Analysis of the Leukemia Inhibitory Factor Receptor Functional Domains by Chimeric Receptors and Cytokines†

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ABSTRACT: In contrast to other hematopoietic cytokine receptors, the leukemia inhibitory factor receptor (LIFR) possesses two cytokine binding modules (CBMs). Previous studies suggested that the NH_2-terminal CBM and the Ig-like domain of the LIFR are most important for LIF binding and activity. Using the recently engineered designer cytokine IC7, which induces an active heterodimer of the LIFR and gp130 after binding to the IL-6R, and several receptor chimeras of the LIFR and the interleukin-6 receptor (IL-6R) carrying the CBM of the IL-6R in place of the COOH-terminal LIFR CBM, we could assign individual receptor subdomains to individual binding sites of the ligand. The NH_2-terminal CBM and the Ig-like domain of the LIFR bind to ligand site III, whereas the COOH-terminal CBM contacts site I. Furthermore, we show that LIFR mutants carrying the IL-6R CBM instead of the COOH-terminal CBM can replace the IL-6R by acting as an α-receptor for IL-6. However, in situations where a signaling competent receptor is bound at IL-6 site I, ligand binding to site III is an absolute requirement for participation of the receptor in a signaling heterodimer with gp130; i.e., a functional receptor complex of IL-6 type cytokines cannot be assembled solely via site I and II as in the growth hormone receptor complex.

Interleukin-6 (IL-6),[1] IL-11, the leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1), the ciliary neurotrophic factor (CNTF), and cardiotrophin-like cytokine [CLC, also designated novel neurotrophin-1/B-cell-stimulating factor-3 (NNT-1/BSF-3)] constitute the IL-6 family of hemato- and neuropoietic cytokines (*1*, *2*). A variety of diseases, including multiple myeloma, rheumatoid arthritis, and cardiac ischemia, are critically affected by IL-6 type cytokines, notably, IL-6 itself (*3*−*7*). Human herpesvirus-8 encodes a viral interleukin-6 analogue, vIL-6, that is secreted in Castleman's disease, pleural effusion lymphoma, and Kaposi's sarcoma (*8*).

IL-6 type cytokines are characterized by a typical four-helix bundle structure that links four α-helices (A−D) by two long loops (A−B and C−D) and one short loop (B−C) so that the four helices are positioned in an up−up−down−down orientation. This fold is also found in other cytokines, including growth hormone (GH), erythropoietin, and interleukin-4 among others (*9*−*12*). On target cells, IL-6 type cytokines induce oligomeric receptor complexes, which share a membrane-spanning 130 kDa glycoprotein, gp130, as a signal transducing β-receptor component. Intracellular signaling is elicited by formation of either a gp130 homodimer (IL-6 and IL-11) or a heterodimer of gp130 and the LIF receptor (LIF, OSM, CNTF, CT-1, and CLC) (*1*, *13*−*15*). OSM can also elicit an active heterodimer of gp130 and the OSM receptor (*16*). Dimerization of these IL-6 type β-receptors leads to phosphorylation of their cytoplasmic tyrosine residues by intracellular Janus tyrosine kinases and subsequent activation of signaling cascades, including activation of STAT3 (signal transducer and activator of transcription 3) and the ras/MAP-kinase pathway (*13*).

GH, erythropoietin, or IL-4 induces signaling dimers of their cognate receptors via two epitopes called site I (COOH-terminal D helix and COOH-terminal A−B loop) and site II (A and C helix) (*10*−*12*, *17*). The signal transducing β-components of IL-6 type cytokine receptor complexes, however, do not dimerize via sites I and II, but via site II and an additional site III (*18*, *19*). Site III is constituted by the COOH-terminal A helix and the NH_2-terminal A−B loop (site IIIA), the B−C loop with adjacent parts of the B and C helix (site IIIB), and the COOH-terminal C−D loop with the adjoining NH_2-terminal D helix (site IIIC) (*20*−*23*). Site II is always engaged by gp130, whereas site III binds to gp130, LIFR, or OSMR. LIF and OSM can directly induce an active gp130−LIFR or gp130−OSMR heterodimer, whereas IL-6, IL-11, CNTF, CLC, and possibly CT-1 need to first bind to small, cytokine specific α-receptors that are not involved in signal transduction and are bound at site I (*1*, *15*, *24*, *25*).

Receptors of the IL-6 family are type I cytokine receptors that are characterized by a cytokine binding module (CBM) constituted by two fibronectin type III (FNIII)-like domains

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[1] Abbreviations: CBM, cytokine binding module; CLC, cardiotrophin-like cytokine; CNTF, ciliary neurotrophic factor; CNTFR, CNTF receptor; CT-1, cardiotrophin-1; FNIII, fibronectin type III; GCSF, granulocyte colony-stimulating factor; GH, growth hormone; GLM-R, gp130-like monocyte receptor; IL-6, interleukin-6; IL-6R, IL-6 receptor, IL-11, interleukin-11; LIF, leukemia inhibitory factor; LIFR, LIF receptor; OSM, oncostatin M; OSMR, OSM receptor; STAT3, signal transducer and activator of transcription 3; vIL-6, viral interleukin-6.

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of 100 amino acid length that are roughly perpendicular to each other (*9*). The NH_2-terminal domain contains four conserved cysteine residues and the COOH-terminal one a conserved WSXWS motif. In contrast to the receptors for growth hormone, erythropoietin, prolactin, and interleukin-4 (*10*−*12*, *17*), the α-receptors of the IL-6 family (IL-6R, IL-11R, and CNTFR) have an additional Ig-like domain at the NH_2 terminus of the CBM. The CBM of the α-receptors mediates cytokine binding (*26*), whereas the α-receptor Ig-like domain is dispensable for biological activity, but appears to be involved in the regulation of receptor shedding and its intracellular trafficking (*27*).

The large IL-6 type β-receptors [gp130, LIF receptor (LIFR, 190 kDa), and OSM receptor (OSMR, 160 kDa)] also contain the structural element composed of an Ig-like domain and a CBM; however, they contain three additional FNIII-like domains between the COOH-terminal CBM and the transmembrane region and possess a cytoplasmic domain with seven tyrosine residues organized in conserved sequence motifs called boxes 1−3 (*1*, *13*, *16*). Recently, a new gp130-like monocyte receptor (GLM-R) was discovered that is most homologous to gp130, but structurally resembles IL-12Rβ1 in that it contains a CBM linked to three FNIII-like domains and a cytoplasmic domain, but no Ig-like domain (*28*, *29*). The ligand or mechanism of activation of GLM-R is unknown. The three membrane-proximal FNIII-like domains of the large receptors of the IL-6 family are not involved in cytokine binding, but mutations or exchanges with other FNIII-like domains can result in completely inactive receptors (*30*, *31*). The LIFR possesses a second CBM at the NH_2 terminus of the Ig-like domain, whereas in the OSMR, the second CBM is reduced to the FNIII-like domain containing the WSXWS motif (*1*, *16*). Conspicuously, formation of neither an active LIFR nor OSMR complex requires an α-receptor for binding, raising the question of the function of the two CBMs of the LIFR in an active receptor complex.

Two CBMs in one receptor also exist in the leptin receptor (*32*), the thrombopoietin receptor (*33*) and KH-97 (the common β-chain of the receptor complexes of IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor) (*34*). The two CBMs are adjacent to each other in KH97 and the thrombopoietin receptor, but recent reports suggest that only the COOH-terminal (KH97) (*35*, *36*) or NH_2-terminal CBM (trombopoietin receptor) (*37*) is involved in ligand binding. A study on soluble deletion mutants of the LIFR concluded that the NH_2-terminal CBM and probably the Ig-like domain are involved in LIF binding (*38*). The Ig-like domain was hitherto thought to determine the species specificity of the LIF receptor (*39*). The COOH-terminal CBM appeared to be essential for correct folding of the Ig-like domain and the NH_2-terminal CBM. A functional LIF receptor complex apparently required the NH_2-terminal domain for LIF binding and a hypothetical superstructure of the LIFR involving a contact between the two CBMs of the LIFR (*38*, *40*). Deletion mutants of the extracellular LIFR domains fused to the transmembrane and cytoplasmic domain of gp130 led to the conclusion that the COOH-terminal CBM has a propensity for homodimerization of the LIFR via a Y/F-XX-R/Q-XR motif to the N-terminal side of the WSXWS motif and potentially for heterodimerization with gp130 through a similar motif in the CBM of gp130 (*40*). In contrast to the prevailing view of the active LIFR complex as a trimer of the LIF, gp130, and the LIFR (*41*), a functional LIFR complex was hypothesized to consist of two copies of each partner, essentially constituting a hexamer.

The IL-6R is presently envisaged as a hexameric complex consisting of two IL-6, IL-6R, and gp130 molecules each (*42*−*44*). The recently determined structure of the viral IL-6−gp130 complex supported models of the IL-6R complex that stipulate an essential contact of the Ig-like domain of gp130 to site III of IL-6 and binding of the gp130 CBM to site II (*1*, *45*, *46*). In line with these models, removal of the Ig-like domain of gp130 abolished signaling in the IL-6 receptor complex (*47*). However, signaling of the LIFR complex was not affected by removal of the Ig-like domain of gp130, suggesting that the LIFR complex is organized differently, possibly as a consequence of the two CBMs in the LIFR.

Using transmembrane deletion mutants of the LIFR and a receptor chimera of the IL-6R and LIFR in which the COOH-terminal CBM of the LIFR was substituted with the CBM of the IL-6R, we have recently shown that the NH_2-terminal CBM and the Ig-like domain are sufficient for assembly of fully active receptor complexes after stimulation with the LIF or OSM (*48*). The Ig-like domain appeared to be a minimal prerequisite for LIF binding. The COOH-terminal CBM of the LIFR appeared to position the NH_2-terminal CBM/Ig-like domain in a way that allowed ligand binding and receptor complex assembly, and its absence did not affect folding of the NH_2-terminal CBM and the Ig-like domain. The available data were condensed to a trimeric model of the LIFR complex that locates the Ig-like domain of the LIFR at site III (A/C) of the LIF (*48*). The model positions the NH_2-terminal CBM at site IIIB and the COOH-terminal CBM at site I. In an active LIFR complex, the cytoplasmic domains of gp130 and the LIFR would thus be at sites I and II, as in the growth hormone receptor complex. However, a reverse orientation of the LIFR with the NH_2-terminal CBM at site I and the COOH-terminal CBM making a contact to the Ig-like domain of gp130 along the lines of a recently suggested tetrameric model of the IL-6R complex (*1*) could not be ruled out on the basis of the existing data. We therefore sought (a) to clarify the position of the individual CBMs of the LIFR with regard to LIF, (b) to investigate whether the CBMs could be perceived as having an α-receptor and a gp130 CBM-like function, and (c) to determine whether a signaling competent receptor solely bound to site I would be sufficient to elicit an active heterodimer with gp130 bound at site II.

EXPERIMENTAL PROCEDURES

Construction of Human LIF Receptor−Human IL-6 Receptor Chimeras. The boundaries of the exchanged and deleted domains of the IL-6 receptor (IL-6R) and the LIFR, respectively, were determined by molecular models of the IL-6R and LIFR built along the structure of the human growth hormone receptor using the WHATIF program package, which was run on an SGI-Indigo2 workstation (*49*). For graphical representation, the program Ribbon was used (*50*). Primers (sequence available on request) were designed on the basis of the results of structural analyses. The chimeras were prepared by PCR−Ligation−PCR (*21*, *51*). The exact borders of the segments swapped or deleted

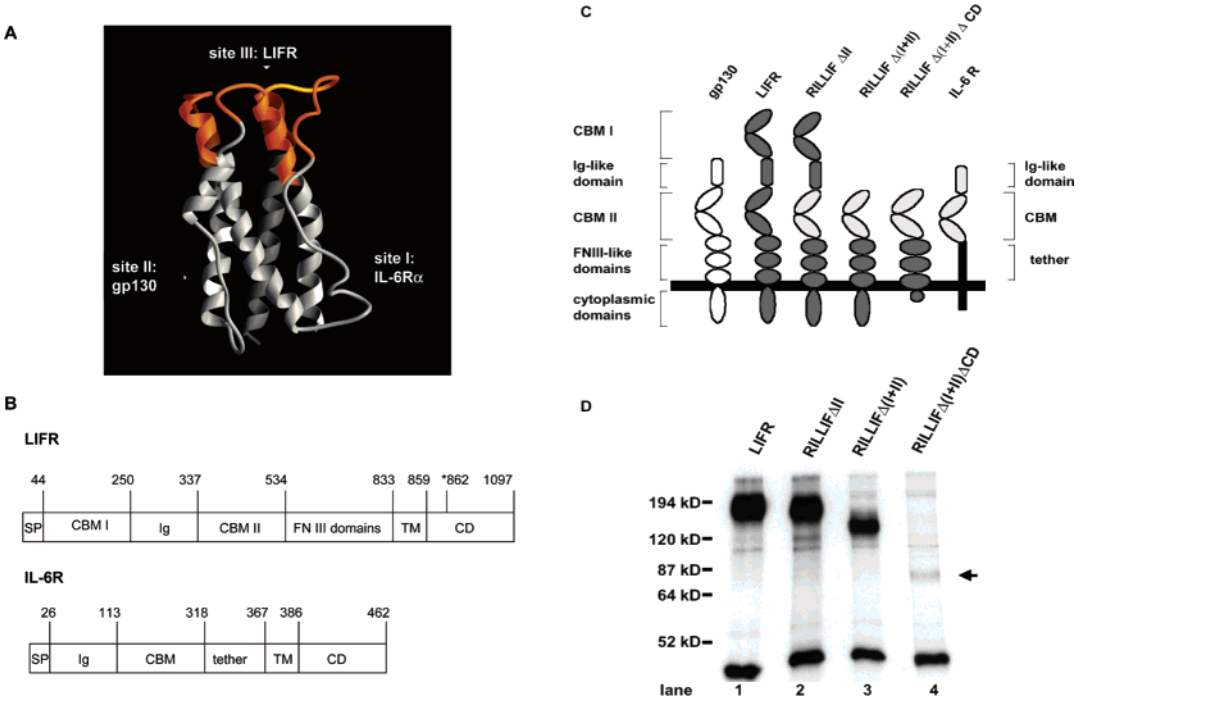

FIGURE 1: Chimeric cytokines and cytokine receptors used in this study. (A) Ribbon model of chimera IC7 (*20*). The IL-6 "backbone" of the chimera is marked in gray and CNTF site III in orange. (B) The IL-6R CBM was mounted on the FNIII-like domains of the LIFR, and in chimera RILLIFΔII, the IL-6R CBM replaced the COOH-terminal CBM of the LIFR. Domain boundaries of the LIFR and the IL-6R were defined by molecular modeling. The number of COOH-terminal amino acids in each domain is designated; the number after the asterisk designates the position of the stop codon in chimera RILLIFΔ(I+II)ΔCD. The numbering follows the amino acid sequence of the LIFR as deposited in GenBank (entry NM_002310), and the numbering of the IL-6R follows the sequence deposited in Swissprot (entry P08887). SP, signal peptide; CBM I, NH_2-terminal CBM; Ig, Ig-like domain; CBM II, COOH-terminal CBM; TM, transmembrane domain; CD, cytoplasmic domain. (C) Schematic representation of the chimeras of IL-6R and LIFR. (D) Expression of the receptor chimeras in transiently transfected COS-7 cells. Transfected cells were radioactively labeled with ^{35}S, and immunoprecipitation was performed with anticytoplasmic LIFR antibody sc659 (lanes 1−3) or antibody PM-1 (lane 4) which recognizes the CBM of the IL-6R (*53*). The autoradiographs were exposed for 36 h. The arrow indicates the band representing mutant RILLIFΔ(I+II)ΔCD.

in the various mutants are defined by the flanking NH_2- and COOH-terminal amino acid residues: RILLIFΔII [PDTPQ (LIFR)/QLSCF (IL-6R); PWTES (IL-6R)/PSKGP (LIFR)], and RILLIFΔ(I+II) [PRRCP (IL-6R)/PEEPQ (IL-6R); PWTES (IL-6R)/PSKGP (LIFR)]. RILLIFΔ(I+II)ΔCD contains a stop codon shortly after the transmembrane domain (see Figure 1B).

Transfection of BaF/3-[gp130] Cells and COS-7 Cells. BaF/3-[gp130] cells were a kind gift of Immunex (Seattle, WA) and were grown in DMEM with glutamax (Life Technologies, Karlsruhe, Germany) supplemented with penicillin (50 units/mL), streptomycin (50 μg/mL), and 10% FCS. BaF/3-[gp130] and COS-7 cells were transfected as described previously (*21*, *48*).

Binding of IL-6 and IC7 to the RILLIFΔ(I+II) Chimera. COS-7 cells were transiently transfected with RILLIFΔ-(I+II). Two days after transfection, the cells were washed with PBS and the indicated amounts of IL-6 and IC7 dissolved in DMEM with 5% FCS added to the cells. These were then incubated on ice for 60 min, followed by two washes with ice-cold PBS. The cells were lysed and the lysates precipitated with the murine anti-IL-6 monoclonal antibody mAB12 (a kind gift of L. Aarden, CLB, Amsterdam, The Netherlands), which recognizes the site II gp130 binding epitope of IL-6 (*52*). The precipitates were subjected to SDS−PAGE and blotted with the anti-LIFR polyclonal serum sc659 (Santa Cruz Biotechnology, Santa Cruz, CA).

Proliferation Assay. Proliferation of transfected BaF/3-[gp130] cells was followed in 96-well microtiter plates. The cells were exposed to test samples of the cytokines for 72 h and subsequently pulse-labeled with [^{3}H]thymidine for 4 h. Proliferation rates were measured by harvesting the cells on glass filters and determining the incorporated radioactivity by scintillation counting. Proliferation assays were performed at least three times in triplicate for each cell line.

Analysis of Receptor Tyrosine Phosphorylation. Transfected BaF/3-[gp130] cells were starved for 4 h in a serum free DMEM/penicillin/streptomycin mixture. After cytokine stimulation (50 ng/mL), the cells were lysed in buffer [50 mM Tris (pH 7.5), 100 mM NaCl, 50 mM NaF, and 3 mM sodium orthovanadate] containing protease inhibitors [0.1 mM PMSF with protease inhibitor cocktail tablets (Roche Molecular Biochemicals, Mannheim, Germany)]. Unless stated otherwise, the lysis buffer contained 1% Brij-96 as a detergent (Sigma, Deisenhofen, Germany). However, in some experiments, a combination of 1% Nonidet P40 (NP-40) and 0.4% sodium deoxycholate was used as a detergent (Sigma). Insoluble material was pelleted, and the supernatants were immunoprecipitated with either anti-gp130 antibody sc655, anti-LIFR antibody sc659 (Santa Cruz Biotechnology), or anti IL-6 receptor antibody PM-1 (*53*). The antibodies sc655 and sc659 recognize the COOH-terminal cytoplasmic domains of gp130 and the LIFR, respectively. The complexes were precipitated with protein A Sepharose (Amersham Pharmacia, Freiburg, Germany), subjected to SDS−PAGE, and transferred to a PVDF membrane (Amersham Pharmacia). The membrane was incubated with monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology,

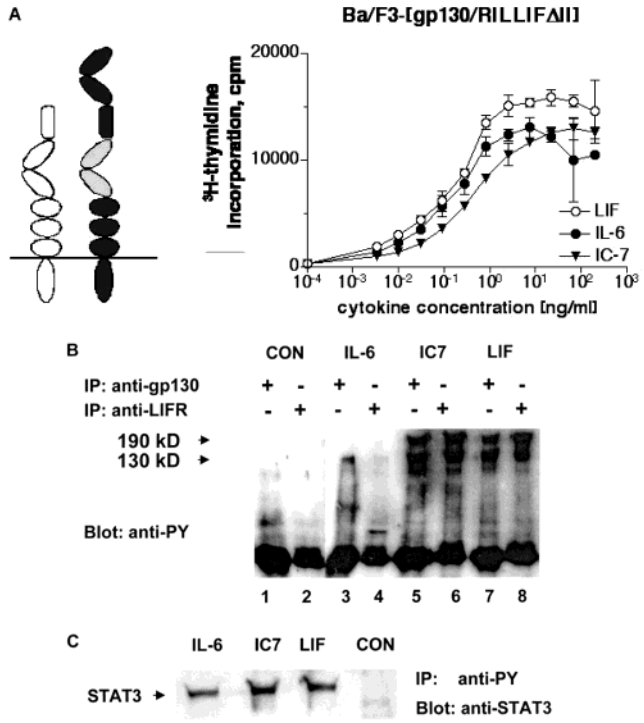

FIGURE 2: Stimulation with IL-6 or IC7 of BaF/3-[gp130] cells transfected with chimera RILLIFΔII. (A) Proliferation of BaF/3-[gp130,RILLIFΔII] cells in response to IL-6 or IC7. (B) Phosphorylation of gp130 and RILLIFΔII after stimulation with the different cytokines. (C) Phosphorylation of STAT3 in IL-6- or IC7-stimulated BaF/3-[gp130,RILLIFΔII] cells.

Lake Placid, NY) before being labeled with a secondary antibody coupled to peroxidase. Subsequently, the membranes were developed using the Amersham ECL plus kit. To assess phosphorylation of STAT3, crude cell lysates were subjected to SDS−PAGE and blotted with an antibody against phosphorylated STAT3 (New England Biolabs, Bad Schwalbach, Germany).

Cytokines. The production of the recombinant cytokines used in this study has been described previously (*21*, *48*).

RESULTS

To elucidate the interaction of the individual CBMs of the LIFR with the different receptor binding epitopes of LIF, we made use of the recently constructed designer cytokine IC7, a chimera of IL-6 and CNTF, in conjunction with chimeras of the IL-6R and the LIFR. Following binding to the IL-6R via site I, IC7 recruits gp130 to site II and the LIFR binds to site III (Figure 1A) (*21*). On cells expressing the IL-6R, gp130, and the LIFR, IC7 displays a biologic activity comparable to that of LIF or IL-6, but it is inactive on cells that do not express the IL-6R (*21*).

A set of chimeras of the IL-6 receptor (IL-6R) and the LIFR was constructed on the basis of the domain boundaries of molecular models of the IL-6R and the LIFR (Figure 1B,C). In chimera RILLIFΔII, the COOH-terminal CBM of the LIFR was replaced with that of the IL-6R, whereas in chimera RILLIFΔ(I+II), the NH_2-terminal CBM and the Ig-like domain of RILLIFΔII were deleted (Figure 1C). Chimera RILLIFΔ(I+II)ΔCD contains a stop codon directly after the transmembrane domain, which eliminates the cytoplasmic domain that stems from the LIFR. Ample expression of the mutant receptors was demonstrated by transient expression of the chimeras in COS-7 cells, radiolabeling, and subsequent autoradiography of the immunoprecipitated proteins (Figure 1D). Although mutant RILLIFΔ(I+II) has a theoretical molecular mass of 89 kDa, it appears as a band slightly below 130 kDa upon SDS−PAGE (Figure 1D, lane 3), which most probably reflects extensive glycosylation. Mutant RILLIFΔ-(I+II)ΔCD (lane 4) consistently appeared as an 80 kDa protein with a weaker band intensity on autoradiography.

We have recently shown that upon stimulation with LIF and OSM, chimera RILLIFΔII forms a receptor complex with gp130 that signals as efficiently as the LIFR−gp130 heterodimer (*48*). Taking into account results obtained with several LIFR deletion mutants, we concluded that only the NH_2-terminal CBM and the Ig-like domain of the LIFR are required for LIF signaling. To identify the cytokine epitope involved in binding to this domain, we tested whether the designer cytokine IC7 was able to elicit functional receptor complexes of RILLIFΔII and gp130. We therefore stably transfected BaF/3 cells with gp130 and chimera RILIFΔII. BaF/3 cells are a murine pre-B cell line which becomes reactive to IL-6 type cytokines upon transfection of IL-6 type receptors (*54*). LIF, IC7, and IL-6 stimulated BaF/3-[gp130,RILLIFΔII] cells with comparable activity (Figure 2A). An analysis of the phosphorylation pattern of the surface receptors of the stimulated BaF/3-[gp130,RILLIFΔII] cells revealed that LIF and IC7 caused phosphorylation of both gp130 and RILLIFΔII as well as phosphorylation of STAT3, whereas only gp130 became phosphorylated after IL-6 stimulation (Figure 2A,B). These results are consistent with an α-receptor-like function of the IL-6R CBM of RILLIFΔII for site I of IC7 and a contact of site III to the NH_2-terminal CBM and the Ig-like domain of the LIFR.

However, the possibilities still remained that (a) the signaling heterodimer of gp130 and RILLIFΔII was formed solely via binding of RILLIFΔII to sites I and II of IC7 without any interaction between site III and the NH_2-terminal domains of RILLIFΔII and (b) a tetrameric complex was formed consisting of IC7, gp130, and two RILLIFΔII molecules, one serving as an α-receptor and the other as a signaling β-receptor. To exclude the first possibility, we transfected BaF/3-[gp130] cells with chimera RILLIFΔ-(I+II). BaF/3-[gp130,RILLIFΔ(I+II)] cells proliferated in response to IL-6 with the same dose dependency as BaF/3-[gp130, IL-6R] cells (*21*), whereas IC7 was totally inactive on this cell line (Figure 3A). IC7 did not elicit phosphorylation of gp130, RILLIFΔ(I+II), or STAT3, whereas IL-6 caused phosphorylation of gp130 and, apparently, also of RILLIFΔ(I+II) (Figure 3B,C). The inactivity of IC7 did not result from a diminished level of binding of IC7 compared to the level of binding of IL-6 to chimera RILLIFΔ(I+II), since RILLIFΔ(I+II) could be precipitated with anti-IL-6 site II antibody mAB12 after addition of IL-6 and IC7, but not in the absence of cytokine (Figure 3D). To test whether gp130 would bind to the site II epitope of IC7 after binding of IC7 to RILLIFΔ(I+II), BaF/3-[gp130,RILLIFΔ(I+II)] cells were stimulated with IL-6 and IC7 and lysed and the lysates precipitated with anti-LIFR antibody sc659 and blotted with anti-gp130 antibody sc655 (Figure 3E). gp130 could be coprecipitated after stimulation with IL-6 and various concentrations of IC7, but not in the absence of cytokine. However, only after stimulation with IL-6 did

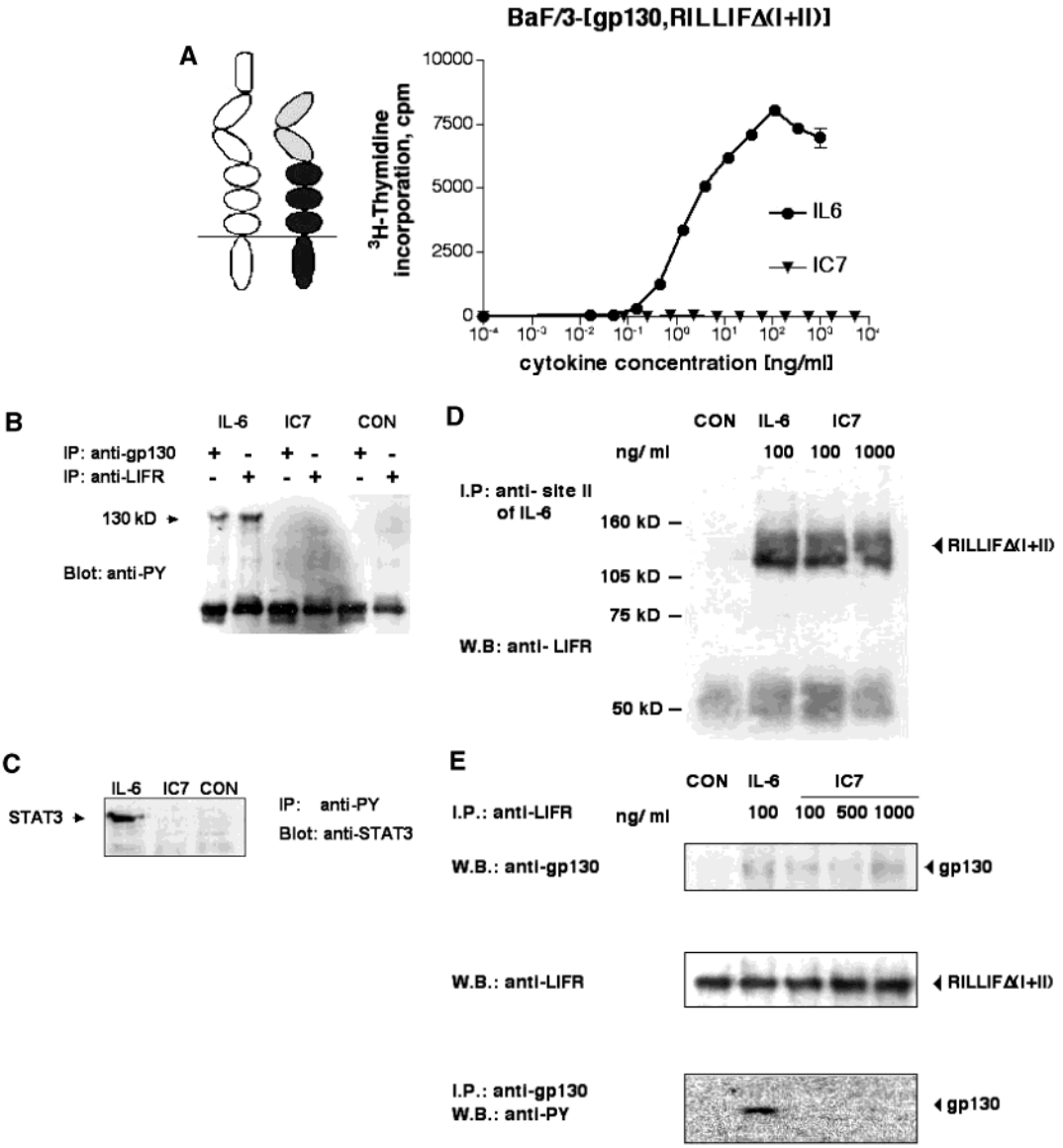

FIGURE 3: Proliferation of BaF/3-[gp130] cells transfected with chimera RILLIFΔ(I+II) after stimulation with IL-6 or IC7. (A) Proliferation of BaF/3-[gp130,RILLIFΔ(I+II)] cells in response to IL-6 and IC7. (B) Phosphorylation of gp130 in IL-6- and IC7-stimulated BaF/3-[gp130,RILLIFΔ(I+II)] cells. (C) Phosphorylation of STAT3 in IL-6- and IC7-stimulated BaF/3-[gp130,RILLIFΔ(I+II)] cells. (D) Binding of IL-6 and IC7 to RILLIFΔ(I+II). (E) Nonproductive heterodimerization of gp130 and RILLIFΔ(I+II) after binding of IC7. After precipitation with an anti-LIFR antibody, the remnant supernatants were subjected to a second immunoprecipitation with an anti-gp130 antibody, and the tyrosine phosphorylation of gp130 was verified.

gp130 become phosphorylated. Thus, chimera RILLIFΔ-(I+II) can replace the IL-6R by acting as an α-receptor for IL-6 and IC7, but heterodimerization of gp130 and RILLIFΔ-(I+II) at sites I and II of IC7 is nonproductive and does not cause phosphorylation of gp130 and RILLIFΔ(I+II).

To ascertain the validity of the second possibility that the RILLIFΔII complex is a tetramer, we reasoned that BaF/3 cells transfected with the LIFR, gp130, and RILLIFΔ(I+II) should proliferate as efficiently as BaF/3-[gp130,RILLIFΔII] cells in response to IC7, if one of potentially two RILLIFΔII molecules in the complex served as an α-receptor IC7 and the other provided the NH_2-terminal LIFR domains as binding partners for IC7 site III. However, whereas BaF/3-[gp130,LIFR,RILLIFΔ(I+II)] cells react strongly to IL-6, they are virtually insensitive to stimulation with IC7 (Figure 4). These results suggest that the receptor complex formed after stimulation of BaF/3-[gp130,RILLIFΔII] cells with IC7 is a trimer of IC7, gp130, and RILLIFΔII.

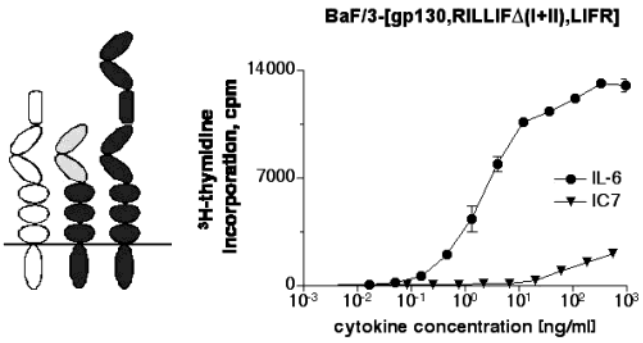

FIGURE 4: Proliferation of BaF/3-[gp130] cells transfected with chimera RILLIFΔ(I+II) and the LIFR after stimulation with IL-6 or IC7.

Since gp130 and RILLIFΔ(I+II) have similar molecular masses on SDS−PAGE (Figure 1D), we were unable to unequivocally attribute the phosphorylated band obtained

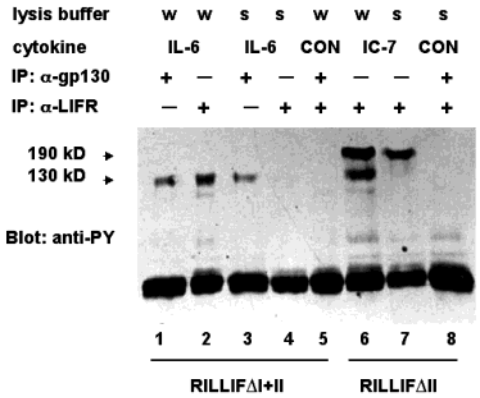

FIGURE 5: Phosphorylation of gp130 and the receptor chimeras in BaF/3-[gp130,RILLIFΔI+II)] and BaF/3-[gp130,RILLIFΔII] cells after stimulation with IL-6 or IC7. w denotes cases in which the lysis buffer contained 1% Brij-96 as the detergent. s denotes cases in which the lysis buffer containing 1% Nonidet P-40 and 0.4% sodium deoxycholate as the detergent.

after stimulation of BaF/3-[gp130,LIFR,RILLIFΔ(I+II)] cells to gp130 or chimera RILLIFΔ(I+II) by co-immunoprecipitation. Phosphorylation of the cytoplasmic domains of both gp130 and RILLIFΔ(I+II) (Figure 3B), however, would mean that in a situation where three signaling competent cytoplasmic domains are clustered in a complex, all of them become phosphorylated. This, however, would imply a change in the site II/site III signaling pattern exclusively observed in IL-6 type cytokines. To clarify this issue, we exploited the observation that the strong detergent NP-40 disrupts membrane-bound receptor complexes more easily than the "weaker" detergent Brij-96 (*55*, *56*). Lysis of IC7-stimulated BaF/3-[gp130,RILLIFΔII] cells with Brij-96 allowed immunoprecipitation of the intact membrane receptor complex of gp130 and RILLIFΔII with anti-LIFR antibody sc-659 (Figure 5, lane 6; Figure 2), whereas the detergent combination of NP-40 and sodium desoxycholate dismantled this complex completely (Figure 5, lane 7). Using the latter "strong" detergents for lysis of IL-6-stimulated BaF/3-[gp130,RILLIFΔI+II] cells, a phosphoryated band of 130 kDa was only detected after immunoprecipitation with the anti-gp130 antibody, but not after immunoprecipitation with the anti-LIFR antibody (Figure 5, lanes 3 and 4). In contrast, a phosphorylated 130 kDa band was detected after immunoprecipitation with both the anti-gp130 and anti-LIFR antibodies, when Brij-96 was used to lyse the cells (Figure 5, lanes 1 and 2). This experiment demonstrates that only gp130, but not the LIFR cytoplasmic domain of chimera RILLIFΔ(I+II), becomes phosphorylated after stimulation with IL-6. Consistent with these results, BaF/3-[gp130] cells transfected with chimera RILLIFΔ(I+II)ΔCD, which lacks the signaling competent cytoplasmic domain of the LIFR and therefore cannot participate in intracellular signaling, could be stimulated with IL-6. IL-6, but not IC7, caused phosphorylation of gp130 and STAT3 on BaF/3-[gp130,RILLIFΔ(I+II)ΔCD] cells (Figure 6A,B). The dose−response curve of IL-6 on BaF/3-[gp130,RILLIFΔ(I+II)ΔCD] cells was almost identical to that on BaF/3-[gp130,IL-6R] cells. Thus, the presence of the three FNIII domains in chimera RILLIFΔ(I+II)ΔCD does not interfere with its capacity to act as an α-receptor for IL-6.

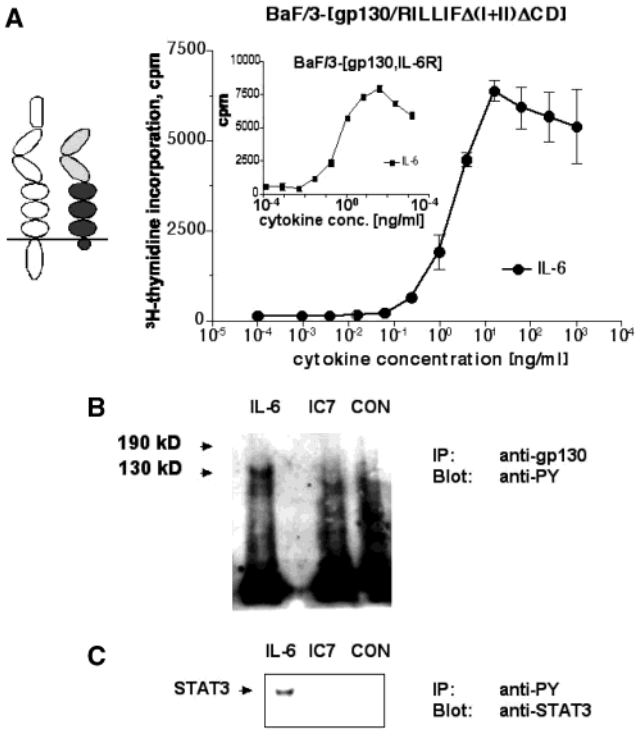

FIGURE 6: Stimulation with IL-6 of BaF/3-[gp130] cells transfected with chimera RILLIFΔ(I+II)ΔCD. (A) Proliferation of BaF/3-[gp130,RILLIFΔ(I+II)ΔCD] cells in response to IL-6. (B) Phosphorylation of gp130 in IL-6-stimulated BaF/3-[gp130,RILLIFΔ-(I+II)ΔCD] cells. (C) Phosphorylation of STAT3 in IL-6- and IC7-stimulated BaF/3-[gp130,RILLIFΔ(I+II)ΔCD] cells. The inset shows the dose−response curve of BaF/3-[gp130,IL-6R] cells after stimulation with IL-6.

DISCUSSION

There are three major findings in this study. (a) Using chimeric receptors and cytokines allowed us to elucidate the interaction of individual cytokine epitopes with receptor subdomains, which very likely reflects the arrangement of individual components in the LIF receptor complex. (b) The FNIII-like domains of the LIFR can substitute for the tether region of the IL-6R without interfering with the assembly of a functional IL-6R complex. (c) Ligand binding to site III is an absolute requirement for participation of the LIFR in a signaling heterodimer with gp130; i.e., a functional receptor complex cannot be assembled solely via sites I and II as in the GH receptor complex.

Previous studies by our group have demonstrated that the Ig-like domain and the NH_2-terminal CBM of the LIFR, but not the COOH-terminal CBM, are esssential for binding and activity of LIF (*48*). This is in accord with binding studies on deletion mutants of the LIFR (*38*, *40*). A model was suggested which stipulates binding of the Ig-like and the NH_2-terminal domains to site III of LIF and a weak, nonessential contact of the COOH-terminal CBM to site I (*48*). IC7 is inactive on BaF/3-[gp130,LIFR] (*21*) and on BaF/3-[gp130,RILLIFΔ(I+II)] cells (Figure 3), whereas it is as active as LIF and OSM on BaF/3-[gp130,RILLIFΔII] cells [Figure 2 (*48*)]. The active RILLIFΔII complex is most likely a trimer, since IC7 is virtually unable to form a signaling gp130−LIFR heterodimer after binding to chimera RILLIFΔ(I+II) (Figure 4). Thus, the presence of the IL-6R CBM and the three NH_2-terminal domains in one receptor

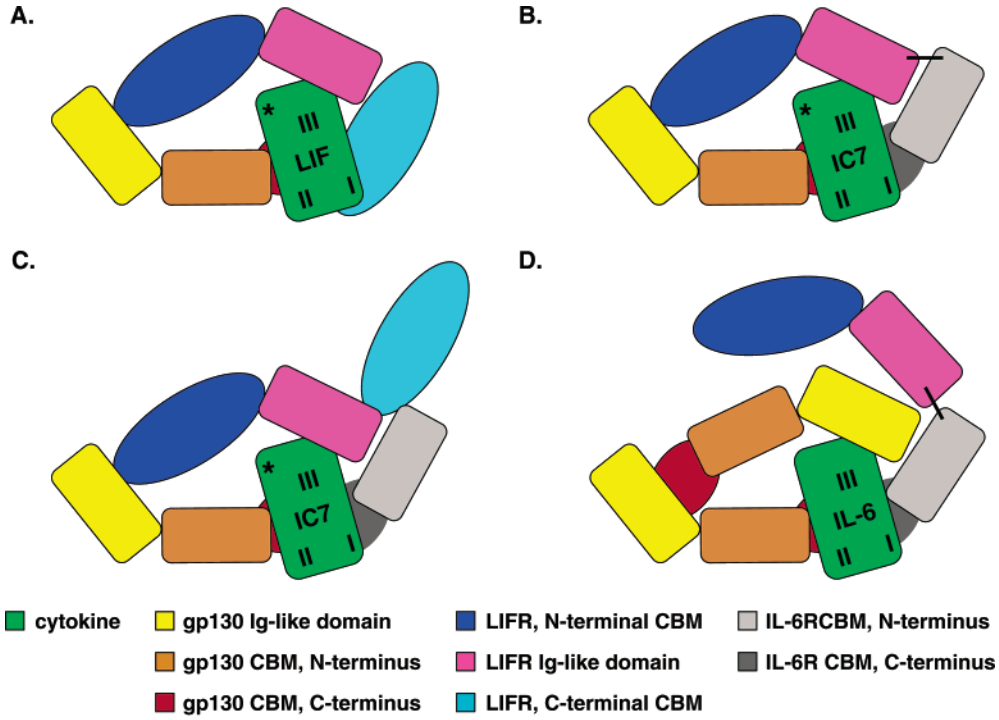

FIGURE 7: IL-6 type cytokine receptor complexes involving the LIFR. The schematic representation of the individual components of the receptor complexes was produced using the crystal structure of the vIL-6−gp130 complex as a template (*45*). The size and orientation of individual domains relative to each other are as in the crystal structure. Domains with an orientation perpendicular to the cell surface are drawn as ovals, and horizontally oriented domains are drawn as rectangles. Cytokines are green; the Ig-like domain of gp130 is yelllow, and the gp130 CBM is light brown and red. The IL-6R CBM is marked in light and dark gray, and the Ig-like domain of the IL-6R was not depicted, since it is dispensable for activity. The COOH-terminal CBM of the LIFR is light blue, the Ig-like domain purple, and the NH_2-terminal CBM dark blue. The position of the Ig-like domain of the LIFR was assumed to be the same as in gp130. (A) Trimeric LIFR complex. The asterisk designates the position of the B−C loop of LIF. (B) Complex of IC7, gp130, and chimera RILLIFΔII. The line between the Ig-like domain of the LIFR (purple) and the gray IL-6R domain indicates that both domains are part of the same receptor. (C) Complex of IC7, the IL-6R, gp130, and the LIFR. The asterisk designates the position of the B−C loop of IC7 which has a strong influence on the activity of IC7. The IC7 complex is also representative of the CNTF receptor complex (*21*). The position of the COOH-terminal CBM of RILLIFΔII is completely hypothetical. (D) Complex of IL-6, gp130, and chimera RILLIFΔII. RILLIFΔII can serve as an α-receptor for induction of a gp130 homodimer by IL-6. The position of the NH_2-terminal domains of RILLIFΔII is completely hypothetical.

seem to be required for activity of IC7. Consequently, the induction of a signaling heterodimer of gp130 and chimera RILLIFΔII by IC7 appears to be compatible with the arrangement depicted in Figure 7B: the COOH-terminal CBM of RILLIFΔII binds to site I of IC7, the Ig-like domain, and the NH_2-terminal CBM to site III and site II is engaged by gp130. Since LIF is as active as IC7 on BaF/3-[gp130,RILLIFΔII] cells (Figure 2), the arrangement of the LIFR complex may be similar to that of the RILLIFΔII complex (Figure 7A). An important corollary of this model is that the link between the Ig-like domain and the COOH-terminal CBM has a surprising degree of flexibility. In the framework of our model, the loss of binding to the LIFR of LIF site III mutants (*23*) results from a disturbed interaction with the NH_2-terminal domains of the LIFR (including the Ig-like domain) which determine LIF binding (*38*, *48*). A reverse arrangement of the LIFR would imply that the COOH-terminal CBM of the LIFR makes the vital contact to site III, which would be inconsistent with the ability of chimera RILLIFΔII to mediate a full LIF response (Figure 2).

A putative Y/F-XX-R/Q-X-R motif in the COOH-terminal CBM of the LIFR has recently been suggested to cause dimerization and self-activation of mutants linking the COOH-terminal CBM of the LIFR to the three FNIII-like domains and the cytoplasmic domain of gp130 [analogous to chimera RILLIFΔ(I+II)] (*40*). However, a comparison with the recently determined NMR structure of gp130 D3 locates the presumed interaction motif to the F β-strand and reveals it as part of a recently described "tryptophan-arginine zipper" required for the structural integrity of the D3 domain (*57*), which is also present in the COOH-terminal CBM of the OSMR and in a rudementary form in the IL-6 receptor and CNTF receptor CBM (K.-J. Kallen and J. Grötzinger, unpublished observation).

An additional configuration, other than that depicted for the LIFR complex in Figure 7 (panels A and B), of a signaling gp130−LIFR heterodimer must, however, exist since both CNTF and IC7 can induce active gp130−LIFR heterodimers (*21*). In this situation, the α-receptors for IL-6 or CNTF, respectively, will displace the COOH-terminal CBM of the LIFR and the FNIII-like domains adjacent to this CBM from site I of the ligand (Figure 7C). The activity of IL-6 on BaF/3-[gp130] cells transfected with chimeras RILLIFΔII, RILLIFΔ(I+II), and RILLIFΔ-(I+II)ΔCD (Figures 2, 3, and 6) demonstrates that the FNIII-like domains of the LIFR can replace the tether region of the IL-6R without interfering with the assembly of a functional IL-6R complex (Figure 7D). However, although IC7 activates BaF/3-[gp130,LIFR] cells with the same dose dependency as IL-6 and LIF after binding to the endogenous IL-6R (*21*), it is virtually unable to form a signaling gp130−

LIFR heterodimer after binding to chimera RILLIFΔ(I+II) (Figure 4). It is noteworthy that in the latter case, the FNIII-like domains of the α-receptor [RILLIFΔ(I+II)] and the LIFR are located on the same site of the cytokine (see Figure 7C,D for orientation), whereas after stimulation with IL-6, the FNIII-like domains of gp130 and the IL-6R−LIFR chimeras are located on opposite sites. Steric interference of the FNIII-like domains of the α-receptor chimeras might therefore hinder the LIFR to assume a "signaling competent position". Such steric effects might also explain why a monoclonal antibody (12D3) directed against the COOH-terminal CBM antagonizes the action of LIF, OSM, CNTF, and CT-1, whereas in combination with a second monoclonal antibody (8C2) also directed against the COOH-terminal CBM, mab 12D3, it displays agonistic activity (*58*). The first antibody alone could displace the LIFR from a signaling competent position such as that in Figure 7A, whereas binding of the second antibody could further push the LIFR into the second signaling competent position (Figure 7C).

A comparison of the orientation of chimera RILLIFΔII in the complex with IL-6 (Figure 7D) and with IC7 (Figure 7B) shows that although bound to the same cytokine epitope, the orientation of chimera RILLIFΔII differs in the two complexes. While this shows that the NH_2-terminal domains of RILLIFΔII do not inhibit binding of gp130 to IL-6 site III and that the interaction of cytokine and α-receptor CBM has some flexibility, the orientation of RILLIFΔII profoundly affects phosphorylation of the cytoplasmic domain. Whereas after stimulation with IC7 the cytoplasmic domains of both gp130 and RILLIFΔII were phosphorylated, only gp130 phosphorylation was detected after stimulation with IL-6 (Figure 2). Similarly, only gp130 became phosphorylated after stimulation of BaF/3-[gp130,RILLIFΔ(I+II)] cells with IL-6, although the complex of gp130 and RILLIFΔ(I+II) was so tightly formed that strong detergents were necessary to disrupt coprecipitation of gp130 and RILLIFΔ(I+II) (Figure 5). However, although IC7 elicited formation of a gp130−RILLIFΔ(I+II) heterodimer at sites I and II, this did not become phosphorylated (Figure 3E), possibly due to the absence of a binding partner for its site III epitope on chimera RILLIFΔ(I+II). These results underline the view that dimerization of receptors per se is not sufficient for receptor activation and that changes in the orientation of dimerized receptors to each other can lead to receptor complexes with high, low, or no activity (*10*, *59*).

Our results demonstrate that the contact of the NH_2-terminal LIFR domains to cytokine site III is all important for the assembly of an active gp130−LIFR heterodimer, whereas binding of receptor chimeras to site I alone is insufficient to elicit such a dimer (Figure 3). This is in obvious contrast with other hematopoietic cytokine receptor complexes such as that of GH, IL-4, and erythropoietin which all signal via a site I/site II pattern and lack the FNIII-like domains of IL-6 type β-receptors (*10*, *12*, *17*). On GCSF, the two identified receptor binding epitopes correspond to sites II and III in the terminology of IL-6 type cytokines (*60*, *61*). The GCSF receptor complex may therefore be assembled in a manner similar to that of the vIL-6−gp130 complex (*45*, *62*). Notwithstanding, on cells expressing chimeric receptors linking the CBM of the GH receptor to the FNIII-like domains of the GCSF receptor, GH induced an active dimer of the mutant receptors via site I and site II interaction (*63*). Thus, a site I/site II signaling pattern is not excluded per se by the presence of the membrane proximal FNIII-like domains in the β-receptor. The precise role of the FNIII-like domains in the assembly of long chain cytokine receptor complexes, in particular, their interaction with the domains directly involved in cytokine binding, is unknown and is a question that remains to be answered.

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