. 6D).

The preparation of IC7 contained trace amounts of LPS which amounted to 30–300 pg/ml at IC7 concentrations (10–50 ng/ml) where maximal biological activity of IC7 was reached. However, it is highly unlikely that our assays were distorted by a direct LPS effect, since SK-N-MC cells only react to IC7 in the presence, but not the absence of the soluble IL-6 receptor (Fig. 2). Furthermore, BAF/3-(gp130,LIFR) cells served as the parent cell line to BAF/3-(gp130,IL-6R,LIFR) cells and in contrast to the latter are unresponsive to IC7 (Fig. 6). These differences cannot be explained by a direct LPS effect.

**DISCUSSION**

Our experiments define a specific LIFR binding epitope on CNTF that corresponds to site III in the terminology established by the growth hormone receptor complex paradigm (13, 29). The epitope consists of amino acid residues located in the C-terminal A-helix, the N-terminal AB loop (Glu36-Met56), the BC loop with adjacent parts of B- and C-helix (Leu91-Ile109), and the C-terminal CD loop with the adjoining N-terminal D-helix (Gly147-Leu162). This accords well with an analysis of the x-ray structure of human CNTF by McDonald et al. (13).
who predicted a LIFR binding epitope on CNTF consisting of the AB, BC, and CD loops. We could successfully substitute one of the two established gp130-binding sites on IL-6, namely site III (33), by this LIFR binding epitope. In contrast to IL-6, the resulting chimera IC7 induced heterodimerization of gp130 and LIFR (Figs. 2, A and B; 3, A and B; and 4A). IC7 induced phosphorylation of gp130 and LIFR as strongly as CNTF (Fig. 2) and only slightly weaker than LIF (Figs. 3 and 4). Biological activity on responsive cells was practically undiminished compared with human LIF (Fig. 6B). Consequently, the transferred LIFR binding epitope of CNTF appears to be complete, i.e., it contains all amino acids necessary for LIFR binding and activation. The successful reconstitution of the complete LIFR binding epitope of CNTF on IL-6 suggests that the receptor recognition sites of hematopoietic and neuropoietic cytokines can be regarded as discontinuous modules which could, in principle, be exchanged between different cytokines.

While IC7 contains the complete LIFR binding epitope our data do not allow to decide whether it is the minimal epitope sufficient for a fully active IL-6/CNTF chimera. The minimal epitope sufficient to stimulate cells via gp130 and LIFR was the combination of CNTF sites IIIA and IIIC in chimera IC5 (Figs. 5A and 6B). At 10-fold higher concentrations than IL-6, IC7, CNTF, and LIF chimera IC5 also induced phosphorylation of the gp130/LIFR heterodimer and the downstream effector STAT3 (Figs. 2 and 3). Consequently, CNTF site IIIB (BC loop, adjacent residues of B-/C-helices) does not appear to be an essential part of the LIFR binding epitope. Nevertheless, the BC loop region is an important part of the LIFR binding epitope, since its presence in IC7 greatly enhances interaction of gp130 and LIFR (Figs. 2 and 3) and biological activity (Fig. 6B) compared with IC5. In contrast, the BC loop seems to be less important for the site III gp130 binding epitope on IL-6, since IC2 which carries the BC loop of CNTF shows only slightly diminished activity compared with IL-6 (Figs. 5B and 6A).

Even at very high concentrations none of the IL-6/CNTF chimeras could elicit biological effects on cells that lacked the IL-6 receptor (Fig. 6, C and D). Likewise, IC5 and IC7 were unable to induce formation of the phosphorylated gp130/LIFR heterodimer in the absence of the soluble IL-6R (Fig. 2). Consequently, binding to the IL-6R is essential for biological activity of the chimeras. Furthermore, the CNTFR cannot replace the IL-6R as receptor α-unit for the chimeras (Figs. 2 and 6D). Contrary to a previous prediction based on structural considerations (21) CNTF amino acids 36–56, which are roughly equivalent to the transferred CNTF site IIIA, may thus not be involved in binding to the CNTFR.

Two residues of CNTF site IIIC, Phe152 and Lys155, are conserved in all members of the IL-6 family that signal via LIFR (34). Mutation of these residues abolished binding of CNTF and LIF to LIFR (30, 34). Di Marco et al. (34) therefore restricted a putative LIFR binding epitope of CNTF to the C-terminal CD loop/N-terminal D-helix. Studies on the binding of chimeric murine and human LIF to human and murine LIFR concluded that six residues of human LIF (Asn57, Ser107, His112, Val113, Val155, and Lys158) located in the short BC loop, the N-terminal C-helix, and the CD loop contribute most of the binding energy to human LIFR (35, 48). With the exception of Asn57 equivalent residues on CNTF are part of the LIFR binding epitope transferred to IC7. The biological inactivity of IC4 (Fig. 5A and 6B) which lacks the CNTF site IIIC is in line with these concepts. However, the inactivity of IC5 reveals that site IIIC alone is unable to recruit the LIFR, whereas the combination of site IIIC and IIIC as in IC5 is active. Thus the C-terminal A-helix and N-terminal AB loop are an indispensable

**FIG. 6.** Proliferative response of transfected BAF/3 cells to the IL-6/CNTF chimeras. A, effect of IL-6, LIF, and the chimeras IC1 to 5 and IC7 on BAF/3 cells transfected with gp130 and IL-6Rα. LIF is symbolized by open squares, the other symbols are as described in the legend to Fig. 1C. B, effect of IL-6, LIF, and the chimeras IC1 to 5 and IC7 on BAF/3 cells transfected with gp130, IL-6Rα, and LIFR. C, effect of IL-6, LIF, and the chimeras IC1 to 5 and IC7 on BAF/3 cells transfected with gp130 and LIFR. D, effect of IL-6, CNTF and the chimeras IC1 to 5 and IC7 on BAF/3 cells transfected with gp130, LIFR, and CNTFR.
and important part of the LIFR binding epitope of CNTF (Figs. 5A and 6B). Contributions of the same region of LIF to LIFR binding might have escaped the approach of Owczarek and Layton due to the high homology of murine and human LIF in their AB loops (35). The importance of site IIIA for binding of IL-6-type cytokines is further indicated by a comparison of IC2 and IC1. Exchange of the BC loop (site IIIB) as in IC2 has an only small effect on biological activity, whereas exchange of site IIIA in IC1 substantially reduces, but does not abolish, bioactivity of IC1 compared with IL-6. The combined sites IIIB and IIIC (BC loop, C-terminal CD loop/N-terminal D-helix) might therefore constitute a minimal epitope for contact to the second gp130 molecule. Unfortunately, our inability to express chimera IC6 leaves open whether combined CNTF sites IIIB and IIIC constitute an epitope sufficient to induce signaling via LIFR.

Chimera IC7 is fully active on cells that express human IL-6Ra, gp130, and LIFR (Figs. 5A and 6B), whereas it is virtually inactive on cells that only possess IL-6Ra and gp130 (Figs. 5B and 6A). This observation indicates that the epitope transferred from CNTF to IL-6 cannot bind gp130 which otherwise would enable chimera IC7 to generate a gp130 homodimer and hence to signal. Therefore, site III of CNTF as defined by our experiments constitutes a specific binding site for LIFR. This finding confutes the existence of a versatile gp130/LIFR binding epitope at site III on CNTF (49) which is a prerequisite of a hexameric CNTFR complex (Fig. 7, A and B).

Immunoprecipitation experiments (18, 20) and gel size exclusion chromatography coupled to sedimentation equilibrium studies (19) indicated that in solution the high affinity receptor complexes of IL-6 and CNTF are hexameric (Fig. 7, A and B). Other authors, however, have argued (22) that the known dimensions of IL-6 and CNTF are hardly compatible with the arrangement of cytokine and cytokine receptors as suggested by the models of Paonessa and de Serio (18, 20). Based on structural considerations Simpson et al. (23) developed a new hexameric model of the IL-6R complex which also accounts for the observation that antibodies against the gp130 Ig-domain neutralize IL-6 bioactivity. In Fig. 7B, we have adapted the Simpson model to the CNTFR complex. Both models of the hexameric CNTFR complex imply that site II and site III are nonspecific binding sites for gp130 and LIFR. In contrast, our data indicate that CNTF site III is a specific LIFR-binding site. It should be noted that the co-immunoprecipitation experiments (18) leading to the concept of a hexameric CNTFR complex were performed with soluble recombinant receptor proteins at cytokine concentrations of around 200 μM. It has been reported that recombinant CNTF forms an antiparallel dimer at concentrations higher than 40 μM with the contact site mainly consisting of amino acid residues from the B- and C-helix (13). In our view the concept of a hexameric CNTFR complex in solution might thus not necessarily reflect the composition of the membrane bound receptor complex.

An alternative tetrameric model of the CNTFR receptor complex is presented in Fig. 7C. Site I is occupied by CNTFR and encompasses the C-terminal D-helix, which is in keeping with the affinity enhancing effect of the D-helix substitutions S166D, Q167A, and V170R (26). Analogously to IL-6 (27) the C-terminal AB loop of LIF is involved in this epitope. gp130 binds to site I while LIFR locates to site III and shields the top of the molecule. This architecture is very reminiscent of the IL-2 receptor complex: the β- and γ-subunits of the IL-2 receptor bind to sites I and II, respectively, whereas site III is occupied by the α-subunit (50). The tetrameric model of CNTFR complex only requires the minimal number of complex components, i.e., CNTF, CNTFRα, gp130, and LIFR in 1:1:1:1 stoichiometry. Such a minimal composition of the receptor complex has recently also been demonstrated for the LIFR complex which is a trimer consisting of LIF and one molecule each of gp130 and LIFR (51).

By transferring the specific LIFR binding epitope from CNTF to IL-6, we created an artificial cytokine with novel biological characteristics, i.e., a cytokine that signals like LIF, but not like the IL-6R for binding. The existence of such an exchangeable LIFR binding module could not be expected with regard to the primary sequence of CNTF and IL-6. It could only be predicted from structural analyses of CNTF and IL-6, since their low sequence identity of only 6% (10) is that of unrelated proteins. A modular structure of receptor recognition site seems to also exist in the neurotrophins, a family of neural growth factors which are structurally unrelated to four-helical bundle cytokines. By combining active domains of the neurotrophins nerve growth factor, brain-derived neurotrophic factor, and neurotrophin 3 into an neurotrophin 3 backbone a synthetic “pan-neurotrophin” was constructed that activates all known neurotrophin receptors (52). However, in contrast to four-helical bundle cytokines the neurotrophins have a high sequence identity of around 50%.
The organizational principle underlying the molecular structure of cytokines and growth factors may therefore be regarded as a set of modules that are discontinuous with regard to the primary amino acid sequence. Functional diversity among cytokines could therefore have arisen from recombination of modules encoded by naturally occurring homologous genes. Both the IL-6 andCNTF gene possess an intron-exon junction near the end of the A-helix, close to the start of site IIIA. However, the IL-6 and CNTF gene possess only two exons, in contrast to the IL-6 gene which consists of four coding exons (10). Thus, the concept of a modular cytokine structure does not simply reflect the organization of the cytokine gene. Nevertheless, our interpretation is strikingly similar to results obtained recently by directed evolution (DNA shuffling) of cephalosporinase genes from four different bacterial species (53). With only a few point mutations, segmental recombination of the four cephalosporinase genes evolved into a powerful molecular biology tool. In principle, modules responsible for receptor recognition should also be identifiable on other cytokines, e.g. IL-2. Exchanging such ligand modules should allow the engineering of new designer cytokines or growth factors almost at will to meet prespecified receptor requirements on target cells and thus exert novel biological activities. Such designer cytokines could be of therapeutic value (44, 54, 55), but will also be useful to analyze the formation of cytokine receptor complexes.

Acknowledgments—We thank Prof. Peter Galle and Dr. Heidi Schooltink for critically reading the manuscript. Dr. Birgit Oppmann kindly provided BAF3/(p130,LIFR) cells and recombinant LIF, recombinant IL-6, and CNTF were expressed by Martina Fischer and Dr. Barbara Krebs. Recombinant soluble IL-6R was a kind gift from Dr. Birgit Oppmann for critically reading the manuscript. Dr. Birgit Oppmann and coworkers generated antibodies to mutant IL-6R and CNTF receptors. Mutant receptors were expressed in the A419 cell line.

REFERENCES

1. Sendtner, M., Gade, E., Rett, M., Holtmann, B., Escaray, J. L., Masu, Y., Carroll, P., Wolf, E., Brem, G., Bruetel, P., and Thoenen, H. (1996)Curr. Biol. 6, 686–694
2. Andersson, K. D., Panayotatos, N., Corcoran, T. L., Lindsay, R. M., and Wiegand, S. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7346–7351
3. Sendtner, M., Schmallerbruch, H., Stockki, K. A., Carroll, P., Kreutzberg, G. W., and Thoenen, H. (1992)Nature308, 502–504
4. Clatterbuck, R. E., Price, D. L., and Koliatsos, V. E. (1993)Proc. Natl. Acad. Sci. U. S. A. 90, 2222–2226
5. Koshimizu, U., Taga, T., Watanabe, M., Saito, M., Shirayoshi, Y., Kishimoto, S., Taga, T., and Kishimoto, T. (1997)Annu. Rev. Immunol.15, 683–720
6. Takakura, Y., Fanelli, A., Ziegler, S. F., Comeau, M. R., Friend, D., Thoma, B., Cosman, D., Park, L., and Salvati, A. (1997) J. Biol. Chem. 272, 4855–4863