Ligand-specific utilization of the extracellular membrane-proximal region of the gp130-related signalling receptors

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The receptor gp130 is used by the interleukin-6 (IL-6)-type cytokines, which include IL-6 and leukaemia-inhibitory factor (LIF). To investigate the role of the three extracellular membrane-proximal fibronectin-type-III-like (FNIII) modules of gp130 and the related receptor for granulocyte colony-stimulating factor (G-CSFR) in cytokine signal transduction we have transfected into murine myeloid M1-UR21 cells the chimaera (GR-FNIII)gp130, which contains the membrane-proximal FNIII modules of the G-CSFR on a gp130 backbone, and its complement, the chimaera (gp130-FNIII)GR. Whereas the binding affinities of ^{125}I-labelled IL-6 to (GR-FNIII)gp130, or of ^{125}I-Tyr1,3-G-CSF to (gp130-FNIII)GR, were similar to wild-type gp130 and wild-type G-CSFR, respectively, ^{125}I-LIF failed to bind with high affinity to (GR-FNIII)gp130. In assays measuring differentiation the (gp130-FNIII)GR cells were fully responsive to G-CSF, whereas the (GR-FNIII)gp130 cells responded fully to the agonistic anti-gp130 monoclonal antibody (mAb) B-S12, but not to IL-6 or LIF. Neutralizing mAbs that recognize the membrane-proximal FNIII modules of gp130 or the G-CSFR differentially interfered with signalling by B-S12, LIF and G-CSF. The data suggest that B-S12 and G-CSF induce the correct orientation or conformation for signalling by the wild-type and chimaeric homodimeric receptors, that the membrane-proximal region of gp130 is important for the correct formation of the signalling IL-6–IL-6 receptor–gp130 complex and that this region is also involved in LIF-dependent receptor heterodimerization and signalling.

Key words: antibody, fibronectin-type-III-like, G-CSF, IL-6, LIF.

INTRODUCTION

The transmembrane protein gp130 is a shared receptor subunit, affinity converter and signal transducer for the interleukin-6 (IL-6)-type cytokines [1]. This cytokine family comprises at least six structurally related members, including IL-6, leukaemia-inhibitory factor (LIF), IL-11, oncostatin M (OSM), ciliary neurotrophic factor and cardiotrophin-1 as well as a viral IL-6 homologue [2,3]. The shared use of gp130 in part explains the many overlapping activities of these cytokines, such as the induction of acute-phase proteins, regulation of haemopoiesis and the proliferation/differentiation of neural cells [1,4].

The activation of intracellular responses in target cells by the IL-6-type cytokines is mediated by dimerization of gp130. LIF and OSM, for example, induce heterodimerization of gp130 to the LIF receptor (LIFR), while OSM also induces heterodimerization of gp130 to the specific OSM receptor (OSMRβ) [5,6]. Activation and homodimerization of gp130 can be induced by IL-6 or IL-11 [together with the specific IL-6 receptor (IL-6R) and IL-11 receptor, respectively] or agonistic anti-gp130 monoclonal antibodies (mAbs) such as B-S12 [7–12]. The most closely related receptor to gp130 is the granulocyte colony-stimulating factor (G-CSF) receptor (G-CSFR) [13,14]. Its ligand, G-CSF, is a growth and differentiation factor primarily for cells of the neutrophil lineage [15,16]. The binding of G-CSF to the G-CSFR induces receptor homodimerization [17,18]. Although G-CSF is structurally related to the IL-6-type cytokines it does not bind to gp130 [2].

The receptors for the IL-6-type cytokines and G-CSF belong to the class-I haemopoietin receptors, a family that is characterized by the presence of a structurally conserved 'cytokine-binding domain' (CBD) in the extracellular region [19]. The extracellular regions of gp130 and the G-CSFR share 46% amino acid similarity and have the same modular structure (see Figure 1) [13,14]. The N-terminal Ig-like module and the CBD of gp130 and the G-CSFR are required for ligand-binding and/or receptor-complex formation [20–24], but the role of the three membrane-proximal fibronectin-type-III-like (FNIII) modules in signalling is poorly understood. It is thought that dimerization of the latter region in the mouse G-CSFR by a small, non-peptidyl compound results in receptor activation, albeit less efficiently than by G-CSF [25].

Among the class-I haemopoietin cytokine receptors, the presence of three membrane-proximal FNIII modules between the CBD and the transmembrane domain is limited to gp130, the G-CSFR, the LIFR and the specific OSM receptor OSMRβ [5,6,13,14]. Because these are receptors with intracellular signalling capacity, it is possible that this region has a structural or functional role in signalling. In agreement with this hypothesis, a mutant G-CSFR lacking the major part of the membrane-proximal region had severely reduced signalling capacity in FDC-P1 cells [> 1000-fold compared with wild-type (wt) G-

Abbreviations used: CBD, cytokine-binding domain; FNIII, fibronectin-type-III-like; G-CSF, granulocyte colony-stimulating factor; G-CSFR, G-CSF receptor; GH, growth hormone; GHR, GH receptor; IL-6, interleukin-6; IL-6R, IL-6 receptor; LIF, leukaemia-inhibitory factor; LIFR, LIF receptor; mAb, monoclonal antibody; OSM, oncostatin M; wt, wild-type.

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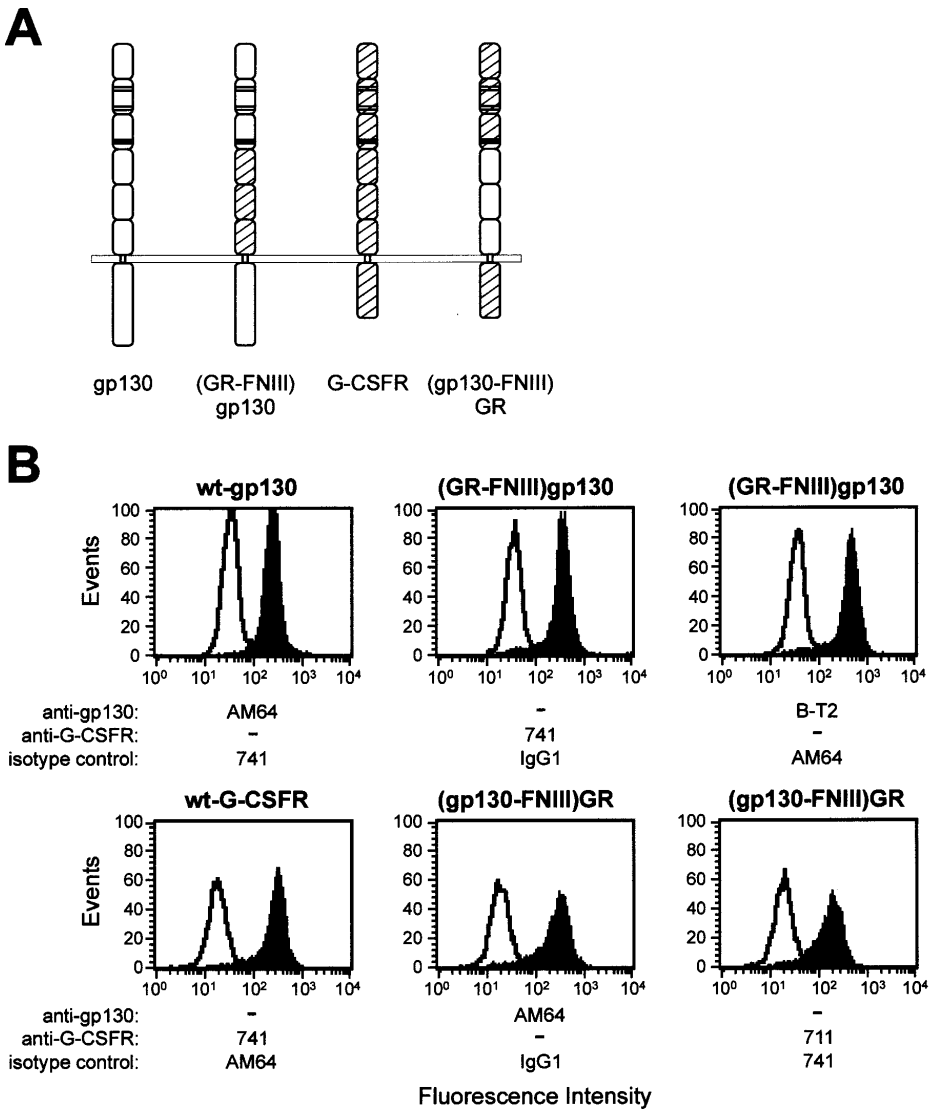

Figure 1 Schematic representation of gp130, the G-CSFR and the gp130/G-CSFR chimaeras

(**A**) Wt gp130 and the G-CSFR (hatched), (GR-FNIII)gp130, a chimaera with the three membrane-proximal FNIII modules of the G-CSFR on a gp130 backbone, and (gp130-FNIII)GR, a chimaera with the three membrane-proximal FNIII modules of gp130 on a G-CSFR backbone, were generated as described in the Experimental Procedures section. The conserved cysteines and the WSXWS motif in the CBDs [19] of the constructs are shown as thin lines and a black bar, respectively. The cell membrane (horizontal bar) and the intracellular and transmembrane domains are indicated. (**B**) The binding of the anti-gp130 mAbs AM64 and B-T2, the anti-G-CSFR mAbs LMM 711 and LMM 741, or an isotype-matched mAb as a negative control were measured by flow cytometry, as described in the Experimental Procedures section. Open histograms, isotype controls. Filled histograms, reactive mAbs. FACScan profiles of one representative cell line expressing wt gp130, (GR-FNIII)gp130, wt G-CSFR or (gp130-FNIII)GR are shown.

CSFR], but G-CSF binding was essentially unaffected [22]. No mutagenesis studies have been reported that address the effects on signal transduction of a corresponding deletion in gp130. However, truncated forms of gp130 lacking the membrane-proximal FNIII modules and the transmembrane and intracellular domains retained the ability to associate with IL-6–'soluble' IL-6R or LIF–'soluble' LIFR, respectively [26,27]. We have recently identified neutralizing mAbs that recognize the membrane-proximal FNIII modules of gp130 or the G-CSFR, further indicating a role for this region in receptor activation [27,28]. Hence, although dispensable for ligand binding, it is likely that the membrane-proximal FNIII modules of gp130 and the G-CSFR may be structurally or functionally required for receptor dimerization and signalling. In the present study, to investigate the role of this region in signal transduction, we have generated the gp130/G-CSFR chimaeras (GR-FNIII)gp130 and (gp130-FNIII)GR, wherein the three membrane-proximal FNIII modules have been interchanged.

EXPERIMENTAL PROCEDURES

Cells

The generation and characterization of the murine myeloid M1-UR21 cell line, which is IL-6- and LIF-unresponsive unless transfected with gp130, have been described previously [20]. The M1-UR21 cells expressing wt gp130, (GR-FNIII)gp130, wt G-CSFR or (gp130-FNIII)GR were maintained in Dulbecco's

modified Eagle's medium/10 % (v/v) foetal bovine serum in a humidified incubator at 37 °C and 10 % CO_2.

Generation and transfection of chimaeric receptors

pBS130BES (containing the cDNA of human gp130) was used for mutagenesis as described previously [20]. pQB3S (containing the cDNA of the human G-CSFR) was used for mutagenesis with the QuikChange site-directed-mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) according to the manufacturer's instructions.

To generate (GR-FNIII)gp130 and (gp130-FNIII)GR the primary sequences of human gp130 and the human G-CSFR were aligned manually. A silent *Nde*I site was introduced at Thr-321–Glu-323 of gp130 using the oligonucleotide 5′-GCAAGTGGGATCACATATGAAGATAGACC-3′ (numbering as in [13]), and a silent *Mun*I site was introduced at Ala-620–Val-622 of gp130 in the predicted transmembrane domain using the oligonucleotide 5′-GGAGAAATTGAAGCAATTGTCGTGCCTGTTTGC-3′. In the G-CSFR, an *Nde*I site was introduced at Thr-329–Glu-331 (our numbering includes the predicted 23-amino acid signal sequence and differs from [14]) using the oligonucleotide 5′-AGCCTGGAGCTGAGAACATATGAACGGGCCCCCACTGTC-3′, and a *Mun*I site was inserted at Ile-628–Leu-630 in the predicted transmembrane domain using the oligonucleotide 5′-GGGTCGGAGCTACACGCAATTGTGGGCCTGTTCGGCCTC-3′, and complementary reverse oligonucleotides. (GR-FNIII)gp130 thus contained residues Met-1–Glu-323 of gp130 followed by Arg-332–His-627 of the G-CSFR and Ala-620–Gln-918 of gp130, whereas (gp130-FNIII)GR contained residues Met-1–Arg-328 of the G-CSFR followed by Thr-321–Val-622 of gp130 and Gly-631–Phe-836 of the G-CSFR. The coding sequences of the constructs were ligated into the *Xba*I site of pEF-BOS [29] and verified by DNA sequencing using a model-370A DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). The restriction enzymes were from Boehringer Mannheim (Mannheim, Germany) and New England Biolabs (Beverly, MA, U.S.A.). Transfection into M1-UR21 cells of wt gp130, (GR-FNIII)gp130, wt G-CSFR or (gp130-FNIII)GR with pGKPuropA encoding the gene for puromycin resistance was done as described previously [20].

^{125}I-Labelling of cytokines and equilibrium-binding assay

Mouse IL-6 was purified as described in [30], mouse LIF was from AMRAD Pharmacia Biotech (Boronia, Victoria, Australia), and human G-CSF was a gift from L. Souza (Amgen, Thousand Oaks, CA, U.S.A.). LIF was radiolabelled using a modified iodine monochloride method [31], IL-6 was radiolabelled using di-^{125}I-Bolton–Hunter reagent (NEN, Boston, MA, U.S.A.) [32], and human Tyr1,3-G-CSF (a G-CSF mutant wherein Thr-1 and Leu-3 have been replaced by tyrosine residues; a gift from A. Shimosaka, Kirin Brewery Co., Gumma, Japan) was radiolabelled using Iodo-Gen [24,33]. The specific activities were determined by self-displacement analysis [34] and varied from 4×10^5 to 15×10^5 c.p.m./pmol. The binding assays were performed essentially as described previously [20] and the data were examined using the curve-fitting program LIGAND [35].

Flow cytometry

The transfected cells were analysed using a FACScan flow cytometer (Becton Dickinson) for binding of the anti-human gp130 mAbs B-T2 [11,36] and AM64 [13], the anti-G-CSFR mAbs LMM 711 and LMM 741 [28] or a non-relevant IgG1 as an isotype-matched control. Fluorescein-conjugated sheep anti-mouse Ig (Silenius Laboratories, Hawthorn, VIC, Australia) was used as a secondary antibody. Non-viable cells were stained with propidium iodide (Sigma–Aldrich, Castle Hill, NSW, Australia) and gated out at analysis. The analysis by flow cytometry of morphological changes upon differentiation was performed essentially as described previously [20], except that 2×10^4 cells were seeded per well. Quadrants were set on the plot of forward scatter versus side scatter so that the lower left (LL) quadrant contained > 97.5 % of cells grown for 4 days without stimulus; the degree of differentiation after 4 days in the presence of stimulus was quantified by determining the percentage of cells in the upper left (UL) + upper right (UR) + lower right (LR) quadrants [20].

Soft-agar assay

The cells were plated into 35-mm culture dishes (Nunc; 150 cells in 1 ml/dish) in Dulbecco's modified Eagle's medium containing 20 % (v/v) foetal bovine serum and 0.3 % (w/v) dissolved bacto-agar (Difco Laboratories, Detroit, MI, U.S.A.) with IL-6, LIF, B-S12, G-CSF or medium as a negative control, in the absence or presence of anti-gp130 or anti-G-CSFR mAbs [13,28]. After 7 days in a humidified incubator at 37 °C and 10 % CO_2, the colonies were counted and scored for differentiation. Colonies with a halo of dispersed cells or composed entirely of dispersed cells were scored as differentiated.

RESULTS AND DISCUSSION

Generation of the constructs, cell-surface expression and ligand binding

Wt gp130 and wt G-CSFR, (GR-FNIII)gp130 (a gp130/G-CSFR chimaera where the three membrane-proximal FNIII modules of the extracellular region of the human G-CSFR replace the equivalent modules of human gp130) and (gp130-FNIII)GR (a chimaera where the three membrane-proximal FNIII modules of the extracellular region of human gp130 replace the equivalent modules of the human G-CSFR) were subcloned into the expression vector pEF-BOS and transfected into murine myeloid M1-UR21 cells (see the Experimental procedures section; Figure 1A). The M1-UR21 cells lack functional endogenous gp130, but express the IL-6R and the LIFR [20]; they also lack endogenous G-CSFR (results not shown).

Independently derived cell lines were analysed for expression of the constructs by flow cytometry using anti-gp130 or anti-G-CSFR mAbs and isotype-matched mAbs as negative controls (Figure 1B). The (GR-FNIII)gp130 transfectants were reactive with anti-gp130 mAbs, as exemplified by B-T2 that recognizes the Ig-like module of gp130 [11,20]. The (gp130-FNIII)GR transfectants were reactive with anti-G-CSFR mAbs, for example the conformation-dependent antibody LMM 711 that recognizes the Ig-like module of the G-CSFR [28]. The cell lines expressing wt gp130 and (gp130-FNIII)GR, but not those expressing wt G-CSFR and (GR-FNIII)gp130, were recognized by the anti-gp130 mAb AM64 that reacts with an epitope located in the region encompassing the three membrane-proximal FNIII modules of gp130 [13,27]. The cells transfected with wt G-CSFR or (GR-FNIII)gp130, but not those expressing wt gp130 or (gp130-FNIII)GR, were also reactive with the anti-G-CSFR mAb LMM 741, which recognizes an epitope in the three membrane-proximal FNIII modules of the G-CSFR [28] (Figure 1B). These results show that all constructs were expressed on the cell surface.

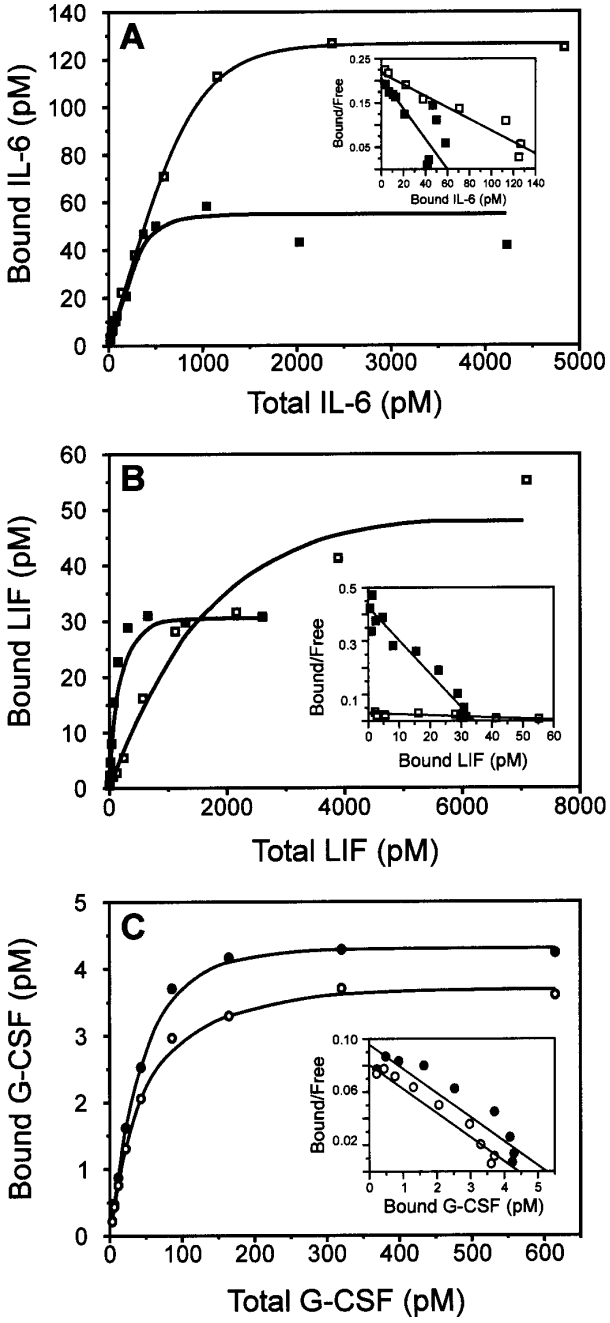

Figure 2 Determination of receptor binding affinities

One cell line expressing (GR-FNIII)gp130 (□) or wt gp130 (■) was assessed for equilibrium binding of (**A**) ^{125}I-labelled IL-6 and (**B**) ^{125}I-labelled LIF. One cell line expressing (gp130-FNIII)GR (○) and wt G-CSFR (●) was assessed for equilibrium binding of (**C**) ^{125}I-labelled Tyr1,3-G-CSF. Insets: Scatchard transformations of the binding data (see Table 1 for a summary of the results). The assays, which were performed at least twice with similar results, are described in the Experimental procedures section.

Scatchard analyses of the equilibrium binding of ^{125}I-labelled IL-6 to the M1-UR21 cells expressing (GR-FNIII)gp130 yielded a single class of binding site with similar affinity ($K_d = 7.2 \times 10^{-10}$ M) to that determined previously on M1-UR21 cells expressing wt gp130 ($K_d = 2.8 \times 10^{-10}$ M [20]; Figure 2 and Table 1). These results on cellular gp130 are in agreement with the finding by Horsten et al. [26] (using soluble forms of gp130) that the membrane-proximal FNIII modules of gp130 are not involved in binding IL-6.

Table 1 Equilibrium binding of ^{125}I-labelled ligands on M1-UR21 transfectants

The data represent the mean values of at least two assays on one independently derived cell line (see Figure 2). The assays were performed as described in the Experimental Procedures section. N/A, not assessed; N/D, not detectable.

	K_d		
Cell line	IL-6 ($M \times 10^{10}$)	LIF ($M \times 10^{10}$)	G-CSF ($M \times 10^{12}$)
(GR-FNIII)gp130	7.2	21	N/A
Wt gp130	2.8*	0.7	N/A
(gp130-FNIII)GR	N/A	N/A	51
Wt G-CSFR	N/A	N/A	55
M1-UR21	N/D*	26*	N/A

* Data from Hammacher et al. [20].

On the M1-UR21 cells expressing (GR-FNIII)gp130, ^{125}I-labelled LIF had one class of binding site with a similar affinity ($K_d = 21 \times 10^{-10}$ M) to that determined previously for non-transfected M1-UR21 cells ($K_d = 26 \times 10^{-10}$ M [20]; Figure 2 and Table 1). The M1-UR21 cells, which express the LIFR but lack functional endogenous gp130, bind LIF with low affinity whereas the wt-gp130-transfected cells bind LIF with high affinity ($K_d = 0.7 \times 10^{-10}$ M [5,20]; Figure 2 and Table 1). Our data show that the membrane-proximal region of the G-CSFR cannot substitute for the corresponding region of gp130 in the formation of the cellular high-affinity LIF–LIFR–gp130 complex. This finding differs from that of Zhang et al. [27], who showed that a truncated form of gp130 lacking the membrane-proximal region as well as the transmembrane and intracellular domains could be chemically cross-linked to form a ternary solution complex with LIF and the ectodomain of the LIFR. Because the affinity of this ternary complex was not investigated, the contribution of the membrane-proximal FNIII region of gp130 in solution-complex formation remains to be determined. Our data using cellular gp130 suggest that the amino acid sequence, the orientation or conformation of the membrane-proximal region of cellular gp130 is important for heterodimerization with the LIFR and high-affinity binding of LIF.

Scatchard analyses of the binding of ^{125}I-labelled Tyr1,3-G-CSF to the (gp130-FNIII)GR- or wt-G-CSFR-transfected cells yielded a single class of binding site ($K_d = 51 \times 10^{-12}$ and 55×10^{-12} M, respectively; Figure 2 and Table 1), similar to previously described results for binding of Tyr1,3-G-CSF to Ba/F3 cells transfected with the wt G-CSFR ($K_d = 47 \times 10^{-12}$ M) [28]. These data are in agreement with previous findings using full-length and soluble G-CSFR that the membrane-proximal FNIII modules of the G-CSFR are not required for binding G-CSF [22,37].

Ligand-specific utilization of the membrane-proximal region in differentiation responses

M1-UR21 cells transfected with wt gp130, but not non-transfected M1-UR21 cells, can be induced by the IL-6-type cytokines to differentiate in soft agar [20]. In the soft-agar assay the non-transfected M1-UR21 cells did not differentiate significantly

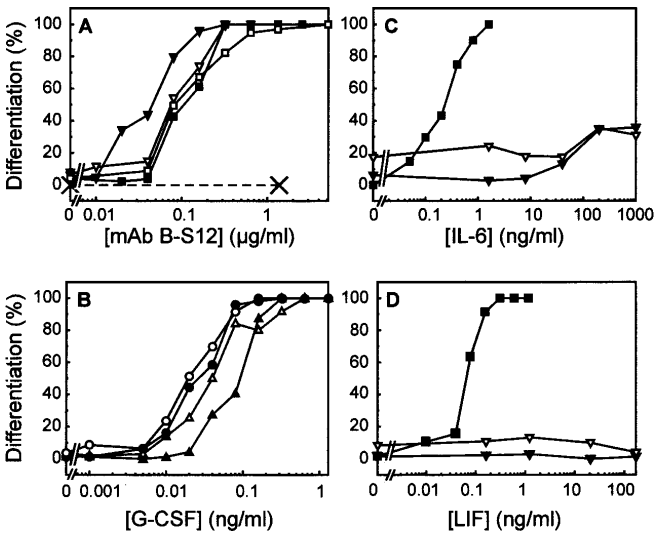

Figure 3 Colony formation in soft agar

Shown is one representative assay with each of mAb B-S12 (**A**), G-CSF (**B**), IL-6 (**C**) and LIF (**D**) using independently derived cell lines expressing (GR-FNIII)gp130 (▼, ▽), wt gp130 (■, □), wt G-CSFR (●, ○) or (gp130-FNIII)GR (▲, △). –X–, Non-transfected M1-UR21 cells. The assays, which were performed at least twice with similar results, are described in the Experimental procedures section.

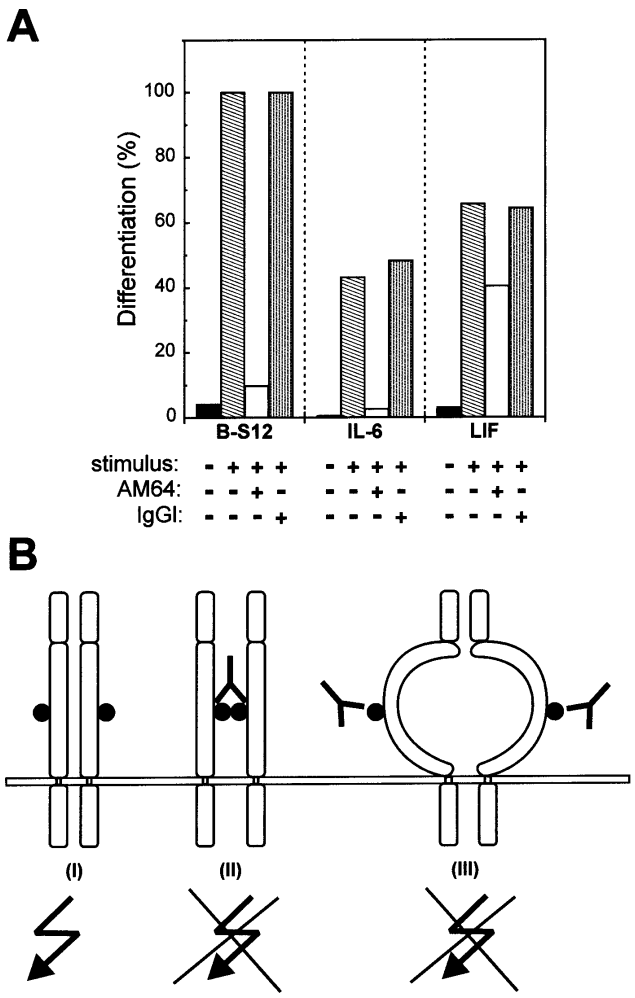

Figure 4 The anti-gp130 mAb AM64 is an inhibitory antibody

(**A**) One cell line expressing wt gp130 was stimulated with 0.32 μg/ml B-S12, 0.2 ng/ml IL-6 or 0.075 ng/ml LIF, in the absence or presence of 5.7 μg/ml mAb AM64 or 5.7 μg/ml of an isotype-matched control mAb (11.4 μg for IL-6), and assessed for colony formation in soft agar, as described in the Experimental Procedures section. (**B**) mAb AM64 interferes with correct gp130 dimerization. The AM64 epitope in the membrane-proximal region of gp130 is depicted as a black dot (the exact location of this epitope remains to be determined). (I) Schematic of the correct positioning for signal transduction of the two gp130 molecules in a gp130 homodimer. The inhibition of IL-6 and B-S12 activities by AM64 may be due to (II) steric hindrance by preventing the correct positioning of the two gp130 molecules in a gp130 homodimer (for example by cross-linking gp130 in the wrong orientation) or (III) interference with a conformational change in gp130 necessary for dimerization and signal transduction.

above background in 1.28 μg/ml mAb B-S12 (a mAb that mimics the effects of IL-6 on several human cell lines [11,12]) (Figure 3A). In contrast, the (GR-FNIII)gp130 and wt gp130 cells fully differentiated in response to B-S12 ($EC_{50} = 0.06$ and 0.09 μg/ml, respectively; Figure 3A). Upon stimulation with G-CSF the (gp130-FNIII)GR-transfected cells differentiated fully, with a similar responsiveness to the wt G-CSFR transfectants ($EC_{50} = 0.05$ and 0.02 ng/ml, respectively; Figure 3B). Non-transfected M1-UR21 cells did not differentiate significantly above background in 0.5 μg/ml G-CSF (results not shown). These data show that B-S12 and G-CSF signalling occur in the presence of the 'inappropriate' membrane-proximal region.

In contrast to B-S12, the (GR-FNIII)gp130-transfected cells had significantly attenuated responsiveness to IL-6, generally reaching 50% of the maximal differentiation of the wt gp130 cells at 1 μg/ml IL-6 (Figure 3C). The EC_{50} of IL-6 on the (GR-FNIII)gp130 cells was thus calculated to be > 1000-fold higher than that on wt gp130 ($EC_{50} = 0.2$ ng/ml). Since (GR-FNIII)gp130 bound IL-6 with similar affinity to the wt gp130 cells (Table 1) and also was responsive to mAb B-S12, which recognizes the C-terminal part of the CBD of gp130 [11], the poor responsiveness to IL-6 was not due to misfolding of the CBD of the chimaera. We conclude that IL-6 signalling is attenuated in the presence of the 'inappropriate' membrane-proximal region.

As expected from the inability of LIF to induce high-affinity receptor-complex formation on the (GR-FNIII)gp130 transfectants, these cells were unresponsive to LIF, whereas the EC_{50} of LIF on wt gp130 cells was 0.07 ng/ml (Figure 3D).

The anti-gp130 mAb AM64 interferes with correct gp130 dimerization

We next investigated whether mAb AM64 was able to interfere with gp130 signal transduction. This mAb has been shown to inhibit high-affinity binding of IL-6 to U266 cells and to recognize the membrane-proximal FNIII modules of gp130 [13,27]. The differentiation in soft agar of the wt gp130 cells in response to B-S12 (0.32 μg/ml) and IL-6 (0.2 ng/ml) was fully inhibited by AM64, whereas the response to LIF (0.075 ng/ml) was partially inhibited (Figure 4A).

Using ELISA and optical-biosensor-based assays, Yasukawa et al. [38] showed that mAb AM64 does not prevent the interaction of the binary complex of IL-6–soluble IL-6R with soluble gp130. From these studies it could be deduced that AM64 is not a neutralizing antibody. However, our results show that AM64 is capable of neutralizing gp130 signalling in a bioassay. The comparison of the effects of AM64 on stimulation by mAb B-S12 and LIF (Figure 4A) suggests that AM64 interferes with gp130 dimerization rather than with the association of the

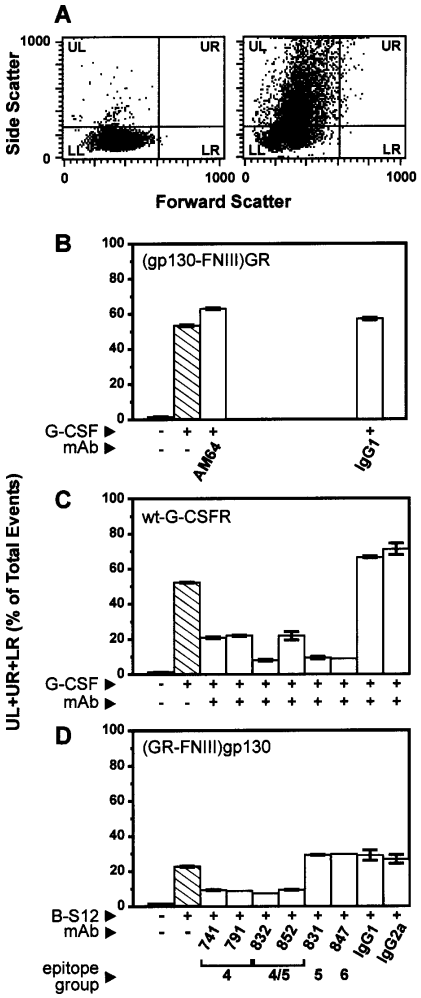

Figure 5 G-CSF- and B-S12-induced differentiation and inhibition by anti-gp130 and anti-G-CSFR mAbs

Flow-cytometry analyses measuring cell size and vacuolization, as described in the Experimental procedures section. (**A**) FACScan profile of one representative (gp130-FNIII)GR transfectant grown for 4 days without G-CSF (undifferentiated cells, left panel), or with 30 ng/ml G-CSF (differentiated cells, right panel). (**B**–**D**) The data represent the sum of the events in the upper left (UL), upper right (UR) and and lower right (LR) quadrants of (**A**) expressed as the percentage of total events. Each bar represents the mean ± range of duplicate samples from one assay; error bars are not shown where duplicate values are within 0.5%. The assays were performed at least twice with similar results. One representative cell line expressing (gp130-FNIII)GR (**B**), wt G-CSFR (**C**) or (GR-FNIII)gp130 (**D**) was stimulated for 4 days with either 30 ng/ml G-CSF (**B** and **C**) or 0.2 μg/ml B-S12 (**D**), in the absence or presence of the anti-gp130 mAb AM64, the anti-G-CSFR mAbs LMM 741, 791, 831, 832, 847 and 852, or the isotype-matched control mAbs B-T2 or LMM 111 (IgG1) and B-T6 or LMM 337 (IgG2a) (all at 5 μg/ml). The same antibodies were used in panels (**C**) and (**D**). The G-CSFR mAbs were assigned to different epitope groups based on their behaviour in antibody cross-inhibition assays. These mAbs recognize the membrane-proximal region of the G-CSFR, but their actual epitopes have not yet been defined [28].

IL-6–IL-6R complex with gp130. AM64 does not compete with B-S12 for binding to gp130 (results not shown). The inhibition of IL-6 and B-S12 activity by AM64 may therefore be due to steric hindrance by preventing the correct relative positioning of the two gp130 molecules in a gp130 homodimer (for example by cross-linking gp130 in the wrong orientation), or AM64 may interfere with a conformational change in gp130 necessary for dimerization and signal transduction (Figure 4B). This would explain why AM64 inhibited B-S12 activity, despite the fact that the membrane-proximal FNIII modules of gp130 are not required for signalling by B-S12 (Figures 3A and 4A), but does not rule out the possibility that part of the membrane-proximal region of gp130 forms part of the dimerization interface.

The mAb AM64 was a significantly more potent inhibitor of B-S12 or IL-6 than of LIF (Figure 4A), possibly because a gp130 homodimer can bind two molecules of AM64, but a gp130 heterodimer only one. Alternatively, in LIF-induced gp130 heterodimers with the LIFR the membrane-proximal FNIII modules of gp130 may have different conformations or orientations than in gp130 homodimers, such that AM64 binds to gp130 but is not able to inhibit LIF signal transduction to the same extent as it inhibits IL-6 signalling. The latter explanation is consistent with the observation that the (GR-FNIII)gp130-transfected cells lacked high-affinity LIF binding, but exhibited an affinity for IL-6 similar to wt gp130 cells (Table 1). Taken together, the data suggest that the correct orientation and/or conformation of the membrane-proximal FNIII modules of gp130 is required for gp130 dimerization and signalling by IL-6 and LIF.

Differences between the orientation and/or conformation of the membrane-proximal regions of gp130 and the G-CSFR in signalling complexes

mAb AM64 recognized the (gp130-FNIII)GR transfectants and was inhibitory for B-S12 and IL-6 on the wt gp130 cells (Figures 1B and 4A). It did not, however, inhibit G-CSF-induced differentiation of the (gp130-FNIII)GR cells, as shown by flow-cytometric determination of cell size and vacuolization (Figures 5A and 5B). A differential effect was also observed when wt G-CSFR- and (GR-FNIII)gp130-transfected cells were stimulated with G-CSF and B-S12, respectively, in the absence or presence of six individual mAbs that recognize three epitopes on the membrane-proximal FNIII modules of the G-CSFR [28]. These mAbs were assigned to different epitope groups based on their behaviour in antibody cross-inhibition assays [28]. Using chimaeras of mouse and human G-CSFR, the mAbs were shown to recognize the membrane-proximal region of the G-CSFR, but their actual epitopes have not yet been defined [28]. All the anti-G-CSFR mAbs, but not the isotype-control mAbs (IgG1 and IgG2a), significantly inhibited differentiation of the wt G-CSFR transfectants induced by 30 ng/ml G-CSF (Figure 5C). All these anti-G-CSFR mAbs recognized non-stimulated (GR-FNIII)gp130 cells (Figure 1B and results not shown), but only the mAbs in the epitope groups 4 (LMM 741 and LMM 791) and 4/5 (LMM 832 and LMM 852) significantly inhibited stimulation with 0.2 μg/ml B-S12. The mAbs belonging to epitope groups 5 (LMM 831) and 6 (LMM 847) did not inhibit B-S12 activity on the (GR-FNIII)gp130-transfected cells (Figure 5D). None of the anti-G-CSFR mAbs competed with B-S12 for binding to (GR-FNIII)gp130 (results not shown).

It has been shown previously that the deletion of the major part of the membrane-proximal region of the G-CSFR significantly attenuates G-CSF signalling [22]. It is thus possible that, rather than this region itself, the presence of a polypeptide 'linker' between the transmembrane domain and the CBD of the G-CSFR is required for G-CSF signal transduction. Our results imply, however, that signalling depends on the correct orientation or conformation of the membrane-proximal FNIII modules in ligand-induced homodimers of the G-CSFR, gp130 and the chimaeras (Figures 3–5), and do not rule out the possibility that this region of the G-CSFR forms part of the homodimerization interface. Deletion of the membrane-proximal region of the G-CSFR could thus result in 'inappropriate' orientation and

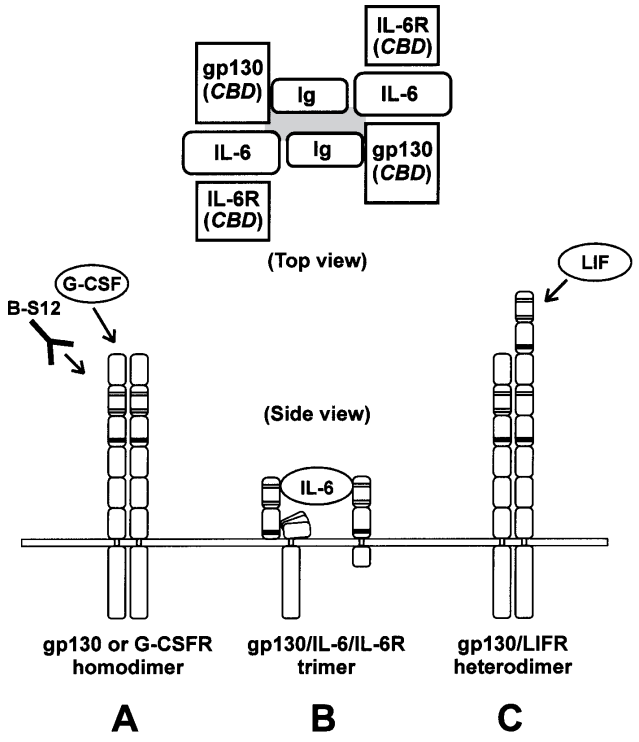

Figure 6 Dimerization models of gp130 and the G-CSFR

The conserved cysteines and the WSXWS motif in the CBDs [19] of the receptors are shown as thin lines and a black bars, respectively. (**A**) Schematic of the homodimer of gp130 induced by mAb B-S12, or the homodimer of the G-CSFR induced by G-CSF, wherein the membrane-proximal FNIII modules may form part of the homodimerization interface. (**B**) Schematic of the IL-6R-dependent gp130 homodimer induced by IL-6. Top view, model of the IL-6–receptor complex, consisting of two GH–GHR-complex-based IL-6–IL-6R–gp130 trimers [2,20,43]; the shaded area indicates a possible location of a gp130-homodimerization region involving the membrane-proximal regions of the two gp130 molecules. Side view, view of the CBDs of gp130 and the IL-6R complexed to IL-6 in one GH–GHR-like trimer. For clarity the N-terminal Ig-like modules of gp130 and the IL-6R are not shown. For the IL-6R, the CBD, the transmembrane and intracellular domains are indicated. Unlike gp130, the IL-6R lacks the three membrane-proximal FNIII modules in its extracellular domain [7,19]; the positioning of the CBDs of gp130 and the IL-6R for binding IL-6 in a GH–GHR-like trimer may therefore require a conformational change in the membrane-proximal region of gp130. This conformational change (indicated by the 'folding' into the plane of the page of the membrane-proximal FNIII modules) is postulated to allow dimerization with the corresponding region of the gp130 molecule in the opposite IL-6–IL-6R–gp130 trimer (see Top view). (**C**) Schematic of the LIF-induced heterodimer of gp130 with the LIFR wherein the membrane-proximal FNIII modules of gp130 may form part of the heterodimerization interface.

dimerization of the cytoplasmic domains with deleterious effects on signal transduction. In the crystal structures of the complexes of erythropoietin or an erythropoietin-mimetic peptide with two soluble erythropoietin receptors, the receptors in each complex had different ligand-dependent orientations relative to each other [39]. Since the efficiency of signalling by this peptide mimetic is at most 5% that of erythropoietin, it appears that optimal intracellular signalling is dependent on defined orientation of receptor dimers [39,40].

B-S12 and G-CSF, like growth hormone (GH), homodimerize their receptors in the absence of additional receptor subunits [11,12,17,18,41], and the presence of the 'inappropriate' FNIII modules did not significantly affect their signalling (Figures 3A and B). Signalling by IL-6, however, was attenuated by the exchange of the membrane-proximal FNIII modules (Fig 3C), suggesting that, on cells, this region of gp130 is required for gp130 homodimerization induced by ligands such as IL-6 that depend on an additional receptor subunit (IL-6R). The membrane-proximal FNIII modules of gp130 may thus also be required for the IL-11-receptor-dependent homodimerization of gp130 by IL-11 [10]. Mutagenesis studies support the notion that, upon binding IL-6, the CBD of the IL-6R dimerizes with that of gp130 in a similar fashion to the two GH receptors (GHRs) in the GH–GHR complex [42,43]. Because the ecto-domain of the IL-6R contains an N-terminal Ig-like module and a CBD (but lacks the three membrane-proximal FNIII modules found in gp130) [7,19], we propose that the membrane-proximal region of gp130 may play a role in the positioning of the CBDs of the IL-6R and gp130. The requirement for the gp130 membrane-proximal FNIII modules by IL-6, but not B-S12, may reflect ligand-specific conformational changes in gp130 or dissimilar orientations of the gp130 molecules relative to each other in the B-S12- and IL-6-induced homodimers (Figure 6).

Like gp130, the LIFR contains three FNIII modules between the transmembrane domain and the membrane-proximal CBD [5,13]. Homology models of the LIFR and gp130 have been analysed to determine the distribution of electrostatic surface potential [44]. The results led to the proposal that, upon binding LIF, the membrane-proximal CBD of the LIFR heterodimerizes with the CBD of gp130 through contacts similar to those seen in the GHR dimer [44]. LIF bound with low affinity to the (GR-FNIII)gp130-transfected cells, implying that the exchange of the membrane-proximal FNIII modules of gp130 for those of the G-CSFR hindered the formation of the signalling high-affinity LIF–LIFR–gp130 complex (Figure 2 and Table 1). Our data using cellular gp130 suggest that the membrane-proximal FNIII region of gp130 forms part of the heterodimerization interface with the LIFR (Figure 6).

Concluding remarks

Using gp130/G-CSFR chimaeras we have investigated the importance in signal transduction of the extracellular three membrane-proximal FNIII modules of gp130 and the G-CSFR. Our results show that the membrane-proximal regions of these receptors are utilized differently by different ligands. Receptor-homodimerizing ligands such as G-CSF and the agonistic anti-gp130 mAb B-S12 are able to induce signalling in the presence of the 'inappropriate' membrane-proximal region. In contrast, signal transduction by IL-6, mediated by IL-6R-dependent gp130 homodimerization, and LIF, mediated by gp130 hetero-dimerization with the LIFR, is attenuated in the presence of the 'inappropriate' membrane-proximal region. Studies using a panel of neutralizing mAbs that recognize the membrane-proximal regions of gp130 and the G-CSFR suggest that these regions are required for the correct orientation or conformation for signalling, and that they may form part of the gp130–gp130 or G-CSFR–G-CSFR homodimerization interfaces.

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