Separate Functions for the Two Modules of the Membrane-proximal Cytokine Binding Domain of Glycoprotein 190, the Leukemia Inhibitory Factor Low Affinity Receptor, in Ligand Binding and Receptor Activation*

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Mathieu-Benoît Voisin‡, Juliette Bitard§§, Sophie Daburon, Jean-François Moreau, and Jean-Luc Taupin¶

From the CNRS UMR 5540, Université de Bordeaux II, 146 Rue Léo Saignant, 33076 Bordeaux, France

The receptor for the cytokine leukemia inhibitory factor (LIF) associates the low affinity binding component gp190 and the high affinity converter gp130. Both are members of the hematopoietic receptors family characterized by the cytokine receptor homology (CRH) domain, which consists of two barrel-like modules of around 100 amino acids each. 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 immunoglobulin-like domain, a membrane-proximal CRH domain called D2, and three type III fibronectin-like repeats. A minimal D1IgD2 fragment is required for binding LIF. By using transmembrane forms of deletion mutants in gp190 ectodomain, we demonstrated that removal of D1 led to spontaneous activation of the receptor and that this property was devoted to a peptidic sequence localized within the last 42 amino acids of the carboxy-terminal module of D2. By using soluble forms of deletion mutants made by progressive truncations from the end of the D1IgD2 fragment, we demonstrated that the carboxy-terminal module of D2 was dispensable for LIF binding and that the correct conformation of the D1Ig fragment required a full amino-terminal module of D2. Therefore, the two constitutive modules of the membrane-proximal CRH domain D2 of gp190 fulfill two distinct roles in gp190 function, i.e. in stabilizing the conformation of gp190 allowing LIF binding and in activating the receptor.

The leukemia inhibitory factor (LIF) low affinity receptor gp190 belongs to the large family of the hematopoietic recep-

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† Both authors contributed equally to this work.

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§ To whom correspondence should be addressed. Tel.: 33-5-57-57-14-71; Fax: 33-5-57-57-14-72; E-mail: jean-luc.taupin@umr5540.u-bordeaux2.fr.

1 The abbreviations used are: LIF, leukemia inhibitory factor; CRH, cytokine receptor homology domain; D1, gp190 membrane-distal cytokine binding domain; D2, gp190 membrane-proximal cytokine binding domain; Ig-like, immunoglobulin-like module; FN, type III fibronectin repeats; OSM, oncostatin M; CNTF, ciliary neurotrophic factor; CT-1, cardiotoxin-1; TPO, thrombopoietin; mAb, monoclonal antibody; IL, interleukin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DHFR, dihydrofolate reductase; STAT3, signal transducer and activator of transcription 3; MTX, methotrexate; ELISA, enzyme-linked immunosorbent assay; gp, glycoprotein; PBS, phosphate-buffered saline; BSA, bovine serum albumin; CHO, Chinese hamster ovary.
played antagonistic or agonistic activity toward the receptor (9). One antagonistic mAb called 1C7 was directed against the D1Ig region and displayed a LIF blocking activity via impairment of LIF/gp190 interaction, whereas another called 12D3 was directed against the D2 domain and displayed a LIF blocking activity via impairment of gp130 recruitment to the LIF-gp190 complex. In addition, a pair combination of anti-D2 mAbs, including mAb 12D3, displayed a potent agonistic activity in the absence of LIF, a phenotype that was never obtained with a large panel of anti-D1Ig mAb (9). Taken together, these findings strongly suggested that the two CRH domains have distinct roles in receptor conformation, LIF binding, and receptor activation.

Here, we attempted to address these questions more specifically for the D2 domain. In a first step, deletion mutants lacking one or several domains of gp190 extracellular region, as well as deletion mutants within D2, were fused to a transmembrane and intracellular transducing region, and their ability to trigger cytokine-dependent or -independent prolongation of Ba/F3 cells was studied. In a second step, progressive deletions were performed in the D2 domain starting from the carboxyl terminus of the shortest LIF-binding receptor (i.e.D1IgD2) backward to the Ig-like domain. These mutants were expressed in soluble form, and their ability to bind LIF and maintain the general conformation of the receptor was analyzed.

**EXPERIMENTAL PROCEDURES**

**Monoclonal Antibodies and Cytokines—**The anti-gp190 mAbs specific for domains D1 (6G8) or D2 (12D3, SC2) have already been described (9, 10). The anti-gp130 mAb B512 was purchased from Diaclone (Besançon, France). The anti-c-Myc mAb 9E10 was purified from cell culture supernatants of the secreting hybridoma provided by Dr. Ramsay (see Ref. 11). Human OSM and murine interleukin (IL)-3 were as supernatants of transiently transfected COS cells. Human LIF was produced in stably transfected CHO cells (12). The cytokines were used as crude supernatants along with control supernatants of nontransfected recipient cells.

**Construction of the gp190 Mutants—**We have already described the generation of the secreted carboxyl-terminally c-Myc-tagged gp190 (sgp190myc) and its deletion mutants D1IgD2myc, IgD2FNmyc, D1Igmyc, D2myc, D2FNmyc, and FNmyc (8). The truncations within the D2 domain were carried out by PCR from gp190, because the homodimerization of the intracellular segment of gp190/130 transmembrane forms of the deletion mutants. We used the pZ plasmid containing a gp130 coding sequence that was mutated to bear an XbaI site for those deleted from the amino terminus, which we fused to the transmembrane and intracellular region of gp130 has also been described (8). The same strategy was used to generate the secreted carboxyl-terminally c-Myc-tagged gp190 (sgp190myc) and its deletion mutants D1IgD2myc, IgD2FNmyc, D1Igmyc, D2myc, D2FNmyc, and FNmyc (8). The creation of the pEDr-mycD2/130 and its deletion mutants D1IgD2myc, IgD2FNmyc, D1Igmyc, D2myc, D2FNmyc, and FNmyc (8). The creation of the secreted carboxyl-terminally c-Myc-tagged gp190 and FNmyc transmembrane deletion mutants of the gp190 ectodomain. The cells were plated in medium containing IL-3. At day 1, G418 was added at 1 mg/ml. Every other day between day 4 and 14, 80% of the culture medium was replaced by fresh medium containing G418 supplemented with or without LIF at 50 ng/ml. At day 16, the cells were washed to eliminate residual traces of IL-3; G418 selection was stopped, and the living cells were maintained in medium with LIF or without any added cytokine. For the transient transfection of COS cells, we used the DEAE-dextran method, as described previously (8). Supernatants were harvested at day 5 after the transfection. For the metabolic labeling experiments, the 35S-substrate was added at day 3 for an overnight incubation before supernatants were harvested. The stable transfection of CHO-DUCKX cells (13) was achieved using the DEAE-dextran method, as described previously (8). The following mutants bearing deletions from D2 carboxyl terminus of gp190 have been described (8). The anti-gp190 mAbs specific for domains D1 (6G8) or D2 (12D3, SC2) have already been described (9, 10).

**Flows Cytometric Detection of Cell Surface Receptors—**Ba/F3 cells were grown in suspension, and adherent CHO cells were released following treatment with PBS containing 25 mM EDTA for 10 min at 37 °C prior to staining. For each staining, 2 £ 106 cells were incubated for 30 min at 4 °C with saturating concentrations (10 μg/ml) of the indicated antibody in 0.1 ml of PBS supplemented with 1% bovine serum albumin (BSA) 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 FITC-conjugated goat anti-mouse IgG. After washing with PBS, the cells were fixed by resuspension in 4% paraformaldehyde (v/v) and analyzed by flow cytometry with a three-color FACSCalibur flow cytometer (Becton-Dickinson, Mountain View, CA) using the CellQuest software.

**Immunoprecipitation Experiments—**Metabolic labeling of the recombinant protein expressed in CHO cells was performed as follows. Cells were starved for 2 h in 4 ml of Dulbecco’s modified Eagle’s medium without methionine and cysteine supplemented with 2 mM glutamine and 5% dialyzed fetal calf serum. Then 200 μCi of L-[35S]methionine/cysteine (Translabel, ICN, Orsay, France) were added per dish. When appropriate, anti-D2 mAbs were then added for 1 h, the cells were harvested by centrifugation to remove debris, and stored at 4 °C until use. For the preparation of cell lysates, the supernatant was discarded 4 h after the labeling had begun; the cell layer was washed with PBS, and the cells were lysed in 4 ml of an isotonic lysis buffer (50 mM Tris, 150 mM NaCl, 0.2% Nonidet P-40, pH 8.0) in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml...
aprotinin, and 10 μg/ml leupeptin) for 10 min on ice. Then the cell lysate was harvested, centrifuged to remove debris, and stored at −80 °C until use. For the immunoprecipitation, 1 ml of CHO supernatant or lysate was pre-cleared one or four times, respectively, with 0.05 ml of a 50% suspension of protein-A-Sepharose beads (Aff-Gel protein A, Bio-Rad) for 3 h at 4 °C under continuous rolling. Beads were washed by centrifugation, and supernatants were incubated with 20 μg of the indicated mAb for 2 h under similar conditions. Immune complexes bound to protein A were sedimented by rapid centrifugation, and beads were washed three times with 1 ml of lysis buffer. Bead pellets were resuspended in 0.025 ml of sample loading buffer containing 0.1 M dithiothreitol and boiled for 5 min. Proteins were separated by SDS-PAGE on 10% gels and visualized by fluorography.

**Proliferation Experiments with Transfected Ba/F3 Cell Lines**—The proliferation assays were conducted as follows. Ba/F3 cells were washed three times with RPMI, and then cells (5 × 10^6 per well, in 50 μl in duplicate) were incubated in the presence of 50 μl of 3-fold dilutions of LIF or OSM or of IL-3 as a positive control and culture medium without any added cytokine as a negative control, as indicated. After 3 days at 37 °C, 0.015 ml of a 5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) in PBS was added to each well. After 4 h at 37 °C, 0.11 ml of a mixture of 95% isopropl alcohol + 5% formic acid was added to the wells, and the absorbance values were read in a Titerlek Multiskan microplate reader (Labsystems). The blank consisted of eight wells containing only culture medium.

**ELISAs for gp190**—The sandwich ELISA for soluble gp190 measurement has been described previously (15). It uses mAb 6G8 as the capture mAb and biotinylated 10B2 mAb as the tracing mAb. Both mAb are directed at the D1Ig fragment of gp190, and the assay has a detection limit of 0.5 ng/ml. To estimate the relative affinity of the soluble deletion mutants for LIF, we set up a sandwich ELISA as follows. All steps were performed at room temperature, and plate washes were performed in PBS containing 0.05% Tween 20. The anti-gp190 mAb 6G8 was used as a capture mAb. After saturation with PBS containing 1% BSA for 1 h, plates were washed once and then incubated with a saturating and constant concentration of 200 ng/ml wild-type gp190myc or its mutants for 2 h and washed three times. Serial dilutions of CHO-derived human LIF ranging from 0.256 to 4000 ng/ml were added for 2 h. After 3 washes, plates were incubated with the biotinylated non-blocking anti-LIF mAb 1F10 at a final concentration of 2 μg/ml for 1.5 h and washed again 3 times. The plates were then incubated with peroxidase-labeled streptavidin for 30 min and washed 3 times. The plates were revealed by the addition of the substrate tetramethylbenzidine (Sigma) and read at 450 nm after the addition of half a volume of 1 x sulfuric acid.

**Analysis of STAT3 Phosphorylation**—When grown in the presence of IL-3 or in cytokine-free medium, Ba/F3 cells were washed twice with serum- and cytokine-free RPMI medium. The cells were activated by incubation for 10 min at 4 °C, the supernatant was harvested, and the total protein concentration was determined using the bicinchoninic acid method (Sigma) using BSA as a standard. The cell lysate (10 μg of proteins/lane) was boiled for 5 min and separated by SDS-PAGE on 8% gels and then transferred to a nitrocellulose membrane (Amersham Biosciences). The membrane was blocked with 0.1% Tween 20/TBS-buffered saline (TBST) supplemented with 5% (w/v) skimmed milk overnight at 4 °C. The membrane was then incubated in TBST supplemented with 5% (w/v) milk with a 1:2000 dilution of rabbit anti-STAT3 serum (R & D Systems Europe, Abingdon, UK) or a 1:1000 dilution of a rabbit anti-phospho-STAT3 antibody (Cell Signaling Technology, Ozyme, St-Quentin-en-Yvelines, France) for 3 h at room temperature. After three washes in TBST, the membrane was incubated with a horseradish peroxidase-labeled goat anti-rabbit immunoglobulin polyclonal antibody (Zymed Laboratories Inc., CliniSciences, Montreouge, France) for another hour at room temperature. The membrane was washed again three times in TBST, and the proteins were visualized using the chemiluminescence ECL system (Amersham Biosciences).

**DHFR Protein Complementation Assay**—The ability of the DHFR reconstituted from its separated fragments to bind its ligand methotrexate was studied as described in Remy et al. (16). DHFR-lacking CHO DUCKX cells, both parent and stable transfectants, were plated in 24-well plates at 3 × 10^4 cells/well and cultured in medium containing dialyzed serum for 22 h at 37 °C in the absence of added nucleosides and in the presence of 10 μM of FTC-labeled mithromethane (FMTX, Molecular Probes, Leiden, The Netherlands). The cell layer was gently washed once in PBS and incubated for 30 min in the absence of FMTX to allow the efflux of unbound FMTX from the cell cytoplasm. The cell layer was washed four times on ice with ice-cold PBS, and the cells were detached with trypsin in PBS. After neutralization of trypsin with 1:10 volume of serum, FMTX-DHFR intracellular complexes were analyzed by flow cytometry.

**RESULTS**

**Proliferative Capacity of Deletion Mutants within gp190 Ectodomain**—A series of deletion mutants within the gp190 ectodomain, which lacked one or several of its constitutive D1, Ig, D2, or FN modules, namely D1IgD2, IgD2FN, D2FN, D1IgFN, and FN, were obtained fused to gp130 transmembrane and intracellular region (Fig. 1). They were transfected alone or in combination with full-length gp130 in Ba/F3 cells. The transfectants were selected by progressive replacement of IL-3, which is required to sustain parental Ba/F3 cells proliferation, by plain culture medium without any added cytokine or by culture medium containing LIF. Table I summarizes the results of these stable transfection experiments, in terms of ability for the deletion mutants to confer long term proliferation of the Ba/F3 cell recipient. At least four transfections were performed, with up to six for the remaining deletion mutants that were unable to allow cell growth after four attempts. As already reported, in the presence of LIF, gp130 alone never led to cell proliferation, because gp190 is required for LIF binding. Chimeric gp190/130, which contained the full gp190 ectodomain, only triggered cell proliferation in the presence of gp130 and LIF. The mutants D1IgD2/130, D1IgFN/130, and FN/130 were not able to sustain cell survival and proliferation under any of the conditions tested. In contrast, the IgD2FN, D2FN, and D2 deletion mutants reproducibly conferred long term cell proliferation, which was independent of gp130 or LIF, because it occurred even upon transfection of the gp190 mutant alone. We also designed a construct where the entire ectodomain of gp190, but not its signal peptide encoding sequence, was removed and replaced by a c-Myc tag. This construct, called Myc/130 was for 5 min under the same conditions. As can be seen in Table I, it did not lead to gp130- or LIF-dependent or -independent proliferation of Ba/F3 cells. Therefore, the spontaneous proliferation occurring with several of the deletion mutants could not be attributed to the transmembrane and intracellular region of gp130 by itself, which was then considered to be unable to trigger a productive signal on its own.
TABLE I

Generation of Ba/F3 cell lines following stable transfection of the gp190 deletion mutants

| Conditions for transfection and cytokine selectiona | gp190 deletion mutant |
|-----------------------------------------------|---------------------|
|                                                | gp190/130 | D1IgD2/130 | IgD2FN/130 | D2FN/130 | D2/130 | D1IgFN/130 | FN/130 | myc/130 |
| No gp130 plasmid, no cytokine                | 0        | 0          | 0         | +        | +       | +          | 0      | 0      |
| No gp130 plasmid, LIF                       | 0        | 0          | 0         | +        | +       | +          | 0      | 0      |
| gp130 plasmid, no cytokine                  | 0        | 0          | 0         | +        | 0       | +          | 0      | 0      |
| gp130 plasmid, LIF                          | 0        | +          | 0         | +        | 0       | +          | 0      | 0      |

a The gp190 deletion mutant was transfected together with the G418 resistance plasmid encoding gp130 (gp130 plasmid) or with the empty G418 resistance plasmid (no gp130 plasmid), and cells were selected in the presence of G418 and by progressive replacement of IL-3 with cytokine-free medium (no cytokine) or medium containing LIF (LIF).

b 0, no stable cell line obtained from six independent transfections; +, stable cell line obtained in at least three out of four transfections.

We then verified by flow cytometry using specific anti-gp190 mAb that the truncated gp190 mutants were expressed on the cell surface for the stable cell lines raised (Fig. 2, left panels). Parent murine Ba/F3 cells were not stained with the anti-D1 monoclonal antibody (mAb) 6G8, the anti-D2 mAb 8C2, or the anti-gp130 mAb B-S12, all specific to the human receptor chains. As expected, the Ba/F3 gp190/130+gp130 cell line was stained with these three antibodies demonstrating the expression of both receptor chains. For the IgD2FN/130, D2FN/130, and D2/130 Ba/F3 cell lines, the D2 domain but not the D1 domain of gp190 nor the gp130 could be detected on the surface of transfected cells, suggesting a direct relationship between receptor expression at the cell surface and proliferation. We then analyzed in a 3-day MTT proliferation assay the cytokine dependence of the established Ba/F3 transfected cell lines toward murine IL-3, human LIF, human OSM, or culture medium devoid of any exogenously added cytokine and compared them with non-transfected parental Ba/F3 cells (Fig. 2, right panels). As expected, non-transfected Ba/F3 cells proliferated in a dose-dependent manner only with IL-3 and died in the absence of cytokine, whereas Ba/F3 gp190/130+gp130 were able to grow in response to IL-3, LIF, and OSM and died in the absence of these growth factors. In contrast, the Ba/F3 cell lines expressing D2/130, D2FN/130, or IgD2FN/130 were completely insensitive to all these cytokines, indicating that they had already reached their maximal proliferation potential in the basal state. This result was confirmed by daily counting of a known starting number of each of these cell lines in plain culture medium over a period of 6 days, because we found that these cell lines proliferated at very close rates with a doubling time of around 20 h, which is similar to the proliferation rate of the LIF-dependent gp190/130+gp130 Ba/F3 cell line (results not shown).

As the D1IgD2/130, FN/130, D1IgFN/130, and Myc/130 deletion mutants might be abnormally processed intracellularly and degraded, and/or not stable on the cell surface, thereby abrogating any signaling functions, we stably transfected these mutants in CHO cells and analyzed by flow cytometry the presence of these molecules at the cell surface of the emerging cell lines (Fig. 3). For the FN/130 chimera, in the absence of mAb specific for the FN region, the construct was slightly modified to include a c-Myc tag between the signal peptide and the intracellular region of gp130. Because only two mAbs specific for D2 are available (8C2 and 12D3), both conformation-dependent, we also inserted between the signal peptide and the beginning of the mutated D2 fragments the c-Myc epitope. Three constructs bearing deletions from the carboxyl terminus of D2, one called mycD2(332–431)/130, which encompassed the full amino-terminal module of D2 (see Fig. 1), and two called mycD2(332–459)/130 and mycD2(332–499)/130, which also encompassed fragments of the carboxyl-terminal module of D2, were designed. We also designed two constructs bearing deletions from D2 amino terminus, one called mycD2(432–541)/130, which encompassed the full carboxyl-terminal module of D2, and a shorter one called mycD2(500–541)/130. As a positive control, we used mycD2/130 composed of the entire D2 segment. These constructs were stably transfected alone in Ba/F3 cells, and IL-3 was progressively replaced by culture medium devoid of any added cytokine. The results of these experiments are summarized in Table II. Stably transfected cell lines were reproducibly obtained with mycD2/130 and with the deletion mutants mycD2(432–541)/130 and mycD2(500–541)/130 in all the transfections performed (n = 3 experiments). For these, the gp190 truncation mutant could be detected by flow cytometry with the anti-D2 mAb 8C2 and/or the anti-c-Myc mAb 9E10 (Fig. 4, panel A). The proliferation of the cells was not influenced by the addition of LIF, OSM or IL-3 to their culture medium in a 3-day MTT assay (Fig. 4, panel B) or by counting cells daily as already described (results not shown), and was comparable in growth rate to that obtained with D2/130 (compare Fig. 4, panel B, with Fig. 2). In contrast, deletion mutants mycD2(332–431)/130, mycD2(332–459)/130, and mycD2(332–499)/130 did not confer autonomous cell proliferation of Ba/F3 cells because no Ba/F3 cell lines could be obtained (Table II). When stably transfected in CHO cells, the truncation mutant mycD2(332–459)/130 could be detected by mAb 8C2 and 9E10, whereas mycD2(332–431)/130 and mycD2(332–499)/130 could not, suggesting that they were not correctly expressed (results not shown).

We hypothesized that the spontaneous growth of Ba/F3 cells expressing D2/130 and mycD2(500–541)/130 occurred via constitutive activation of the gp130 signaling pathway. To demonstrate this, we analyzed the phosphorylation state of the signal transducer and activator of transcription 3 (STAT3) in these...
cell lines in the absence of any added cytokine. As controls, we used parent Ba/F3 cells grown in IL-3 and Ba/F3 gp190/130 + gp130 cells, which were washed to remove the LIF-containing culture medium and cultured for 2 days in the presence of IL-3. After final washes, the cells were incubated with a cytokine-free medium or with LIF for 2 h before lysis and immunoblotting of phospho-STAT3 as well as STAT3 which served as an internal control of the amount of protein loaded on the gels (Fig. 4, panel C). STAT3 was not phosphorylated in parent Ba/F3 cells, even upon stimulation with LIF. In Ba/F3 gp190/130 + gp130 cells, phospho-STAT3 was strongly induced upon incubation with LIF, whereas small amounts of phospho-STAT3 could still be detected in the presence of IL-3, which could represent residual activity after the 48-h starving period. In contrast, phospho-STAT3 was readily strongly detectable in Ba/F3 expressing D2/130 or mycD2(500–541)/130 in the absence of stimulation by an exogenous cytokine. Because these cell lines have been raised in the absence of any cytokine known to activate STAT3, we concluded that STAT3 was constitutively activated in these cell lines and that this occurred via an autonomous activation of the signaling pathway normally triggered by the intracellular region of gp130.

FIG. 2. Analysis of membrane expression of gp190 deletion mutants and analysis of cytokine dependence for proliferation of the stably transfected Ba/F3 cell lines. For flow cytometry staining, non-transfected (parent) Ba/F3 cells or cells transfected with gp190/130 + gp130, with D2FN/130, or with D2/130 were labeled with anti-gp130 mAb B-S12 (dotted line, only for parent and gp190/130 + gp130 cells), with anti-D1 mAb 6G8 (thin line), with anti-D2 mAb 8C2 (thick line), or with an irrelevant antibody (dashed line), before analysis by flow cytometry. For proliferation assays, each cell line was incubated with 3-fold dilutions of either murine IL-3 (filled circles), human LIF (filled squares), human OSM (open triangles), or plain culture medium (open squares) for 3 days before analysis of cell viability with the colorimetric MTT assay at 570 nm. Stock solutions were at 50 ng/ml for LIF, whereas IL-3 and OSM were in the form of a COS supernatant diluted 1:200.
a minimal fragment of 42 amino acids of D2 encompassing its two most carboxyl-terminal $\beta$-strands was sufficient to confer cytokine- and gp130-independent growth by gp190 ectodomain deletion mutants in Ba/F3 cells, via a constitutive activation of the gp130-triggered STAT3 signaling pathway.

The Ability of D2 to Trigger Autonomous Cell Growth Occurred via Receptor Homodimerization—To analyze whether the ability of D2/130 to trigger autonomous cell proliferation occurred via spontaneous homodimerization of the D2 fragment, we replaced in the D2/130 and myc/130 chimeras the intracellular region of gp130 by each of the DHFR fragments 1–3, respectively, amino-terminal and carboxyl-terminal, as described by Remy et al. (16) for the erythropoietin receptor. This system allows the reconstitution of the enzymatic activity from its two inactive fragments, but only when the proteins to which they are fused can interact with each other. The resulting pairs of constructs D2/DHFR-(1,2) and D2/DHFR-(3) on one side and myc/DHFR-(1,2) and myc/DHFR-(3) on the other as a negative control were co-transfected in the DHFR-deficient CHO-DUCKX cell line. As a positive control, we used the pEDr plasmid that encodes a full-length functional DHFR. Cells were selected by culturing for 2 weeks in nucleoside-free medium. From a total of six transfections, stable cell lines were obtained with the full-length DHFR-positive control and D2-containing constructs in all the transfection experiments performed. In this latter case only, the cells expressed D2 on the cell surface, as shown by flow cytometry staining (Fig. 5, panel A). In contrast, no cell line could be obtained with the D2 chimeric constructs when transfected separately nor with the myc/DHFR-(1,2) and myc/DHFR-(3) constructs when transfected together (0 of 6 transfections), although these chimeric proteins could be observed separately on the cell surface of transiently transfected HEK cells by flow cytometry staining with the 9E10 mAb, thereby ruling out an anomaly in protein expression (results not shown). This result confirmed that the DHFR fragments did not tend to dimerize spontaneously, as already shown (16).

Table II

| Mutant name       | Fragment of D2 | Autonomous proliferation of Ba/F3 cells |
|-------------------|----------------|----------------------------------------|
| mycD2/332–431/130 | N-terminal module | 0$^b$                                  |
| mycD2/332–459/130 | N-terminal module + AB | 0                                      |
| mycD2/332–499/130 | N-terminal module + ABC/E | 0                                      |
| mycD2/432–541/130 | C-terminal module | +                                      |
| mycD2/500–541/130 | FC from C-terminal module | +                                      |
| mycD2/130        | Full D2        | +                                      |
| myc/130          | None           | 0                                      |

$^a$ N- and C-terminal are, respectively, the amino- and carboxyl-terminal modules of CRH D2. Letters in italics depict the putative $\beta$-strands in the carboxyl-terminal module of D2, as they are in the carboxyl-terminal module of the unique gp130 CRH (23).

$^b$ 0, no stable cell line obtained from three independent transfections; +, stable cell line obtained in all 3 transfections performed.

For the Ability of D2 to Trigger Autonomous Cell Growth Occurred via Receptor Homodimerization, Mock-transfected CHO cells or cells transfected with gp190/gp130, with D1IgD2/130, with D1IgFN/130, with mycFN/130, or with myc/130 were labeled with anti-D1 mAb 6G8 (thin line), with anti-D2 mAb 8C2 (thick line), or with the anti-c-Myc antibody 9E10 (dashed line) before analysis by flow cytometry.
ing efflux of the unbound chemical, the cell suspensions were analyzed by flow cytometry for residual fluorescence, a probe for the folding and reconstitution of the functional enzyme from its separate fragments (Fig. 5, panel B). Parent CHO cells did not significantly retain fMTX, whereas cells expressing DHFR or the D2 chimeric constructs efficiently did. Therefore, both
the establishment of stable CHO cell lines and fMTX binding showed that D2 had a propensity to homodimerize.

A Role for the Amino-terminal Module of D2 in Receptor Conformation and Ligand Binding—We reported previously (8) that D2 was required to stabilize the conformation of the minimal LIF-binding receptor D1IgD2myc via interactions with the D1 fragment. To analyze which part(s) of D2 was (were) required for this, we constructed a series of 14 deletion mutants containing the intact D1Ig domain fused to truncations of the D2 domain from its carboxyl terminus upstream to the Ig-like module. Following alignment of the protein sequence of D2 with that of the unique gp130 CRH domain, the truncations were designed to take place at the end of the putative β-strands of gp190 D2, which were inferred from a comparison with the known three-dimensional structure of the unique gp130 CRH (Fig. 6). All the constructs were fused carboxyl-terminally in-frame to the c-Myc epitope recognized by mAb 9E10.

All these mutants were stably transfected in CHO cells, and the production of the protein was assessed in the cell culture supernatants using immunoprecipitation with the anti-c-Myc mAb 9E10 after 35S metabolic labeling, as well as by using a sandwich ELISA specific for gp190 based on two conformation-dependent mAbs directed against distinct epitopes in the D1Ig region (Fig. 7, panel A). All the constructs were fused carboxyl-terminally in-frame to the c-Myc epitope recognized by mAb 9E10.

All these mutants were stably transfected in CHO cells, and the production of the protein was assessed in the cell culture supernatants using immunoprecipitation with the anti-c-Myc mAb 9E10 after 35S metabolic labeling, as well as by using a sandwich ELISA specific for gp190 based on two conformation-dependent mAbs directed against distinct epitopes in the D1Ig region (Fig. 7, panel A). The combination of both methods would enable the detection of all the mutants produced and give information about their conformation. As shown previously (8) by immunoprecipitation, mutant D1IgD2myc was efficiently secreted, whereas mutant Δ332, which contained the D1Ig fragment but lacked any sequence from D2, was not detectable. None of the mutants within the amino-terminal module of D2 (i.e. Δ345, Δ355, Δ371, Δ381, Δ391, Δ398, and Δ415) could be immunoprecipitated from cell culture supernatants, and none could be measured by ELISA. In contrast, all the mutants within the carboxyl-terminal module of D2 (right panel) were immunoprecipitated from supernatants with mAb 9E10 and were present at levels consistent with those measured by ELISA. Among these, the shortest two (i.e. Δ448 and Δ459) were produced at levels in the same order of magnitude as D1IgD2myc, whereas the longer mutants were secreted in much lower amounts (10 times less for Δ472 and 100 times less for Δ489, Δ499, and Δ514). Among the whole series of deletion mutants, the shortest secreted mutant that could be detected by both methods was Δ431, which encompassed the full amino-terminal module of D2 and was produced in amounts comparable with D1IgD2myc.

To verify that the mutants were efficiently expressed by the cells, they were immunoprecipitated from cell lysates with the anti-c-Myc mAb 9E10 or the anti-D1 mAb 6G8 (Fig. 7, panel B). All could be recovered with mAb 9E10, including the deletion mutants within the D2 amino-terminal module. However, none of the deletion mutants within the D2 amino-terminal module were recognized by the conformation-dependent mAb 6G8. These results strongly suggested the following: 1) deletions within the amino-terminal module of D2 led to profound conformational alterations precluding the secretion of the mutants, and 2) the amino-terminal module of D2 is required for a correct conformation of D1Ig.

We then analyzed the LIF binding capacity of Δ431, Δ448, and Δ459, the shortest truncation mutants with the highest...
secretion levels, by comparison with D1IgD2myc. For this purpose, we set up a sandwich ELISA based on the capture of the receptor fragment at a saturating concentration by the non-blocking anti-D1 mAb 6G8, followed by the incubation with serial dilutions of LIF. The LIF bound to the receptor is subsequently detected using as a tracer the non-neutralizing...
suggest that gp190 also exists as a preformed homodimer in the absence of ligand. We previously raised this hypothesis following the determination of binding stoichiometries of anti-human gp190 antibodies by the Scatchard method. In this work, we noticed that several mAbs bound to 2-fold less receptors than other mAbs on the surface of cells expressing either recombiant or natural gp190 molecules, which could suggest that certain epitopes might be masked, possibly due to receptor homodimerization (17). If so, the stoichiometry of the LIF signaling receptor complex would be more complex than the currently acknowledged heterodimer of gp190 and gp130 (18, 19), because it could associate two gp190 and at least one gp130.

Our results also suggest that the carboxyl-terminal module of D2 has a different task in the receptor function. It is able to trigger cytokine- and gp130-independent proliferation of Ba/F3 cells when fused to the transmembrane and intracellular region of gp130 via a spontaneous homodimerization. A more thorough analysis of this module showed that this property lies within the carboxyl-terminal module of D2, and more precisely within a 42-residue-long fragment at the carboxyl terminus of this module. This short stretch contains a (Y/F)XXR/QXR consensus sequence, where X can be any residue, which has been found to exert a similar function in the β-common receptor chain using a similar approach of progressive truncation in the receptor ectodomain (4). This sequence is upstream of the conserved WSXWS motif and makes the F β-strand within the CRH carboxyl-terminal module. In the unique CRH of gp130, this sequence is also found and in a similar position. It is striking that the unique gp130 CRH is closer to gp190 CRH D2 than to D1, both at the amino acid sequence level and in a predictive model of D2 spatial conformation (20). Especially within this 42-amino acid-long stretch, 19 residues are identical (45.2 versus 25.7% over the whole CRH domain), and the consensus motif pointed out above differs by only 1 amino acid between gp130 and D2 (YVFRIR for gp130 and YTFRIR for gp190 D2). Because spatial modeling suggested that D2 could directly interact with the gp130 CRH (20) and because we suggested that interaction between gp190 and gp130 in the high affinity signal-transducing LIF receptor could occur via this highly conserved motif. However, more work is needed to confirm this hypothesis, because in the experiments reported in the present work the occurrence of spontaneous homodimerization of D2 precluded the analysis of heterodimerization with gp130. The situation may be even more complex, because in the case of the D2FN/130 mutant, the presence of gp130 abrogated its capacity to trigger autonomous proliferation. We have no clues to explain this, but it remains possible that non-productive heterodimerization of both chains could occur. In this regard, recent work (21, 22) documented an important role for the FN region of gp130 in the receptor function.

It is intriguing to note that none of the D2-containing receptor mutants that triggered autonomous signaling contained the D1 CRH, because neither gp190/130 nor D1ligD2/130 chimeras were able to stimulate cell proliferation when transfected alone. Therefore, these results suggest that the dimerization motif on the gp190/130 and D1ligD2/130 receptors is not free to interact with another chain and that D1 is also involved in this restriction. If gp190 does exist as an homodimer, spontaneous homodimerization via D2 could be prevented simply by virtue of the distance between the carboxyl-terminal modules of D2 monomers resulting from their spatial locations in the dimer, as is the case for the β-common. Indeed, the present available model of the ligand-receptor α-chain-β-common complex does not favor a ligand-induced movement of these modules toward each other but on the contrary suggests that the low affinity
complex between the ligand and its specific α-chain could sneak into the cavity delineated by the interlocked β-common chains (7). In the absence of the membrane-distal CRH domain, these structural constraints disappear allowing for the two membrane-proximal gp190 CRHs to interact freely between each other, leading to constitutive signaling. In the presence of LIF, a possible scenario could be that after the cytokine has bound to gp190, a conformational adjustment in the D2 domain is induced that unmasks its dimerization motif. Simultaneously, recruitment of gp130 by the LIF-gp190 complex could trigger a similar change in gp130 unique CRH, allowing the two motifs to interact with each other, and bring the downstream regions of the receptor in close enough proximity to lead to the transduction of the activation signal. Therefore, as suggested for the β-common model, the LIF-gp190-gp130 functional complex would contain two molecules of each partner. Further experiments are needed to evaluate this hypothesis.

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Separate Functions for the Two Modules of the Membrane-proximal Cytokine Binding Domain of Glycoprotein 190, the Leukemia Inhibitory Factor Low Affinity Receptor, in Ligand Binding and Receptor Activation
Mathieu-Benoît Voisin, Juliette Bitard, Sophie Daburon, Jean-François Moreau and Jean-Luc Taupin

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