Identification of Agonistic and Antagonistic Antibodies against gp190, the Leukemia Inhibitory Factor Receptor, Reveals Distinct Roles for Its Two Cytokine-binding Domains*

The receptor for the cytokine leukemia inhibitory factor (LIF) associates the low affinity binding component gp190 and the high affinity converter gp130, both of which are members of the family of hematopoietic receptors characterized by the cytokine receptor homology (CRH) domain. The gp190 is among the very few members of this large family to contain two CRH domains. The membrane-distal one (herein called D1) is followed by an Ig-like domain, a membrane-proximal CRH domain called D2, and three type III fibronectin repeats. We raised a series of monoclonal antibodies specific for the human gp190. Among them was the blocking antibody 1C7, which was directed against the D11g region and which impaired the binding of LIF to gp190. Another blocking antibody, called 12D3, was directed against domain D2 and interfered with the reconstitution of the high affinity receptor complex, independently of the interaction between LIF and gp190. The blocking effect of these two antibodies concerned four cytokines known to use gp190, i.e. LIF, oncostatin M, ciliary neurotrophic factor, and cardiotrophin-1. Among 23 antibodies tested alone or in combination (two anti-D2 and 21 anti-D11g), only the mixture of the two anti-D2 antibodies displayed agonistic activity in the absence of the cytokine. Taken together, these results demonstrate that the two CRH domains of gp190 play different functions in ligand binding and receptor activation.

The leukemia inhibitory factor (LIF) low affinity receptor gp190 belongs to the large family of the hematopoietic receptors that are characterized by a consensus cytokine receptor homology (CRH) domain, the so-called cytokine-binding domain, consisting of two modules of around 100 amino acids each. The amino-terminal one contains several conserved disulfide bonds, whereas the carboxyl-terminal one comprises the consensus WSXWS amino acid sequence. The extracellular region of gp190 is unusual in that it has two CRH domains, which we called D1 for the amino-terminal membrane distal one and D2 for the membrane proximal one. D1 and D2 are separated by an Ig-like module of around 100 amino acids, and D2 is followed by three 100-amino-acid-long type III fibronectin modules, the FN region (1). gp190 participates in the high affinity receptor complex for five human cytokines (reviewed in Ref. 2), namely LIF, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), and the very recently discovered neurotrophin-1/B cell-stimulating factor 3 (3).

Given the structural complexity of gp190, very few data relative to the function of each module are available to date. Other receptor chains in humans also have two CRH domains, such as the common β-chain for IL-3, IL-5, and GM-CSF (the β-common), the thrombopoietin receptor, the leptin receptor, and the OSM-specific receptor that lacks the amino-terminal half of the first CRH. In the β-common and the thrombopoietin receptor, the two CRH are adjacent to each other. In these cases, the available data suggest that only the membrane proximal one for the β-common (4, 5) or the membrane distal one for the thrombopoietin receptor (6) is involved in ligand interactions. In the case of gp190, OSM-specific receptor, and leptin receptor, an Ig-like module is intercalated between the two CRH, with potentially important consequences on receptor function. The Ig-like module could act as a spacer or a hinge between the two CRH, giving flexibility for both of them either to participate to ligand binding or to interact with each other, as we recently suggested for human gp190 in a recent work (7). Alternatively, it could also directly participate in ligand binding, and we recently described a ligand-binding site for human LIF at its carboxyl terminus (7). Taking advantage of the finding that murine gp190 displays a 30–100 times higher affinity for human LIF than human gp190 does (8), it has been demonstrated using human-murine chimeric gp190 that the Ig-like module was responsible for the species specificity of gp190 for LIF. In this study, a human gp190 where the Ig-like module was replaced with its murine homolog also displayed a high affinity for human LIF (9). Interestingly, the LIF-binding site we identified also belonged to the Ig-like module as defined in the chimeras made by these authors. Therefore, the complex modular architecture of gp190 most probably reflects specific functions for the Ig-like module as well as for each of the CRH domains. In addition, there could be important differences with respect to gp190 engagement between the cytokines that share gp190.

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Published, JBC Papers in Press, October 17, 2001
Published for publication, June 13, 2001, and in revised form, October 17, 2001
Published, JBC Papers in Press, October 17, 2001, DOI 10.1074/jbc.M105476200

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Printed in U.S.A.

DOI: 10.1074/jbc.M105476200

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* This work was supported by the Ligue Nationale contre le Cancer (Comités de la Gironde, de la Dordogne, des Pyrénées-Atlantiques) and by the Association pour la Recherche sur le Cancer. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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We previously obtained a series of mAbs specific for human gp190 (10). One of them (1C7) displayed a LIF blocking activity via impairment of LIF/gp190 interaction and was directed against a conformation-dependent epitope localized within D11g (7). In this report, we describe the functional properties of another blocking antibody, called 12D3, which is directed against the D2 domain. Comparison of these two anti-gp190 mAbs between each other and with the available data on gp190 gives new insight into gp190 structure-function relationships.

**EXPERIMENTAL PROCEDURES**

**Monoclonal Antibodies**—The anti-gp190 mAb 12D3 was produced in the laboratory according to the protocol described in Ref. 10. The obtaining and the mapping of mAbs 1C7, 10B2, and 8C2 have already been described (10). The lack of anti-gp130 cross-reactivity of the anti-gp190 mAbs was ascertained by testing them on a CHO cell line stably expressing human gp130 but not gp190. In all of the assays performed in this study, the mAbs were used after purification from mouse ascites by affinity chromatography on a protein A column followed by extensive dialysis against PBS buffer and sterilization by filtration, as described (10). The anti-gp130 mAb B-R3 was purchased from Diacclone (Besançon, France).

**Obtaining and Expression of the gp190 Mutants**—Obtaining the secreted Myc-tagged gp190 deletion mutants (D11gD2myc and D2FNmyc) from stably transfected CHO cells has been described elsewhere (7). Obtaining the chimeric transmembrane gp190/130 consisting of the extracellular region of gp190 fused to the transmembrane and intracellular domains of gp130, the generation of LIF-dependent Ba/F3 cells lines expressing wild-type gp190 and gp190 (Ba/F3 gp190 + gp130), or gp130 and chimeric gp190/130 receptors (Ba/F3 gp190/130 + gp130) has also been described previously (7). These cell lines were maintained in RPMI supplemented with 5% fetal calf serum, 2 mM l-glutamine, and 50 ng/ml CHO-recombinant human LIF. The Ba/F3 cell line expressing gp130 and the IL-6 low affinity receptor gp80 was obtained in a similar fashion, with a selection of transfected cells in the presence of the IL-6 ligand, as described (7). Several transfected CHO cell supernatants, the following cytokines were used: murine IL-3 was in the form of a supernatant from a transfected COS cell line. The proliferation of transfected Ba/F3 cells expressing wild-type gp130, Ba/F3 gp190/130, or IL-3 as a control were 3-fold serially diluted and directly added to the washed cells. For the blocking experiments conducted with anti-gp190 mAbs, 2-fold dilutions of mAb were preincubated (20 μl/well) with the washed Ba/F3 cells (70 μl/well) for 30 min at 37 °C to saturate the receptors before the addition of 10 μl/well of a subsaturating concentration of cytokine (final concentrations, 100 pg/ml for LIF, 5 ng/ml for OSM, 5 ng/ml for CT-1, 1 μg/ml for CNTF, or 2 units/ml for murine IL-3). For the proliferation experiments involving the agonistic mAb combination, the washed Ba/F3 cells were directly incubated with the antibodies alone or in combination by pairs, at fixed concentration and/or 2-fold serially diluted, as indicated.

**Flow Cytometric Detection of Cell Surface Receptors**—For each staining, 2 × 105 cells were incubated for 30 min at 4 °C with saturating concentrations (10 μg/ml) of indicated antibodies. The indicated antibodies were incubated with 20% PBS supplemented with 1% bovine serum albumin and 0.1% human polyclonal IgG (w/v, both from Sigma). The cells were then washed twice with the same buffer and incubated for 30 min at 4 °C with the fluorescein isothiocyanate-conjugated goat anti-mouse IgG. After washing with PBS, the cells were resuspended in 0.14 ml of PBS containing 1% formaldehyde (v/v) and analyzed by flow cytometry with a three color FACScalibur flow cytometer (Becton-Dickinson, Mountain View, CA) equipped with the CellQuest software.

**Radioiodination of LIF and Binding Experiments**—E. coli-derived human LIF (PeproTech Inc., Rocky Hill, NJ) was iodinated according to the chloramine T method as described (12). LIF was labeled at a specific radioactivity of around 35,000 cpm/nmol. The plastic-adherent human choriocarcinoma JAR cell line was cultured in Dulbecco’s modified Eagle’s medium supplemented with 8% fetal calf serum, harvested by treatment with trypsin, then washed three times, and resuspended in PBS containing 0.5% bovine serum albumin. The cells (105/well in 96-well round bottom plates) were incubated with increasing concentrations of iodinated LIF in the presence or in the absence of a constant and saturating concentration of the nonlabeled competitor mAb (100 ng/ml for 9E10, 8C2, B-R3, 12D3, and 105 ng/ml for 1C7) in a final volume of 50 μl/well. Because of its very low affinity for gp190, a higher concentration of 1C7 was required (13). Nonspecific binding was evaluated by including a 100-fold excess of unlabeled LIF. Incubation was carried out under agitation for 90 min at 4 °C. Cell bound and unbound fractions were separated by centrifugation through a dibutyltiosalicylate cushion at 15,000 rpm for 10 min. The binding data were subjected to regression analysis using a one-site equilibrium-binding equation (Orafti, Erathosinus Software, Staines, UK). To measure the affinity of mAbs 12D3 and 8C2 for human gp190, CHO cells stably expressing gp190 (14) were used. The mAbs were labeled with iodine, and the experiments were conducted essentially as previously described (13).

**RESULTS**

**Anti-human gp190 mAb 1C7 and 12D3 Abrogate the Proliferation Induced by LIF, OSM, CT-1, and CNTF**—We have generated 35 monoclonal antibodies specific for the human gp190 cytokine receptor, some of them having already been described (10). Only two, the previously described 1C7 (10) and the recently generated 12D3, could inhibit the LIF-induced proliferation of transfected Ba/F3 cells expressing wild-type gp190 and gp130 in a MTT assay. When incubated in the presence of subsaturating concentrations of each of four cytokines sharing gp190, the cytokines LIF, OSM, CNTF, and CT-1, both antibodies were effective in a dose-dependent manner to differentiate effects (Fig. 1, middle and right panels). In contrast, none of them did affect IL-3-induced proliferation, and the control anti-gp190 mAb 8C2 (Fig. 1, left panel) had no effect on the proliferation induced by any of these five cytokines. Therefore, the blocking effects of 1C7 and 12D3 were specific to gp190 receptor function. The
inhibitory activities of mAbs 1C7 and 12D3 were roughly similar toward all cytokines tested: CHO-derived LIF (molecular mass, 43 kDa), E. coli-derived OSM (molecular mass, 26 kDa), and E. coli-derived CNTF (molecular mass, 23 kDa), with IC₅₀ values of around 2–4 and 0.5–1 μg/ml for mAbs 1C7 and 12D3, respectively. In comparison, both mAbs displayed an approximately six times better blocking efficacy toward CT-1 (molecular mass, 22 kDa), because its IC₅₀ was around 0.5 and 0.1 μg/ml for 1C7 and 12D3, respectively. This discrepancy could not be explained by a difference in molarity of the cytokines used in the assay, because the molecular mass of CT-1 was very close to that of OSM or CNTF.

1C7 and 12D3 Epitope Localized in Distinct Segments of the gp190 Extracellular Region—Using Myc-tagged soluble deletion mutants of gp190, we previously established that 1C7 recognized an epitope localized within the D1Ig region of gp190 (7). A similar approach was undertaken to map the region recognized by 12D3. The two deletion mutants, D1IgD2myc and D2FNmyc, were stably expressed in CHO cells and metabolically labeled. The supernatants were harvested and immunoprecipitated with 1C7, 12D3, or the anti-c-Myc 9E10 mAb as a positive control, and the immunoprecipitates were analyzed by SDS-PAGE and autoradiography (Fig. 2). As expected, the 9E10 antibody could immunoprecipitate both tagged receptor fragments, whereas the 1C7 antibody could immunoprecipitate D1IgD2myc but not D2FNmyc. mAb 12D3 was found to immunoprecipitate both fragments, demonstrating that it recognized an epitope within D2, the only domain shared by the two receptor mutants. Therefore, the two blocking antibodies, 1C7 and 12D3, recognized distinct domains within the extracellular region of gp190.

1C7 and 12D3 Exert Their Blocking Effects via Distinct Mechanisms—We previously demonstrated that mAb 1C7 competed with LIF for binding to gp190 in an immunoprecipitation assay (7). In this assay, supernatants of CHO cells producing a secreted Myc-tagged form of the extracellular part of gp190 (herein called sgp190myc) were coincubated with metabolically radiolabeled CHO-derived LIF to allow for the ligand-receptor complex to form. The complexes were then immunoprecipitated by the antibodies under test and electrophoresed by SDS-PAGE, and the presence of LIF was revealed by autoradiography. In the presence of 1C7, no LIF could be immunoprecipitated (7). To investigate whether mAb 12D3 exerted its blocking effects via a similar mechanism, the same assay was carried out with 12D3, and the results were compared with those obtained with the blocking mAb 1C7, the nonblocking mAb 10B2 and the anti-c-Myc mAb 9E10 (Fig. 3). As expected, LIF could be immunoprecipitated by the positive control antibodies anti-c-Myc 9E10 and the nonblocking anti-gp190 10B2, demonstrating that the low affinity ligand-receptor complex was efficiently formed under these experimental conditions. Also as expected, the anti-D1Ig mAb 1C7 did not precipitate at all of the LIF-gp190 complexes because of a competition between the ligand and the antibody. In contrast to mAb 1C7, the blocking anti-D2 mAb 12D3 did precipitate LIF as efficiently as mAbs 10B2 or 9E10. Immunoprecipitation of LIF by 9E10, 10B2, and 12D3 mAbs required gp190, because no LIF was detected in the absence of the receptor. Therefore, the blocking anti-D2 mAb 12D3 exerted its inhibitory activity without impairing the formation of the ligand-receptor low affinity complex, contrary to the anti-D1Ig mAb 1C7.

12D3 Acts Via Impairment of gp130 Recruitment into the High Affinity Receptor Complex—The mode of action of mAb 12D3 was further examined by studying its effect on the binding of iodinated E. coli-derived LIF to the surface of the human chorioncarcinoma JAR cell line, which constitutively expresses natural gp190 and gp130 receptor subunits (Fig. 4 and Table I). The effect of 12D3 was compared with that of nonblocking mAbs (anti-D2 8C2 and irrelevant antibody) and of other block-
FIG. 4. The anti-gp190 blocking mAb 12D3 inhibits the recruitment of gp130 into the high affinity LIF receptor complex, on JAR cell line (Scatchard plots). Serial dilutions of iodinated LIF were incubated with a fixed concentration of an irrelevant mAb (filled circles), the nonblocking anti-D2 mAb 8C2 (filled triangles), the blocking anti-D1Ig mAb B-R3 (filled squares), the blocking anti-D1Ig mAb 1C7 (empty triangles), or the blocking anti-D2 mAb 12D3 (empty circles). All mAbs were unlabeled and were used at 100 nM, except 1C7, which was at 667 nM, given its low affinity for gp190. $F_{uc}$, unbound fraction; $F_b$, bound fraction.

TABLE I

<table>
<thead>
<tr>
<th>Competitor mAb</th>
<th>Number of LIF binding sites $K_d$ for LIF (pm)</th>
<th>Kind of affinity of LIF for gp190</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2074 ± 106</td>
<td>95 ± 9, High</td>
</tr>
<tr>
<td>8C2</td>
<td>2290 ± 217</td>
<td>93 ± 40, High</td>
</tr>
<tr>
<td>1C7</td>
<td>0</td>
<td>0, No affinity</td>
</tr>
<tr>
<td>B-R3</td>
<td>2770 ± 367</td>
<td>968 ± 278, Low</td>
</tr>
<tr>
<td>12D3</td>
<td>2271 ± 253</td>
<td>583 ± 171, Low</td>
</tr>
</tbody>
</table>

The anti-gp190 blocking mAb 12D3 prevents the recruitment of gp130 into the high affinity LIF receptor complex, on JAR cell line (Scatchard plots). Serial dilutions of iodinated LIF were incubated with a fixed concentration of an irrelevant mAb (filled circles), the nonblocking anti-D2 mAb 8C2 (filled triangles), the blocking anti-D1Ig mAb B-R3 (filled squares), the blocking anti-D1Ig mAb 1C7 (empty triangles), or the blocking anti-D2 mAb 12D3 (empty circles). All mAbs were unlabeled and were used at 100 nM, except 1C7, which was at 667 nM, given its low affinity for gp190. $F_{uc}$, unbound fraction; $F_b$, bound fraction.

The anti-gp190 blocking mAb 12D3 shows agonistic activity when combined with anti-D2 mAb 8C2. It has been reported, using chimeras consisting of the GM-CSF receptor ectodomain linked to the transmembrane and intracellular region of either gp190 and gp130, that the gp190 intracellular domain, in contrast to the gp130 intracellular domain, was not able to transduce a proliferative signal in Ba/F3 cells upon GM-CSF addition (16). Therefore, to set up a cellular system enabling the detection of anti-gp190 antibodies able to stimulate cell growth, we generated a gp190/130 chimera in which the transmembrane and intracellular region of gp190 was replaced by the corresponding region of gp130. Following transfection in Ba/F3 cells in combination with wild-type gp130, we obtained a cell line that became dependent for its survival and proliferation of LIF, OSM, CNTF or CT-1. Preliminary experiments showed that there was no significant difference in the sensitivity of this cell line to these cytokines as well as to IL-3, as compared with a Ba/F3 cell line that expressed wild-type gp190 together with gp130 (results not shown). This demonstrated that the chimeric gp190/130 receptor behaved identically to the wild-type full-length gp190.

The gp190/130 + gp130 Ba/F3 cell line was therefore used to test the functional activity of 23 anti-gp190 mAbs (21 directed against D1Ig and two directed against D2) covering the major epitopes recognized by our set of anti-gp190 antibodies. These mAbs were tested alone or in pair combinations. Of the 253 possible combinations tested, only one displayed an agonistic effect, which strikingly consisted of the association of the two anti-D2 mAbs, 8C2 (nonblocking), and 12D3 (blocking). Fig. 5 displays a few of the combinations tested, especially those including the anti-D1Ig 1C7 blocking antibody, which was never found to be able to trigger an agonistic signal, regardless of the combination tested. When used alone, neither 8C2 nor 12D3 by itself exerted any stimulatory effect on these cells, in a range of concentrations extending from 60 to 0.009 μg/ml (results not shown and Fig. 6B).

The agonistic activity of this antibody combination was dose-dependent and reached a plateau at 60% of the maximal proliferative response induced by LIF. The maximal effect of the mAb combination required a concentration of at least 1 μg/ml for each mAb, and the half-maximum effect required around 0.2 μg/ml of each mAb (Fig. 6A). In contrast, mAb combinations 8C2 + 1C7 and 12D3 + 1C7 tested under similar conditions were not active. The mAb combination 8C2 + 12D3 was also tested with a fixed concentration of one antibody (20 μg/ml) and serial dilutions of the other (20 μg/ml to 4.9 ng/ml) (Fig. 6B). When the concentration of mAb 8C2 was decreased below 2.5 μg/ml, the agonistic activity dropped as rapidly as when both mAbs were concomitantly decreased (Fig. 6, compare A and B), because the half-maximum effect was obtained with 20 μg/ml of 12D3 and 0.31 μg/ml of 8C2. In contrast, in the presence of mAb 8C2 at a constant concentration of 20 μg/ml, the maximal agonistic effect of the mAb combination progressively increased with decreasing concentrations of 12D3 from...
20 to 0.31 μg/ml. In these experimental conditions, mAb-induced proliferation was still at 60% of LIF-induced maximum proliferation when 8C2 and 12D3 were at 20 and 0.078 μg/ml, respectively, and half of mAb-induced maximum proliferation required only 0.027 μg/ml of 12D3. This result suggested that either 12D3 had a ~10–15-fold higher intrinsic potency than 8C2 in the agonistic combination or that 8C2 had a much lower affinity for gp190 than 12D3.

To solve this question, the binding affinities of mAbs 8C2 and 12D3 for gp190 were measured on CHO cells stably expressing human gp190 (14), with iodinated mAb, followed by Scatchard analysis. Both mAbs recognized similar numbers of receptor sites and displayed very similar $K_d$ values ($K_d = 5.6 \pm 0.5$ nM for 8C2 and $K_d = 5.8 \pm 1.2$ nM for 12D3) (results not shown). To increase the agonistic effect of this mAb combination, we added an anti-mouse IgG polyclonal antiserum that could cross-link antibodies. No further effect could be noticed, regardless of the concentrations used. Similar cross-linking of mAbs 8C2, 12D3, 1B2, or 1C7 alone did not lead to any agonistic signal (results not shown).

To confirm that this agonistic activity was specifically due to gp190, the 8C2 + 12D3 combination was tested on the IL-6-sensitive Ba/F3 cell line expressing wild-type gp130 and gp80 but lacking gp190. No activity was seen, demonstrating that the effect of the antibody combination required the presence of gp190 (Fig. 6C). We also used the Ba/F3 gp190 + gp130 cell line expressing gp130 together with wild-type gp190 instead of the chimeric gp190/130 receptor. Again, the 8C2 + 12D3 combination did not display any agonistic activity on the Ba/F3 cell line expressing the wild-type gp190 instead of the chimeric gp190/130 (Fig. 6C). To rule out a putative involvement of gp130 in the agonistic effect, we used the B-R3 anti-gp130 blocking mAb (Fig. 6D). At a concentration (5 μg/ml) where it substantially inhibited the LIF-induced proliferation of the Ba/F3 gp190/130 + gp130 cell line, it did not impair at all the ability of the agonistic mAb combination to stimulate cell growth when added simultaneously. Therefore, the agonistic effect required the chimeric gp190/130 protein and was independent of gp130.

**Selection of Stable Ba/F3 Transfectants with mAb Combination 8C2 + 12D3** —We reasoned that mAb combination 8C2 + 12D3 should be able to raise stable Ba/F3 transfectants expressing the gp190/130 chimeric protein in the absence of gp130. Ba/F3 cells were transfected with the construct encoding gp190/130, and the cells were selected by replacing murine IL-3 with mAbs 8C2 and 12D3 at 3 μg/ml each or with LIF. Stable cell lines could be obtained only when the antibody selection was used. As expected, these cells expressed on the membrane the gp190 receptor ectodomain but not the gp130 receptor, as shown by flow cytometry (Fig. 7A, right panel). In contrast, Ba/F3 cells stably transfected with gp190/130 and gp130 and selected in the presence of LIF displayed both receptor chains on the membrane (Fig. 7A, left panel). We tested the proliferative capacity of the Ba/F3 cells expressing gp190/130 in response to LIF and mAbs combination 8C2 + 12D3 and compared it with that of cells coexpressing gp190/130 and gp130 (Fig. 7B, left panel). The cells expressing gp190/130 but not gp130 did not proliferate at all in response to LIF at concentrations up to 2 μg/ml, whereas they exerted sensitivity to mAbs 8C2 + 12D3 very close to that of cells expressing both chains. As described previously for Ba/F3 gp190/130 + gp130, they did not respond to each mAb alone, and 12D3 was more potent than 8C2 in the agonistic effect (Fig. 7B, right panel). These results confirm that gp130 has no role in the agonistic activity of mAbs 8C2 and 12D3 and that the intracellular region of gp130 is required for them to trigger proliferation of Ba/F3 cells.

**DISCUSSION**

In a previous publication (7), we reported on the identification of a LIF-binding site in the carboxyl terminus of the Ig-like module. We also showed that the deletion of D2 led to the disruption of the D1 conformation, whereas the deletion of D1 did not alter D2 conformation but abolished LIF binding. These results strongly suggested that 1) direct interactions between the two CRH domains of gp190 were needed for stabilization of the full receptor ectodomain, allowing for the constrained Ig-like domain to bind LIF, and that 2) the two CRH domains of gp190 played distinct functional roles. In line with this, computer-assisted alignment of the amino acid sequences for each of the gp190 CRH D1 and D2 with the unique CRH from gp130 showed that D2 is more closely related to gp130 CRH than is D1, and homology modeling suggested that D2 and gp130 CRH share a very similar spatial conformation (17). Based on this observation, it was hypothesized that these domains may probably directly interact with each other in the high affinity receptor (17), as do receptors acting via homodimerization upon ligand binding.

The present work describes the functional properties of the anti-D2 mAb 12D3 confirms these assumptions. mAb 12D3 displays antagonistic activity toward LIF via inhibition of the recruitment of the gp130 high affinity converter and not via...
impairment of LIF binding to gp190, as does anti-D11g mAb 1C7. The blocking effect of both blocking mAbs is shared by the four cytokines tested known to use gp190, i.e. LIF, OSM, CT-1, and CNTF, demonstrating that ligand-induced gp190/gp130 receptor activation involves common steps shared by these four cytokines. Blocking antibodies specific for distinct epitopes of gp130 and exerting selective inhibitory activity against one or some cytokines using gp130 have also been reported (18, 19), leading to the conclusion that not all gp130 ligands share the same binding sites. This might also be the case for gp190 because the LIF-binding site we identified previously at the carboxyl terminus of the Ig region did not impinge on OSM-dependent proliferation of the Ba/F3 cells transfected with this gp190 mutant together with gp130 (7). Anti-gp130 mAbs exist that, similarly to 12D3, inhibit the formation of the high affinity receptor for all of the cytokines using both gp190 and gp130, as well as IL-6 and IL-11, which require gp130 but not gp190. One of these is mAb B-R3, whose epitope on gp130 has been identified and lies within the amino-terminal module of the unique gp130 CRH (19). The antagonistic activity of mAb 12D3 could be due to the specific blocking of the contact interface between gp190 and gp130, by binding directly to this region on gp190 or indirectly through a steric hindrance impairing the moving of both chains toward each other. Unfortunately, we did not succeed so far in localizing the epitope of mAb 12D3 using a series of deletion mutants within the D2 domain, suggesting that it is highly conformation-dependent.

Monoclonal antibodies have been generated that can replace at least partially the cytokine in its ability to activate a cytokine receptor. This is the case for growth hormone receptor (20), erythropoietin receptor (21), prolactin receptor (22), and gp130 (19, 23–25). In all cases but gp130, the agonistic effect could be exerted by such a monoclonal antibody used alone. In the case of gp130, an agonistic effect occurred only when certain mAbs were used as pairs, and such mAbs often exerted cytokine blocking activity when used alone. For all of these receptors, the experimental data strongly suggest that the agonistic effect is a consequence of mAb-induced productive dimerization of the receptor chains, which is in agreement with the mechanism of action of the respective cytokines. In our case, among the 23 anti-gp190 mAbs we tested, covering all of the epitopes recognized, none of them displayed any agonistic activity when used alone. When tested in combination by pairs, only the pair associating the two anti-D2 mAbs we raised displayed agonistic properties, and it comprised the blocking mAb 12D3. The agonistic effect was totally independent from gp130 and required the gp190/130 receptor, because it did not occur with a Ba/F3 cell line expressing gp130 together with wild-type gp190, a result that strengthened the previous finding described with the GM-CSF receptor/gp190 chimeras, that the intracellular region of gp190 is not able to transduce a proliferative signal upon dimerization, in contrast to gp130 intracellular region (16).

It is noteworthy that as for gp130, a pair of mAbs is required to activate gp190. One possible explanation is that the ectodomains of these receptors have a more complex architecture in comparison with the simpler and prototypic growth hormone receptor, erythropoietin receptor, and prolactin receptor, which consist of only a CRH domain. Especially, gp130 and gp190 both contain a FN region between the CRH and the transmembrane span, which may also play an important role in activation, at least in the case of gp130 (26, 27). Therefore, because of these additional structural constraints, a single antibody may not be able to activate gp130 or gp190. There is also experimental evidence to argue that the mere mAb-induced dimerization of membrane receptors is not sufficient to trigger a
signal and that appropriate conformational changes of particular receptor epitopes are required (20, 25). In agreement with this, we found that mAbs 8C2 and 12D3 displayed comparable affinities for gp190 but distinct potencies in their participation to the agonistic effect, because around 10 to 15 times less 12D3 than 8C2 was required to obtain comparable proliferation.

In contrast to the two anti-D2 mAbs, it is remarkable that none of the 21 anti-D1 Ig mAbs representing as many epitopes displayed any agonistic signal when tested by pairs between each other or with any of the two anti-D2 mAbs. We conclude from these experiments that mobilizing the D1 Ig region is not sufficient to trigger a productive activation of the receptor. If triggering effectively occurs via receptor homodimerization, this may be due to inadequate spatial orientation of receptor chains with regard to each other, which a critical parameter governing the ability of the receptor to deliver a signal into the cell interior (28, 29). Furthermore, the ability for a dimerized receptor to transduce a signal critically depends on the capacity of the ligand to get the intracellular regions of the monomers into a close enough proximity (30). Therefore, in our model where D1 directly interacts with D2, probably leading to a shived structure, a dimerization of the receptor via D1 Ig may not be able to fulfill this requirement because the distance between the downstream parts of the two concerned ectodomains would remain too important to trigger a signal, in contrast to what happens with the anti-D2 mAbs.

Recently, crystallographic analysis of soluble forms of the IL-3/IL-5/GM-CSF β-common receptor chain or of the erythropoietin receptor in the absence of ligands (31, 32). These results were confirmed for the erythropoietin receptor on the cell surface (30, 33). The β-common receptor chain is close to gp190 in that it also contains two CRH domains, although it does not face (30, 33). The presence of preassociated dimers in the absence of ligands (31, 32). These results contrast to what happens with the anti-D2 mAbs.

In conclusion, our findings demonstrate that D1 and D2 play fundamentally different roles in the dynamics of gp190 engagement toward the formation of a functional receptor for LIF. However, more work is still needed to delineate the critical epitopes involved in the molecule interactions, to precisely define the steps leading to the formation of the high affinity receptor, and to determine the stoichiometry of the low and high affinity receptors for LIF.

REFERENCES
Identification of Agonistic and Antagonistic Antibodies against gp190, the Leukemia Inhibitory Factor Receptor, Reveals Distinct Roles for Its Two Cytokine-binding Domains

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doi: 10.1074/jbc.M105476200 originally published online October 17, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M105476200

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