Two Different Epitopes of the Signal Transducer gp130 Sequentially Cooperate on IL-6-Induced Receptor Activation

Stefan Pflanz, Ingo Kurth, Joachim Grötzinger, Peter C. Heinrich and Gerhard Müller-Newen

*J Immunol* 2000; 165:7042-7049; doi: 10.4049/jimmunol.165.12.7042
http://www.jimmunol.org/content/165/12/7042

References
This article cites 43 articles, 25 of which you can access for free at: http://www.jimmunol.org/content/165/12/7042.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Two Different Epitopes of the Signal Transducer gp130 Sequentially Cooperate on IL-6-Induced Receptor Activation

Stefan Pflanz, Ingo Kurth, Joachim Grötzinger, Peter C. Heinrich, and Gerhard Müller-Newen

Cytokines are important intercellular mediators in the regulation of immune responses, hemopoiesis, fertility, and the acute phase reaction. They bind to specific cell surface receptors on their target cells and thereby induce receptor oligomerization. This event triggers juxtaposition of the cytoplasmic parts of the respective receptors and initiates the intracellular signal transduction cascade, which involves phosphorylation of diverse signaling proteins and transcription factors (1). Cytokine receptors are usually classified according to structural attributes. The predominant class 1 cytokine receptors are characterized by the presence of at least one cytokine-binding module. Mutants of gp130 that either lack the Ig-like domain D1 (ΔD1) or contain a distinct mutation (F191E) within the cytokine-binding module have been shown to be severely impaired with respect to IL-6 induced signal transduction. After cotransfection of COS-7 cells with a combination of both inactive gp130 mutants, signal transduction in response to IL-6 is restored. Whereas cells transfected with ΔD1 do not bind IL-6/sIL-6R complexes, cells transfected with the F191E mutant bind IL-6/sIL-6R with low affinity. Combination of ΔD1 and F191E, however, leads to high-affinity ligand binding. These data suggest that two different gp130 epitopes, one on each receptor chain, sequentially cooperate in asymmetrical binding of IL-6/IL-6R in a tetrameric signaling complex. On the basis of our data, a model for the mechanism of IL-6-induced gp130 activation is proposed. The Journal of Immunology, 2000, 165: 7042–7049.

Abbreviations used in this paper: CBM, cytokine-binding module; CNTF, ciliary neurotrophic factor; LIF, leukemia-inhibitory factor; OSM, oncostatin M; s, soluble; PVDF, polyvinylidene difluoride.
were cultured in DMEM supplemented with 10% (v/v) FCS, streptomycin (150 μg/ml), penicillin (60 units/ml), and 0.1% sodium azide (PBS-F/azide) at 4°C and subsequently incubated on ice with 1 μg/ml gp130 Ab B-P4 for 30 min. Cells were washed with cold PBS-F/azide and incubated with R-PE-conjugated anti-mouse IgG Fab fragment at a 1:50 dilution for another 30 min. Again, cells were washed with cold PBS-F/azide and then resuspended in 400 μl PBS-F/azide followed by flow cytometry analysis using a FACS calibur (Beckton Dickinson).

**Materials and Methods**

**Enzymes, proteins, Abs, chemicals, and cell culture**

Enzymes were purchased from Boehringer Mannheim (Mannheim, Germany), protein A-Sepharose from Pharmacia (Freiburg, Germany). DMEM and antibiotics were obtained from Life Technologies (Eggenstein, Germany), and FCS from Seromed (Munich, Germany). Radiochemicals were purchased from Amersham International (Amersham, U.K.). Recombinant human IL-6 was expressed in Escherichia coli, refolded, and purified as described by Arcone et al. (16). Soluble IL-6R was prepared as described previously (17). The monoclonal gp130 Ab B-P4 was generated as described elsewhere (18). All other Abs were purchased from Dako (Hamburg, Germany). PBS buffer contained 200 mM NaCl, 2.5 mM KCl, 8 mM Na2HPO4, and 0.1% sodium azide (PBS-F/azide) at 4°C and subsequently incubated.

**Plasmid construction and transfection of cells**

Generation of mutants gp130F191E and gp130ΔD1 as well as Fc and γ constructs has been described previously (13, 19). To obtain both gp130 mutants in the context of a WT cytoplasmic tail, the extracellular parts of the respective γ constructs were cloned into pSVL/gp130 via XhoI/EcoRI digestion. COS-7 cells were transiently transfected applying the DEAE-dextran method. It is important to emphasize that in all transfections equal total amounts of receptor-encoding plasmid-DNA were used; in case of a two-plasmid transfection, the vectors encoding each individual receptor construct were used only in one-half the amount compared with single-plasmid transfection. Efficiency of transfection was analyzed by FACS.

**Flow cytometry**

Cells were collected, washed, and resuspended in PBS containing 5% FCS and 0.1% sodium azide (PBS-F/azide) at 4°C and subsequently incubated on ice with 1 μg/ml gp130 Ab B-P4 for 30 min. Cells were washed with cold PBS-F/azide and incubated with R-PE-conjugated anti-mouse IgG Fab fragment at a 1:50 dilution for another 30 min. Again, cells were washed with cold PBS-F/azide and then resuspended in 400 μl PBS-F/azide followed by flow cytometry analysis using a FACS calibur (Becton Dickinson).

**Reportergene assay using transfected COS-7 cells**

COS-7 cells were transiently transfected with equal total amounts of gp130 expression plasmid were cultured in DMEM for 48 h post transfection and then incubated with a constant amount of sIL-6R (100 nM) in combination with varying amounts of 125I-labeled IL-6 (67,000 cpm/ng) ranging from 0.125 to 8 nM in binding medium (20 mM HEPES/0.2%BSA in DMEM). After incubation for 12 h at 4°C, supernatants were separated from the cells, the cells were washed twice with PBS, and cell-associated vs free radioactivity was measured using a gamma counter. Specific binding was obtained by subtracting the radioactivity associated with mock transfected COS-7 cells.
**Results**

Two different epitopes involving the Ig-like domain or the CBM of gp130 cooperate in the formation of a signal-transducing gp130 homodimer in response to IL-6

Previous studies demonstrated the importance of two distinct receptor epitopes for IL-6-induced activation of gp130: one epitope is located in the gp130-CBM; and the other involves the Ig-like domain (D1) (12, 13). Disturbances introduced to any of these two epitopes such as mutation of a critical amino acid residue in the CBM (F191) or deletion of D1 abolished gp130-mediated signal transduction after stimulation with IL-6. Thus, efficient receptor dimerization could not be achieved by gp130 molecules defective in either D1 or the CBM. In the present study, we investigated whether both critical gp130 epitopes are required in each of the two homodimer-forming gp130 receptors for efficient signal transduction.

As a first approach, a reporter gene assay was performed using a luciferase reporter gene under the control of the IRF-1 promoter. COS-7 cells were transiently transfected with equal total amounts of plasmid DNA encoding gp130 wild-type, gp130F191E (F191E), gp130ΔD1 (ΔD1), the combination of gp130F191E and gp130ΔD1 (F191E/ΔD1), or empty expression vector (Fig. 1, left). Transfected cells were stimulated with IL-6 (15 ng/ml)/sIL-6R (500 ng/ml) or were left unstimulated. Because COS-7 cells contain small amounts of endogenous gp130, the stimulation had to be suboptimal to minimize reporter induction in the mock transfected cells. When transfected alone, neither of the two mutants F191E and ΔD1 was able to significantly induce the reporter gene (Fig. 2). After transfection of the combination F191E/ΔD1, a restored reporter gene activity could be monitored. Because of the suboptimal stimulation, the overall induction of the IRF-1 promoter was weak even on transfection of gp130 wild-type (1.60 ± 0.09). Nevertheless, the differences in gene induction for control (1.02 ± 0.04), F191E (1.08 ± 0.01), and ΔD1 (1.06 ± 0.02) on the one hand and the combination F191E/ΔD1 (1.32 ± 0.06) on the other hand are significant and were reproducible in three independent experiments. Statistical significance was proved, applying Student’s t test with a probability value of α = 0.05 (data not shown).

To confirm these observations in the context of a rapid signaling response such as STAT activation, COS-7 cells were again transfected with the respective receptor constructs. However, to overcome STAT activation via the small amounts of endogenous gp130 present in COS-7 cells, the receptor constructs used for transfection were further mutated to contain a cytoplasmic part comprising the gp130 box1/2-sequences required for recruitment of Jak kinases followed by the exclusively and strongly STAT1-activating tyrosine motif YDKPH derived from the IFN-γ-receptor (γ constructs, Fig. 1, middle) (11). A comparable cell surface expression of the receptor γ constructs was observed by FACS (Fig. 3A), using the gp130 mAb B-P4. This mAb recognizes an epitope located in the membrane-proximal part of gp130 (24); thus, binding is not affected by either the point mutation or the deletion of D1. The comparable cell surface expression of the three proteins (gp130γ, F191Eγ, and ΔD1γ) indicates that the overall structure of gp130 was not affected by the mutations. Otherwise, the receptors would not be able to appear on the cell surface but as a result of partial misfolding would have been intracellularly degraded.

In FACS analysis, the Ab B-P4 does not discriminate between the two mutants F191Eγ and ΔD1γ. To verify expression of both mutants after cotransfection, cell lysates were prepared, subjected to SDS-PAGE, and analyzed by Western blotting (Fig. 3B). All proteins were detectable at electrophoretic mobilities corresponding to their anticipated molecular masses, demonstrating that cotransfection of F191Eγ and ΔD1γ indeed led to coexpression of both mutants. ΔD1γ shows an increased electrophoretic mobility correlating with the deletion of a 100-aa polypeptide containing two potential N-glycosylation sites.

COS-7 cells, transfected with equal total amounts of expression plasmids encoding gp130γ, F191Eγ, ΔD1γ, or a control plasmid were stimulated with IL-6, and a large molar excess of sIL-6R or were left unstimulated. Nuclear extracts were prepared and analyzed by EMSA for STAT1 DNA-binding activities (Fig. 3C, top). Intensities of the radioactive bands corresponding to STAT1DNA complexes were quantified using a PhosphorImager (Fig. 3C, bottom). Transfection of F191Eγ or ΔD1γ alone did not lead to significant STAT1 activation. In each experiment, levels of STAT1, which has been activated by these two individual mutants, range within the background activation observed for the mock transfected control cells. This implies that the potential of gp130 to mediate signal transduction is severely impaired by either of the two mutations. The combination F191Eγ/ΔD1γ, however, elicited about one-half the STAT1 activation mediated through gp130γ. These results are in line with the previous data obtained by the reporter gene assay (see Fig. 2).
An epitope involving the Ig-like domain cooperates with the CBM of gp130 for efficient binding of IL-6/sIL-6R complexes

The restoration of IL-6 sensitivity by combination of two per se inactive receptor mutants pointed to a complementary involvement of two different gp130 epitopes on two receptor chains in the context of a gp130 dimer. This hypothesis was further analyzed in a coimmunoprecipitation assay, investigating the binding of IL-6/sIL-6R to soluble Fc fusion proteins of the gp130 mutants. The extracellular parts of gp130, F191E, and ΔD1 were fused to the Fc part of hIgG1 (Fc constructs, Fig. 1, right). The Fc fusion proteins are covalently dimerized via the cysteine residues responsible for the connection of the two heavy chains in an Ab. This experimental design enabled us to investigate the ligand-binding capacity of our gp130 mutants as preformed dimers in solution.

COS-7 cells were transiently transfected with equal total amounts of the three single expression plasmids sgp130Fc, FIGURE 3. IL-6-induced STAT activation in transiently transfected COS-7 cells. A, COS-7 cells were transiently transfected with the expression vectors as indicated; again equal total amounts of DNA were used. Forty-eight hours after transfection, the cells were incubated with the gp130 mAb B-P4 and stained with a PE-labeled secondary Ab. Mock transfected cells served as a control. Unspecific fluorescence by the secondary Ab alone is represented by the gray peak; the transparent peaks monitor cellular fluorescence intensities after overexpression of gp130 or the respective mutants. B, Total lysates of COS-7 cells were prepared 48 h after transient transfection with the gp130γ construct or the respective mutants as described in A. Mock transfected cells served as a control. Cellular proteins were separated by SDS-PAGE and electroblotted. gp130γ and mutants were detected by immunostaining using the gp130 mAb B-P4 and visualized by ECL. α hgp130, anti-human gp130. C, Analysis of STAT1 activation in COS-7 cells transiently transfected with equal total amounts of cDNA encoding gp130γ, a control plasmid, F191Eγ, ΔD1γ, and the combination F191Eγ/ΔD1γ. Forty-eight hours after transfection with the respective expression plasmids, as indicated, cells were stimulated with IL-6 (12.5 ng/ml) and sIL-6R (500 ng/ml) for 15 min (+) or were left unstimulated (−). Nuclear extracts were prepared and subsequently analyzed by EMSA; a typical autoradiograph is shown (top). Mock transfected cells served as a control. Radioactivity of STAT1-DNA complexes were analyzed using a PhosphorImager. Counts obtained from STAT1 activation were normalized relative to the maximal signal intensity obtained for gp130γ. Error bars indicate SDs derived from three independent experiments (bottom).
sF191EFc, or sΔD1Fc; the combination of sF191EFc/sΔD1Fc; or a control plasmid. The soluble dimeric receptor-Fc fusion proteins can be precipitated directly from the cell supernatants using protein A-Sepharose. These primary precipitates were subsequently incubated with IL-6/sIL-6R, and a coprecipitation experiment was performed. Sepharose-associated proteins were separated by SDS-PAGE under reducing conditions, blotted, and immunodetected using Abs recognizing theFc part of the fusion proteins and hIL-6, respectively. SDS-PAGE under nonreducing conditions retarded the Fc fusion proteins to an apparent molecular mass of >220 kDa, indicating that the soluble Fc fusions were indeed expressed as cysteine-bridged dimers (data not shown). spg130Fc potently coprecipitated IL-6 in the presence of sIL-6R, whereas sF191EFc showed only weak coprecipitation capacity, and the deletion mutant sΔD1Fc was unable to coprecipitate detectable IL-6 quantities (Fig. 4). This experimental design rules out the possibility that one ΔD1 initially binds IL-6/sIL-6R very weakly and therefore cannot survive the time span required for the second ΔD1 to join the complex; the ΔD1 mutant itself apparently is not capable of binding to IL-6/sIL-6R.

The amount of IL-6 that was coprecipitated by the combination of sF191EFc and sΔD1Fc ranges between the amounts observed with spg130Fc and sF191EFc. As demonstrated by the immunodetection of Fc, the sum of precipitated receptor fusion proteins in the combination sF191EFc/sΔD1Fc (Fig. 4, top, lane 6) did at least not exceed the levels of precipitated single proteins sF191EFc and sΔD1Fc (Fig. 4, top, lanes 4 and 5). Thus, the increase in coprecipitated IL-6 by sF191EFc/sΔD1Fc cannot be attributed to higher expression levels of the combination compared with sF191EFc. Moreover, from a statistical point of view, coexpression of sF191EFc and sΔD1Fc will lead to three different species of cysteine-bridged Fc dimers in the cellular supernatants: 25% (sF191EFc); 25% (sΔD1Fc); and 50% dimers as sF191EFc/sΔD1Fc. Therefore, the IL-6 amounts shown in lane 6 must have been coprecipitated by a significantly lower number of Fc dimers compared with lane 4, because sΔD1Fc dimers were found to be completely inactive (lane 5). These results point to a remarkably increased IL-6/sIL-6R-binding capability of the combination sF191EFc/sΔD1Fc compared with sF191EFc or sΔD1Fc.

High affinity binding of IL-6/sIL-6R can be restored by combining F191E with ΔD1

Binding of IL-6/sIL-6R complexes to the mutants was further investigated using membrane-bound receptors. gp130 has been found to convert the low affinity binding between IL-6 and IL-6R into a high affinity ternary complex (4). COS-7 cells were transiently transfected with equal total amounts of plasmids encoding F191Eγ, ΔD1γ, or the combination F191Eγ/ΔD1γ. The γ-constructs were chosen because they lead to enhanced surface expression because of the lack of the gp130 dileucine motif required for efficient internalization of the receptor (25). Mock transfected cells served as a control. As described above, cell surface expression of all transfected constructs was analyzed by FACS and by Western blot analysis of total cell lysates (data not shown, but comparable with those in Fig. 3, A and B). The IL-6/sIL-6R-binding capacities of the two gp130 mutants and their combination during expression on the cell surface was studied by an equilibrium binding assay using radiolabeled IL-6 (125I-labeled IL-6). To saturate the initial low affinity equilibrium between IL-6 and sIL-6R, transfected cells were incubated with 125I-labeled IL-6 in the presence of a large molar excess of sIL-6R. By this means the three-components system (IL-6, sIL-6R, gp130) was reduced to two components ([IL-6/sIL-6R], gp130), regarding the complex of IL-6/sIL-6R as an activated heterodimeric cytokine.

Scatchard analysis of the binding data is shown in Fig. 5. Strong overexpression of ΔD1 does not lead to a significant enhancement of IL-6-binding capacity because the bound radioactivity barely exceeds the radioactivity associated with mock transfected cells (the data in Fig. 5 result from subtraction of the low levels of

**FIGURE 4.** Soluble ternary complex formation assay using IL-6/sIL-6R with spg130Fc, sF191EFc, sΔD1Fc, and the combination sF191EFc/sΔD1Fc in a coprecipitation experiment. COS-7 cells were transfected with equal total amounts of expression vectors encoding the three individual gp130 variants and the combination sF191EFc/sΔD1Fc, respectively. Sixty hours after transfection, the Fc fusion proteins were precipitated from the cell supernatants using protein A-Sepharose. Sepharose-bound proteins were incubated with IL-6/sIL-6R as indicated. The ternary complexes formed were precipitated, separated by SDS-PAGE, and transferred to PVDF membranes. Proteins were visualized by ECL using the Abs indicated. α hgp130, anti-human gp130; α hIL-6, anti-human IL-6.

**FIGURE 5.** Scatchard analysis of 125I-labeled IL-6 binding to transfected COS-7 cells. COS-7 cells (2.5 × 10⁴) transfected with equal total amounts of the expression plasmids encoding F191Eγ (□, dotted line), ΔD1γ (■), or the combination of F191Eγ and ΔD1γ (▲, solid line) were incubated with varying amounts (0.125–8 nM) of [125I]-IL-6 in the presence of an excess of sIL-6R (100 nM). After incubation for 12 h at 4°C, free and cell-bound radioactivity was measured. Specific binding was obtained by subtraction of the low levels of radioactivity associated with mock transfected cells. Each point of data represents the mean value of two independent experiments.
125I-labeled IL-6-binding to mock transfected cells). Overexpression of F191EY leads to the formation of a huge amount of low affinity binding sites. This coincides with the low level of IL-6/sIL-6R binding by sF191EFe (Fig. 4). The combination of F191E with ΔD1γ, however, results in the formation of both low and high affinity binding sites.

From the Scatchard plot the $K_d$ of the high affinity interaction was calculated to be in the range of 350–450 nM. This value fits well with the data obtained from cells expressing gp130 endogenously (210–380 nM) as well as from COS-7 cells transfected with gp130 wild type (330–650 nM). The number of high affinity binding sites critically depends on transfection efficiency. From the data in Fig. 5, a number of $\approx 57,000$ high affinity binding sites was calculated. This is in agreement with previous observations obtained from COS-7 cells transfected with gp130 wild type (12,000–51,000 sites/cell). For the low affinity interaction, quantitative evaluation of the Scatchard data is limited because for practical reasons 125I-labeled IL-6 could not be applied at near saturation concentrations. Nevertheless, the data allow a good estimation of the low affinity interaction with a $K_d$ of 13–18 nM. Thus, the affinity is reduced by a factor of 33–45.

Taken together, the binding data are in line with the findings described above supporting the concept that two different gp130 epitopes on two receptor chains cooperate in high affinity ligand binding and activation of the signal transducer.

**Discussion**

Initiation of cellular signal transduction via gp130 activation requires a gp130 dimer of a distinct conformation because dimerization per se was shown to be necessary but not sufficient for receptor activation (28). The appropriate dimerization is achieved by the specific interaction of gp130 with the IL-6/IL-6R complex. Recent studies applying site-directed mutagenesis as well as investigation of gp130 deletion mutants revealed that one epitope participating in the interaction with IL-6/IL-6R involves the Ig-like domain (in the following referred to as epitope A; this epitope is lacking in the ΔD1 mutant) and the other is located in the CBM (referred to as epitope B; this epitope is destroyed in the F191E mutant). The data presented here show that functional homodimerization of gp130 carrying mutations in either epitope A or epitope B is not possible (Figs. 2 and 3C). The combination of one epitope A-defective (ΔD1) with an epitope B-defective (F191E) gp130 mutant, however, restored receptor activity (Figs. 2 and 3C) and led to high affinity binding of IL-6/sIL-6R (Fig. 5). Accordingly, epitopes A and B are both required to allow IL-6-induced activation, but one pair of complementary epitopes on two different receptor chains is sufficient to constitute an active gp130 dimer. Thus, gp130 epitopes A and B cooperate inter- but not intramolecularly on IL-6-induced receptor activation.

Site-directed mutagenesis of IL-6 led to the identification of three distinct epitopes (sites) responsible for the interaction with IL-6R (site I) and the two gp130 molecules (sites II and III) (30). Similarly positioned receptor-engaging epitopes have been described for the closely related cytokines IL-11 (31, 32) and CNTF (33), which signal via gp130/gp130 homodimers and gp130/LIFR heterodimers, respectively. The initial event in the formation of a signal transducing IL-6/IL-6R/gp130 ternary complex on the cell surface is the contact of site I of IL-6 with IL-6R, which is a prerequisite for the engagement of gp130 because neither IL-6 nor IL-6R alone have a detectable affinity for gp130. How the two gp130 molecules join the IL-6/IL-6R complex is currently not known. Previous studies demonstrated that the Ig-like domain of gp130 is not needed for LIF- as well as OSM-induced activation in the context of a gp130/LIFR heterodimer (12, 34). Instead, in these complexes the Ig-like domain of the LIFR contributes to ligand binding (35, 36). In another report, the authors altered the receptor specificity of IL-6. They created an IL-6/CNTF cytokine hybrid by transferring site III of CNTF into the IL-6 molecule (IC7), thereby switching the receptor specificity from gp130/gp130/IL-6R in IL-6 to gp130/LIFR/IL-6R in IC7 (37). Taken together, these findings suggest that site II of IL-6 engages epitope B. This implies that epitope A is involved in recognition of site III.

Destruction of epitope A by deletion of the Ig-like domain (ΔD1 mutant) generated a gp130 molecule that did not respond to IL-6 (Figs. 2 and 3) and that was not able to bind IL-6/sIL-6R via its functional epitope B (Figs. 4 and 5), whereas destruction of epitope B (F191E mutant) by site-directed mutagenesis generated a gp130 that was biologically inactive (Figs. 2 and 3C) but retained residual binding activity to IL-6/sIL-6R via its functional epitope A (Figs. 4 and 5). Therefore, we conclude that the interaction of IL-6 site III with gp130 epitope A is a prerequisite for interaction between site II and gp130 epitope B, eventually constituting a nonsymmetrical IL-6/IL-6R/gp130 ternary complex which is capable of initiating signal transduction. A model for IL-6-induced activation of gp130 by sequential engagement of the three receptor chains IL-6R (via site I), first gp130 (via site III/epitope A), and the second gp130 (via site II/epitope B) is shown in Fig. 6. We assume that epitope A might form contacts between site III and parts of the IL-6R as well, because IL-6 alone does not show a detectable affinity to gp130. Of course, we cannot exclude that

![FIGURE 6](http://www.jimmunol.org/Downloaded_from)
conformational changes of site III that are possibly induced by binding of IL-6R to site I could account for this effect. However, the IL-6-induced signal transduction via gp130 seems to be a defined sequential three-step process, in which each step is a prerequisite for the subsequent events.

Thus far, the structure of the growth hormone/growth hormone receptor complex was a paradigm for cytokine receptor complex assembly (38). In this complex, as well as in the more recently solved structure of the erythropoietin-erythropoietin receptor complex (39), sites I and II of the cytokine bind to identical epitopes of the two receptors, thereby forming the active receptor homodimer. Our findings demonstrate that within the family of class I cytokine receptors the mode of ligand recognition differs significantly, depending on the ectodomain architecture of the receptor and the binding site topology of the cytokine.

The question of the stoichiometry for IL-6/IL-6R/gp130 ternary complexes has long been subject to discussion (40). By using soluble receptor proteins, two groups have provided experimental evidence for a hexameric complex consisting of two molecules of each IL-6, sIL-6R, and sgp130 (14, 15). The data led to the proposal of two models for the architecture of the (IL-6/sIL-6R/sgp130) ternary complex (14, 30). Apart from differences in minor details, both models postulate contacts between IL-6 site I to the respective sIL-6R and, more importantly, demand two topologically separated contact epitopes on each sgp130: one engaging site II of one IL-6; and a second providing contacts to site III of the other IL-6. Thus, four epitopes on two sgp130 provide a double bridging of the two separate IL-6/sIL-6R units thereby assembling a hexameric complex. Alternatively, on the basis of structural considerations, a model for a tetrameric ternary complex consisting of one IL-6, one IL-6R, and two gp130 has been suggested (41). This model postulates three contacts between a single IL-6 and its receptors: IL-6 site I with IL-6R; site II with the CBM of the first gp130; and site III with the second gp130, each receptor contacting IL-6 only once. Unlike the postulate for the soluble hexameric ternary complexes, our data suggest that there does not necessarily exist a requirement for four intact gp130 epitopes with respect to IL-6-induced gp130 activation on the cell surface. We were able to show that epitope A on one gp130 and epitope B on another gp130 are sufficient to initiate IL-6 induced signal transduction. From this point of view, at least for low concentrations of IL-6/sIL-6R, the formation of a hexameric ternary complex on the cell surface seems unlikely. Because in the tetrameric complex each gp130 molecule provides a free binding site, in the presence of high IL-6 doses the formation of higher order complexes cannot be excluded (40).

Regarding our experiments investigating gp130 activation on cells, the levels of cellular responses initiated through the F191E or ΔD1 mutant range within the background response observed for control cells (0%), whereas gp130 gave the 100% response under the respective experimental conditions. The combination F191E/ΔD1 did significantly reduce the cellular response but did not reach a level comparable to that of gp130 wild type. The stimulation via the combination F191E/ΔD1 amounts to ~50% (see Figs. 2 and 3C). Provided that the receptor mutants are surface expressed in comparable amounts, this finding cannot be explained by assuming diffusible receptor monomers on the plasma membrane: After the complex of IL-6/sIL-6R has been caught by epitope A of gp130, the intermediate low affinity ternary complex would be completed by association of a second gp130 providing the intact epitope B (ΔD1), leading to a 100% response.

Studies on the gp130/OSMR heterodimer (42) and more recent investigations on erythropoietin receptor homodimerization (43) suggest that cytokine receptors may appear as preformed dimers on the cell surface. On ligand binding, these receptor dimers switch from an inactive to an active conformation. If gp130 is predimerized in an inactive conformation independently of the ligand, from a statistical point of view four different dimers are conceivable regarding the combination of gp130 epitopes, A/A, B/B, A/B, and B/A. The former two combinations were shown to be biologically inactive, the latter two combinations are biologically active but represent only one-half of the total receptor population, which limits the expected response to 50%.

It remains to be shown whether a preformed gp130 dimer actually exists on the cell surface. In any case, the understanding not only of ligand-receptor interactions but also of receptor activation mechanisms provides a rational basis for drug design to pharmacologically interfere with the earliest event of signal transduction.

Acknowledgments

We thank Andreas Timmermann, Heike Hermanns, and Drs. Fred Schaper, Lutz Graeve, and Serge Haan for critical reading of the manuscript and Dr. John Wijdenes (Diaclone, France) for the supply of gp130 mAb B-P4.

References
