Signal transducer gp130: biochemical characterization of the three membrane-proximal extracellular domains and evaluation of their oligomerization potential

Stefan PFLANZ, Thomas KERNEBECK, Bernd GIESE, Andreas HERRMANN, Michael PACHTA-NICK, Jürgen STAHL, Axel WOLLMER, Peter C. HEINRICH, Gerhard MÜLLER-NEWEN¹ and Joachim GRÖTZINGER

Department of Biochemistry, RWTH Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany

Glycoprotein 130 (gp130) is a type I transmembrane protein and serves as the common signal-transducing receptor subunit of the interleukin-6-type cytokines. Whereas the membrane-distal half of the gp130 extracellular part confers ligand binding and has been subject to intense investigation, the structural and functional features of its membrane-proximal half are poorly understood. On the basis of predictions of tertiary structure, the membrane-proximal part consists of three fibronectin-type-III-like domains D4, D5 and D6. Here we describe the bacterial expression of the polypeptides predicted to comprise each of these three domains. The recombinant proteins were refolded from solubilized inclusion bodies *in vitro*, purified to homogeneity and characterized

by means of size-exclusion chromatography and CD spectroscopy. For the first time the prediction of three individual membrane-proximal protein domains for gp130 has been verified experimentally. The three domains do not show intermediate-affinity or high-affinity interactions between each other. Mapping of a neutralizing gp130 monoclonal antibody against D4 suggested a particular functional role of this domain for gp130 activation, because above that an intrinsic tendency for low-affinity oligomerization was demonstrated for D4.

Key words: cytokines, dimerization, receptors, signal transduction.

INTRODUCTION

Intercellular communication mediated by cytokines has a crucial role in complex physiological processes such as inflammation and haemopoiesis as well as in the control of the acute-phase response and fertility. Signal transduction from cytokines to intracellular compartments is achieved by specific plasma-membrane-spanning cytokine receptors [1]. These receptors are often classified by their structural similarities. Class I cytokine receptors contain the characteristic cytokine-binding module (CBM) which comprises two fibronectin type III (FNIII)-like domains: the N-terminal domain contains a set of four conserved cysteine residues, whereas the C-terminal domain contains a sequence motif of the consensus WSXWS (single-letter amino acid codes). This type of cytokine receptor is activated by helical cytokines consisting of a bundle of four α -helices [2]. The cytoplasmic parts of cytokine receptors do not exhibit kinase activity; instead, they are constitutively associated with tyrosine kinases of the Jak (Janus kinase) family. Cytokine binding to the extracellular part of the receptors juxtaposes the intracellularly associated Jaks, leading to their transphosphorylation and activation. Subsequently, activated Jaks phosphorylate tyrosine residues located in the cytoplasmic tails of the receptors, which thereby become docking sites for transcription factors of the STAT ('signal transducer and activator of transcription') family. The recruited STATs are also phosphorylated at the receptors and translocate to the nucleus to drive the transcription of their respective target genes [3].

Unlike the receptors for growth hormone or erythropoietin, whose extracellular parts consist of only a single CBM, other members of the class I cytokine receptor family such as gp130, leukaemia inhibitory factor receptor (LIFR), leptin receptor,

granulocyte colony-stimulating factor receptor (G-CSFR), interleukin 12 receptor β 1 (IL-12R β 1) and IL-12R β 2 contain additional domains. On the basis of predictions of tertiary structure, gp130 contains an N-terminal Ig-like domain (D1) followed by the CBM (D2, D3) and three FNIII domains (D4, D5, D6) in its extracellular part [4]. FNIII domains consist of seven antiparallel β -strands in two opposite β -sheets, a three-stranded β -sheet (A, B, E) and a four-stranded β -sheet (C, D, F, G). These predictions were confirmed for domains D2 and D3 by X-ray crystallography [5] and for D3 by NMR spectroscopy [6].

gp130 is the common signal-transducing receptor subunit for the interleukin (IL)-6-type cytokines IL-6, IL-11, ciliary neurotrophic factor, oncostatin M (OSM), LIF, cardiotrophin 1 [7] and the recently discovered NNT-1/BSF-3/CLC [8,9]. Whereas the membrane-distal extracellular half of gp130 (D1–D3) was shown to be necessary and sufficient for binding of IL-6 and IL-11 [10–12], the architecture and function of its membrane-proximal half are not well understood. However, despite the fact that this part of the receptor lacks direct involvement in ligand binding, it seems none the less to be critical for agonist-induced gp130 activation [13,14]. To analyse the structural and functional features of this part of the receptor, the predicted three membrane-proximal domains were expressed individually in *Escherichia coli*. The recombinant proteins were refolded from solubilized inclusion bodies *in vitro*, purified and characterized by size-exclusion chromatography and CD spectroscopy. We were able to prove the prediction of three independent β -sheet protein domains constituting the membrane-proximal half of gp130. These three domains do not show intermediate-affinity or high-affinity interactions between each other, whereas D4 exhibits a potential for low-affinity oligomerization. Mapping of an inhibitory gp130 monoclonal antibody (mAb) against D4

Abbreviations used: CBM, cytokine-binding module; FNIII, fibronectin type III; G-CSFR, granulocyte colony-stimulating factor receptor; IL, interleukin; LIFR, leukaemia inhibitory factor receptor; mAb, monoclonal antibody; OSM, oncostatin M; PDGFR, platelet-derived growth factor receptor; TCCR, T-cell cytokine receptor.

¹ To whom correspondence should be addressed (e-mail mueller-newen@rwth-aachen.de).

suggests a functional role for this domain in the activation of gp130.

EXPERIMENTAL

Enzymes, antibodies, chemicals and cell culture media

Enzymes were purchased from Roche/Boehringer Mannheim (Mannheim, Germany), and Protein A-Sepharose was obtained from Pharmacia (Freiburg, Germany). Chemical cross-linker bis(sulphosuccinimidyl) suberate (BS³) was purchased from Pierce (Rockford, IL, U.S.A.). Cell culture media and antibiotics were obtained from Life Technologies (Eggenstein, Germany), fetal calf serum was provided by Seromed (Berlin, Germany). The gp130 mAbs B-S12-G7 and B-P4 were a gift from Diaclone (Besançon, France). Other antibodies were purchased from Dako (Hamburg, Germany). The frequently used PBS contained 200 mM NaCl, 2.5 mM KCl, 8 mM Na₂HPO₄ and 1.5 mM KH₂PO₄. Supernatant of baculovirus-infected insect cells expressing soluble gp130 was prepared as described previously [15].

Construction of expression vectors for D4, D5 and D6

Parts of gp130-cDNA were amplified by PCR with gp130-pSVL vector (supplied by Dr T. Taga and Dr T. Kishimoto, Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan) as a template. *Nco*I and *Bam*HI sites were introduced into the 5' and 3' primers respectively to permit the convenient cloning of the amplified DNA fragments in the *Nco*I and *Bam*HI sites of the prokaryotic expression vector pET3d (Novagen). The primers used were as follows. D4: sense, 5'-ggg atc acc atg gaa gat aga cca tct aaa-3'; anti-sense, 5'-agg gtg agg atc cta aaa gtc aca ggc agg-3'; D5: sense, 5'-atc cct gcc atg gac ttt caa gct act cac-3'; anti-sense, 5'-aac agt agg atc ctc gga agg tgg tgc ttg-3'; D6: sense, 5'-cca cct tcc atg gga cct act gtt egg aca-3'; anti-sense, 5'-gac tat ggg atc cta ttc tcc ttg agc aaa-3'; restriction sites are underlined. PCRs were performed in a total volume of 100 µl of PCR buffer [20 mM Tris/HCl (pH 8.3)/1.5 mM MgCl₂/25 mM KCl/0.01% gelatin, containing each dNTP at 125 µM] with 250 ng of template DNA, 4 units of *Taq* DNA polymerase (AGS, Heidelberg, Germany) and 20 pmol of the respective sense and anti-sense primer pairs. PCRs were performed with 30 cycles of denaturation (1 min, 93 °C), primer annealing (2 min, 50 °C) and elongation (2 min, 72 °C). The amplified DNA was purified by agarose-gel electrophoresis, subsequently cut with *Nco*I and *Bam*HI (Boehringer, Mannheim) and ligated into the *Nco*I/*Bam*HI-digested pET3d vector. Plasmid DNA was prepared from transformed XL1-Blue bacteria (Stratagene) and the sequence of the inserts was verified by automated DNA sequencing (Prism Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit from PerkinElmer; Applied Biosystems 310-Sequencer).

Expression of recombinant proteins in *E. coli*

BL21(DE3)pLysS cells were transformed with the expression vectors [16] encoding D4, D5 and D6. Protein expression of D4 and D6 was performed as described for D3 [17]; D5 was expressed in minimal medium as described for D3 [6]. Cells were harvested by centrifugation (4000 g, 10 min) and resuspended in PBS. Lysis of bacteria was achieved by a single freeze-thaw step followed by four cycles of sonication (2 min) and centrifugation, resulting in a preparation of pre-purified inclusion bodies. Supernatants and precipitates were analysed by SDS/PAGE. Inclusion bodies were solubilized in 6 M guanidinium chloride (pH 4.0) to a final concentration of approx. 1 mg/ml; 20 mM dithiothreitol was

added for D4 and 100 mM dithiothreitol for D5. The solubilized inclusion bodies were incubated for 1 h at 37 °C and if necessary stored at -20 °C.

Purification and refolding procedures

D4

A Superdex 75 (16/60) column (Pharmacia) was equilibrated with refolding buffer [20 mM sodium acetate (pH 4.0)/200 mM NaCl/0.5 mM EDTA], loaded with 0.5 ml of solubilized inclusion bodies and run at a constant flow rate of 1 ml/min. Fractions (3 ml) were collected and analysed by SDS/PAGE. After dialysis against PBS and the concentration of D4-containing fractions with a Centricon-3 device (Amicon), rechromatography was performed in PBS under identical flow conditions. With this method 1 mg of purified protein was obtained from 0.3 litre of bacterial culture.

D5

Solubilized inclusion bodies (10 µl) were refolded by a rapid 1:100 dilution with refolding buffer [20 mM sodium phosphate (pH 6.0)/200 mM NaCl/0.5 mM EDTA]; the refolded protein was subsequently dialysed for 72 h against PBS containing 2 mM GSH and 0.2 mM GSSG. Eventually, pure D5 was isolated by applying size-exclusion chromatography on a Superdex 75 (16/60) column equilibrated with PBS/0.5 mM EDTA. With this method 1 mg of purified protein was obtained from 0.4 litre of bacterial culture in minimal medium.

D6

Similarly to D5, solubilized inclusion bodies (10 µl) were refolded by a rapid 1:100 dilution with refolding buffer [20 mM sodium phosphate (pH 7.5)/200 mM NaCl/1 mM EDTA]; the refolded protein was subsequently dialysed for 72 h against PBS. Subsequently, pure D6 was prepared by size-exclusion chromatography on a Superdex 75 (16/60) column equilibrated with PBS/0.5 mM EDTA. With this method 1 mg of purified protein was obtained from 0.1 litre of bacterial culture.

All of these procedures were performed at 4 °C.

N-terminal sequencing

A 200 pmol sample of each of the three purified proteins was used for determination of the N-terminal amino-acid sequence with a Knauer 910 protein sequencer (Knauer, Berlin, Germany). The phenylthiohydantoin derivatives were identified on-line by reverse-phase HPLC.

Size-exclusion chromatography

Size-exclusion chromatography was performed with an equilibrated Superdex 75 (16/60) column at a constant flow rate of 1 ml/min. The column was calibrated with a mixture of four proteins of known molecular mass: BSA (69 kDa), ovalbumin (46 kDa), myoglobin (17 kDa) and aprotinin (6 kDa).

CD spectroscopy

CD measurements were performed on an AVIV (Lakewood, NJ, U.S.A.) 62DS CD spectrometer, equipped with a temperature control unit calibrated with a 0.1% aqueous solution of D-10-camphorsulphonic acid as described by Chen and Yang [18]. The spectral bandwidth was 1.5 nm. The time constant ranged between 1 and 4 s and the cell path length between 0.1 and 10 mm.

Calculation of protein concentrations

Protein concentrations were calculated from absorption spectra in the range 240–320 nm by the method of Waxman [19].

Chemical cross-linking

Refolded D4, D5 and D6 (each 50 mg/l) in PBS/0.05 % (v/v) Triton X-100 were incubated for 60 min with a 500-fold molar excess of chemical cross-linker BS³ at 4 °C. Cross-linking was stopped by the addition of a concentrated Tris solution.

Construction of eukaryotic expression vectors

Construction of the pSVL vectors encoding gp130 and gp130ΔD4 was described earlier [13,20].

Cell culture and transient transfection of COS-7 cells

Simian monkey kidney cells (COS-7) were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) fetal calf serum, 100 mg/l streptomycin and 60 mg/l penicillin. The transient transfection of COS-7 cells was performed with the DEAE-dextran method. Cells were cultivated for 24 h after transfection; receptor expression was then monitored by FACS as described [13].

RESULTS

Expression of D4, D5 and D6

E. coli cells were transformed with expression vectors encoding the predicted gp130 domains D4, D5 and D6 (see Table 1 and Figure 7). After protein expression, bacteria were collected and lysates were prepared. After sonication of bacterial lysates, soluble and insoluble fractions were separated by centrifugation and analysed by SDS/PAGE. All three proteins were found almost exclusively as insoluble inclusion bodies (Figure 1, lanes 2, 5 and 8). The weak protein bands visible in the lanes of the respective soluble fractions (Figure 1, lanes 1, 4 and 7) either reflect a small amount of initially expressed soluble forms of D4, D5 and D6 or might be due to incomplete separation. The

Figure 1 Analysis of proteins by SDS/PAGE

Soluble (lanes 1, 4, 7) and insoluble (lanes 2, 5, 8) fractions of bacterial lysates derived from the expression of D4, D5 and D6 were loaded on an SDS/15 % (w/v) polyacrylamide gel. After electrophoresis, protein bands were detected by staining with Coomassie Brilliant Blue. Lanes 3, 6 and 9 show the three proteins after performance of the respective purification protocols. The electrophoretic mobilities of marker proteins are indicated at the left.

Table 1 Properties of D4, D5 and D6

Amino acid residues that differ from the wild-type gp130 sequence as a result of cloning requirements are underlined.

Property	D4	D5	D6
Number of amino acids	100	102	97
Calculated molecular mass per molecule (kDa)	11.4	11.6	10.9
N-glycosylation sites	3	0	2
Cysteine residues	1	3	0
N-terminal sequence	<u>MEDRP</u> ...	<u>MDFQA</u> ...	<u>MGPTV</u> ...
C-terminal sequence	... <u>PACDF</u>	... <u>QAPPS</u>	... <u>FAQGE</u>

electrophoretic mobilities monitored for D4, D5 and D6 correspond well to the calculated molecular masses of the three proteins (Table 1).

Refolding and purification of recombinant proteins

For each of the three gp130 membrane-proximal domains an *in vitro* refolding protocol was established (for details see the Experimental section). Refolded proteins were purified by size-exclusion chromatography. Aliquots of purified D4, D5 and D6 were analysed by SDS/PAGE as shown in Figure 1 (lanes 3, 6 and 9). To prove the identity of the proteins, the amino acid sequences of their N-termini were determined. The sequences obtained (MEDRPS for D4, MDFQAT for D5, MGPTVR for D6; compare with Table 1) correspond to those expected from the cloned cDNA species.

Rechromatography of purified proteins

Samples of each of the three refolded and purified proteins were dialysed against PBS, incubated for 4 h at 4 °C and subsequently rechromatographed on a calibrated size-exclusion chromatography column equilibrated with PBS. Whereas D5 and D6 were eluted in a single sharp peak, indicative for a uniform population of pure protein, rechromatography of D4 showed a two-peak profile (Figure 2). The retention times monitored for D5 and D6 were 83 and 84 min respectively, matching monomeric globular proteins of a calculated molar mass of 10–12 kDa.

For D4 two peaks were observed: a smaller peak at a retention time of 72 min, representing approx. 10 % of the total protein loaded, and a larger peak that was eluted after 86 min, representing approx. 90 % of the protein loaded. These retention times would correspond to a small dimeric and a larger monomeric D4 population. Further investigation of D4 revealed that the relative intensity of the peak that eluted earlier increased compared with the later peak with increasing storage time after refolding. Moreover, rechromatography of fractions from the first peak retained the one-peak profile with a peak maximum after 72 min (results not shown). This indicates that there is no dynamic equilibrium between the two D4 populations. SDS/PAGE analysis under non-reducing conditions revealed that the peak that was eluted earlier did not represent impurities but contained a disulphide-bridged D4 dimer (results not shown).

With regard to secondary structure predictions the cysteine residue in the C-terminal extension of D4 is not located in a β -strand but is part of the interdomain linker (compare with Table 1). It therefore lacks shielding amino acid residues in the isolated D4 and owing to its flexibility is accessible to other cysteine residues for oxidative coupling. For all experiments described below, monomeric D4 was used immediately after preparation by size-exclusion chromatography.

Figure 2 Size-exclusion chromatography of D4, D5 and D6

After performance of the respective refolding and purification protocols, the three proteins were dialysed against PBS and run on an equilibrated size-exclusion column. The retention times of marker proteins are indicated as arrows along the top of each panel together with their molecular masses (in kDa).

Figure 3 Far-UV CD spectra of D4, D5 and D6

CD measurements were performed with an AVIV 62DS CD spectrometer, equipped with a temperature control unit. Spectra from D5 and D6 were recorded at 20 °C; because D4 is temperature sensitive, its spectrum was recorded at 4 °C. The spectral bandwidth was 1.5 nm. The time constant ranged between 1 and 4 s and the cell path length between 0.1 and 10 mm. All spectra are indicative of a protein in a folded state consisting mainly of β -sheets.

CD spectroscopy of D4, D5 and D6

The three refolded and purified proteins were analysed by CD spectroscopy to obtain information on secondary structure elements (Figure 3). Spectra from D5 and D6 were recorded in the far UV at 20 °C. Because D4 was extraordinarily temperature sensitive (it denatures irreversibly above 10 °C), the far-UV spectrum was recorded at 4 °C. Calculations of the secondary structure composition [21] were performed on the basis of the far-UV CD spectra obtained. All three proteins were in fact independent folding units containing β -sheets as predominant secondary structure elements (Table 2).

Investigation of potential interactions between domains

In the context of a gp130 dimer it is conceivable that protein–protein interactions occur not only between ligand and receptor but also between different parts of the two receptor chains. Individual domains of a multi-domain receptor deliver prom-

Table 2 Secondary structure elements for D4, D5 and D6 calculated from the CD spectra in Figure 3 by the method of Sreerama et al. [21]

Domain	Proportion (%)			
	α -helix	β -strand	Turn	Others
D4	9	45	26	20
D5	14	39	26	21
D6	0	52	24	24

ising tools to study potential inter-receptor interactions that are restricted to certain regions. Thus three dual combinations (D4/D5, D4/D6 and D5/D6) and the triple combination D4/D5/D6 were incubated for 4 h in PBS at 4 °C, each domain at a concentration of 50 mg/l (approx. 5 μ M). Subsequently, the domain mixtures were loaded on a calibrated size-exclusion column to investigate the possible formation of protein complexes.

The elution profiles observed (Figure 4) corresponded to a direct superposition of the profiles for the respective individual domains rather than for larger protein complexes (compare with Figure 2). Apart from the small peak representing the population of dimeric D4 (see above), no peaks corresponding to complexes comprising two or more domains were detectable in this assay. A second approach with surface plasmon resonance did not reveal heterologous interactions between D4, D5 and D6 either (results not shown). Again, the proteins were applied at up to 5 μ M for these experiments. In general, plasmon resonance studies are adequate for detecting dissociation constants in the range of the applied protein concentrations. These results clearly indicate the lack of intermediate-affinity (in the nanomolar and lower micromolar ranges) or higher-affinity interactions between the domains constituting the membrane-proximal extracellular half of gp130.

Chemical cross-linking of individual domains

Chemical cross-linking is a widely used method for obtaining experimental access to low-affinity interactions. Once interacting proteins have been covalently linked by the bivalent chemical reagent, they are trapped and thereby withdrawn from the equilibrium. Thus chemical cross-linking studies on D4, D5 and

Figure 4 Size-exclusion chromatography of domain mixtures D4/D5, D4/D6, D5/D6 and D4/D5/D6

The four combinations of domains, each at 50 mg/l in PBS, were each incubated for 4 h at 4 °C before application to an equilibrated size-exclusion column. The retention times of marker proteins are indicated as arrows along the top of each panel together with their molecular masses (in kDa).

D6 were performed in order to detect potential homologous domain interactions.

The three purified gp130 domains, each adjusted to a concentration of 50 mg/l (approx. 5 μ M) in PBS/0.05 % (v/v) Triton X-100, were incubated separately in the absence or presence of the cross-linking reagent BS³ at 4 °C and analysed by SDS/PAGE. A ladder of oligomeric complexes of increasing masses was clearly visible after the incubation of D4 with the cross-linker (Figure 5). The molecular masses observed for these complexes corresponded to monomeric, dimeric, trimeric, tetrameric and pentameric D4 and even higher-order oligomers. D5 and D6 did not show comparable oligomers.

It is noteworthy that the oligomerization obviously did not occur as a consequence of disulphide bridging between individual domains, because D4 contains only a single cysteine residue (see above) and thus would be capable of forming disulphide-bridged dimers but no higher-order oligomers. Moreover, D5, which certainly would have the potential for disulphide-bridged oligomerization by means of its three cysteine residues, did not display oligomeric forms after cross-linking under identical experimental conditions. Thus the observed potential for non-covalent oligomerization is apparently specific for D4. However, the possibility cannot be excluded that owing to protein expression in *E. coli* the observed oligomerization of D4 might have been facilitated by the absence of N-linked glycosylation on three asparagine residues (Table 1). However, for D6 the absence of N-glycosylation on two sites did not lead to oligomerization under identical experimental conditions, suggesting specificity in the observed D4–D4 contacts.

These findings imply that we would not be able to extend this experimental approach to investigating heterologous domain

Figure 5 Investigation of homo-oligomerization of D4, D5 and D6 by applying chemical cross-linking

The three domains, each adjusted to a concentration of 50 mg/l (approx. 5 μ M) in PBS/0.05 % (v/v) Triton, were incubated in the absence (–) or presence (+) of a 500-fold molar excess of the chemical cross-linker BS³ at 4 °C for 60 min. The reaction was stopped by the addition of Tris buffer. From each sample total protein was precipitated by trichloroacetic acid and separated by SDS/PAGE [15 % (w/v) gel]. Protein was detected by staining with Coomassie Brilliant Blue. The electrophoretic mobilities of marker proteins are indicated at the left.

interactions (as done previously by size-exclusion chromatography): because of the very similar molecular masses of D4, D5 and D6 we would not be able to discern whether a retarded protein complex on SDS/PAGE contained homologous or

Figure 6 Epitope mapping of B-P4 by Western blot and FACS analysis

(A) Equal amounts of purified D4, D5 and D6 were run on two SDS/15% polyacrylamide gels. The first was immunoblotted with the gp130 mAb B-P4 for detection (upper panel). Soluble gp130 from supernatants of baculovirus-infected insect cells served as a control (sgp130). The second gel was stained with Coomassie Brilliant Blue as a control for equal loading of D4, D5 and D6 (lower panel). The electrophoretic mobilities of marker proteins are indicated at the left of each panel. (B) COS-7 cells were mock-transfected (top row), transfected with gp130 (middle row) or transfected with the deletion mutant gp130 Δ D4 (bottom row). At 24 h after transfection, the cell-surface expression of receptors was monitored by applying the gp130 mAbs B-P4 (left column) and B-S12-G7 (right column) as primary antibodies. An anti-mouse phycoerythrin-conjugated Fab₂ was used for fluorescence staining. Grey peaks represent background fluorescence caused exclusively by the phycoerythrin-conjugated Fab₂; open peaks represent cellular fluorescence monitored after incubation with the respective mAb and the phycoerythrin-conjugated Fab₂. The weak signal elicited by mock-transfected cells was due to endogenously expressed gp130.

heterologous domain oligomers as soon as D4 was present in the protein mixtures.

Epitope mapping of the gp130 mAb B-P4

The monoclonal gp130 antibody B-P4 has been shown to inhibit gp130 activation induced by IL-11 [22] and IL-6 [23]. B-P4 has previously been mapped to the membrane-proximal half of the extracellular part of gp130 [13]. This implies that B-P4 should not be capable of interfering directly with ligand binding because the membrane-distal half of gp130 is sufficient for binding IL-6 and IL-11 [10,11]. Moreover, B-P4 precipitates IL-6/sIL-6R/sgp130 ternary complexes [13], confirming that this antibody does not disturb ligand binding. Because D4, D5 and D6 together represent the entire membrane-proximal half of the gp130 extracellular region, we attempted to map B-P4 to one of these three gp130 domains. Purified D4, D5 and D6 from *E. coli*, as well as soluble gp130 (sgp130) from supernatants of baculovirus-infected insect cells, were subjected to SDS/PAGE, electroblotted and immunostained with B-P4. We found that the mAb B-P4 recognized both sgp130 and D4 and showed no cross-reactivity with D5 or D6 (Figure 6A).

Because D4 was recognized under denaturing conditions by Western blotting, we verified the domain mapping with native proteins. COS-7 cells were transiently transfected with a control plasmid (mock), gp130 or a D4-deletion construct of gp130 (gp130 Δ D4). The surface expression of the respective receptor constructs was monitored by FACS with the gp130 mAbs B-P4 and B-S12-G7 (Figure 6B). gp130 Δ D4 was not recognized by B-P4, whereas B-S12-G7 bound to overexpressed gp130 Δ D4 on the cell surface. Overexpressed wild-type gp130 was recognized by both mAbs. These results underline the fact that the mAb B-P4 recognizes an epitope in D4 under both denaturing and native conditions. Therefore B-P4 seems to bind to a linear rather than a conformational epitope.

DISCUSSION

gp130, the common signal transducing receptor for the IL-6-type cytokines (IL-6, IL-11, LIF, OSM, ciliary neurotrophic factor, cardiotrophin 1 and NNT-1/BSF-3/CLC), belongs to the class I family of cytokine receptors. Whereas the extracellular part of other members of the class I cytokine receptor family such as growth hormone receptor ('GHR') or erythropoietin receptor ('EpoR') consist of only a single CBM, the architecture of gp130 is more complex [1]. From predictions of tertiary structure, the extracellular portion of gp130 contains, besides the CBM (D2, D3), an N-terminal Ig-like domain (D1) and three additional C-terminal FNIII domains (D4, D5, D6) [4]. A similar architecture with regard to the three membrane-proximal extracellular FNIII domains was predicted for the related receptors LIFR, OSMR β , G-CSFR, IL-12R β 1, IL-12R β 2 and WSX-1/T-cell cytokine receptor (TCCR) (Figure 7). The N-terminal half of the extracellular part of gp130, which comprises the domains D1–D3, has been studied in great detail, establishing its important role in ligand binding [24]. The structural and functional features of the membrane-proximal extracellular half are less well understood. However, it has recently been shown that each of the membrane-proximal domains, although not directly involved in ligand-binding, is required for the activation of gp130 by IL-6 and IL-11 [13].

In the present study all three predicted domains constituting the membrane-proximal extracellular half of gp130 were expressed individually in *E. coli*. They were refolded from inclusion bodies *in vitro* and purified to homogeneity to study the

Figure 7 Comparison of predicted cytokine receptor architectures

Schematic representation of the related haemopoietic cytokine receptors WSX-1/TCCR, IL-12R β 1, IL-12R β 2, G-CSFR, gp130, OSMR β and LIFR, all based on predictions of tertiary structure. Receptor domains predicted to have an FNIII-like fold are shown as hexagons; those predicted to have an Ig-like fold as black circles. Horizontal bars within the hexagons represent the characteristic features of the CBMs: conserved cysteine residues (thin bars) or the WSXWS sequence (fat bars). gp130 is marked by an arrow.

structural assembly and functional features of this part of gp130. For the first time the prediction of three individual membrane-proximal domains of gp130 has been experimentally verified by size-exclusion chromatography (Figure 2) and CD spectroscopy (Figure 3). Each of the three proteins represents an independent folding unit and all consist of β -strands as the predominant secondary structure element. Our results also strengthen the reliability of predictions of tertiary structure made for other haemopoietic cytokine receptors such as LIFR, OSMR β , G-CSFR, IL-12R β 1, IL-12R β 2 and WSX-1/TCCR (Figure 7), all of which are closely related to gp130.

Studies on the potential heterologous interactions between D4, D5 and D6 by size-exclusion chromatography (Figure 4) revealed that the three domains do not show intermediate-affinity or higher-affinity interactions between one another. With respect to the functionally required inducibility of gp130 activation by cytokines, this result makes sense: interactions occurring in the non-ligand-binding part of a receptor necessarily have to be weak to be overcome by ligand-enforced interactions. For D4, but not for D5 or D6, a potential for specific low-affinity homologous oligomerization was demonstrated (Figure 5). D4–D4 interactions led to the assembly of at least four different oligomeric species (dimers, trimers, tetramers and pentamers) and most probably even higher-order oligomers. This observation implies the existence of at least two distinct inter-domain contact epitopes on the surface of D4. The assumption of only one contact epitope could explain the formation of dimers but not of higher-order oligomers.

Although evidence for a functional role of D4 in gp130 activation has been presented [13], it is not yet clear whether a

D4-driven oligomerization is required for the function of the entire receptor. Considering full-length gp130 on the cell surface, different models that might account for the importance of D4–D4 contacts are imaginable: (1) homologous D4 interactions support the coupling of ligand-induced receptor dimerization to signal transduction by adjusting more C-terminally located parts of gp130 to a certain conformation required for signalling; (2) a D4-driven gp130 oligomerization conformationally stabilizes the receptor on the cell surface and a disturbance of this stabilization leads to a strong decrease in gp130 sensitivity towards its ligands; or (3) D4 pre-oligomerizes gp130 in an inactive conformation but at the same time maintains the three N-terminal receptor domains in such a manner that these can easily bind the ligand. Conformational changes induced by the ligand are able to overcome the inactive preassociated state and switch on signal transduction.

Of course, various combinations of these three possibilities can also be envisaged. Experimental evidence supporting the first model has been presented for platelet-derived growth factor receptor (PDGFR). Although PDGFR is a tyrosine kinase receptor and is therefore of limited similarity to the gp130 signal transduction mechanisms, its extracellular part was also predicted to consist of multiple β -sheet domains. Whereas ligand binding was shown to be restricted to the D2 and D3 domains of the PDGFR, efficient receptor dimerization was demonstrated to be strongly contingent on D4–PDGFR [25,26]. A mAb directed against D4–PDGFR aborted signal transduction but did not affect ligand binding [25]. Thus as well as the receptor epitopes accommodating the ligand, certain receptors additionally require interfaces for inter-receptor contacts that guarantee the efficient

coupling of ligand binding to signal transduction. In the present study the epitope of an mAb (B-P4) neutralizing IL-6 and IL-11 that does not interfere with ligand binding [13] was mapped to a single gp130 domain. Using two different methods we showed that B-P4 recognizes a gp130 epitope located in D4 (Figure 6). Thus a very similar inhibitory mechanism is imaginable for the cytokine-induced activation of gp130, with B-P4 disturbing essential D4–D4 contacts on binding a ligand.

The second possibility might be supported by the observation of a lowered cell-surface expression of gp130ΔD4 in comparison with gp130 (Figure 6B). A suboptimal conformational stability of a receptor would automatically result in an increased intracellular degradation on expression in mammalian cells and at the same time would negatively affect the sensitivity of the receptor for interaction with its ligands on the cell surface.

The third model mentioned tempts us to speculate on the mechanism of transition between free and ligand-bound gp130. In principle, a preformed receptor oligomer would have to meet two important criteria. On the one hand, the interaction must be of low affinity to be easily overcome by the ligand-enforced interaction permitting receptor activation. On the other hand, it must prevent random activation in the absence of ligand. From that point of view, D4 could possibly be envisaged as a modular switch that actively keeps the receptor in an off-state in the absence of ligand and at the same time provides an environment of preformed receptor dimers to facilitate efficient ligand binding and subsequent signal transduction. Experimental evidence for this type of mechanism had recently been described for the tumour necrosis factor receptor: a receptor domain that is not directly involved in ligand binding provides contacts for receptor self-association and thereby leads to ligand-independent receptor oligomerization [27]. Deletion of D4 in the context of a soluble gp130 (sgp130ΔD4) was shown not to affect IL-6/sIL-6R binding. However, when gp130ΔD4 was expressed on the cell-surface, impaired binding of IL-6/sIL-6R as well as abrogation of signal transduction was observed in comparison with wild-type gp130 [13]. Hence D4 might be important for a certain adjustment of the receptor on the cell surface before ligand binding. Substantial refinements of biochemical and biophysical methods will be necessary for a complete understanding of the functionality of gp130 and its related receptors.

We thank Dr G. Buse for N-terminal sequencing analysis, and Dr J. Wijdenes (Diacclone, Besançon, France) for the gift of gp130 mAbs B-P4 and B-S12-G7. This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 542) and by the Fonds der Chemischen Industrie (Frankfurt, Germany).

REFERENCES

- Wells, J. A. and de Vos, A. M. (1996) Hematopoietic receptor complexes. *Annu. Rev. Biochem.* **65**, 609–634.
- Bazan, J. F. (1990) Structural design and molecular evolution of a cytokine receptor superfamily. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6934–6938.
- Ihle, J. N. (1995) Cytokine receptor signaling. *Nature (London)* **377**, 591–594.
- Hibi, M., Murakami, M., Saito, M., Hirano, T., Taga, T. and Kishimoto, T. (1990) Molecular cloning and expression of an IL-6 signal transducer, gp130. *Cell* **63**, 1149–1157.
- Bravo, J., Staunton, D., Heath, J. K. and Jones, E. Y. (1998) Crystal structure of a cytokine-binding region of gp130. *EMBO J.* **17**, 1665–1674.
- Kernebeck, T., Pflanz, S., Müller-Newen, G., Kurapkat, G., Scheek, R. M., Dijkstra, K., Heinrich, P. C., Wollmer, A., Grzesiek, S. and Grötzinger, J. (1999) The signal transducer gp130: solution structure of the carboxy-terminal domain of the cytokine receptor homology region. *Protein Sci.* **8**, 1–8.
- Heinrich, P. C., Behrmann, I., Müller-Newen, G., Schaper, F. and Graeve, L. (1998) IL-6-type cytokine signalling through the gp130/JAK/STAT pathway. *Biochem. J.* **334**, 297–314.
- Senaldi, G., Varnum, B. C., Sarmiento, U., Starnes, C., Lile, J., Scully, S., Guo, J., Elliott, G., McNinch, J., Shaklee, C. L. et al. (1999) Novel neurotrophin-1/B cell-stimulating factor-3: a cytokine of the IL-6 family. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 11458–11463.
- Elson, G. C. A., Lelièvre, E., Guillet, C., Chevalier, S., Plun-Favreau, H., Froger, J., Suard, I., Benoit de Coignac, A., Delneste, Y., Bonnefoy, J.-F. et al. (2000) CLF associates with CLC to form a functional heteromeric ligand for the CNTF receptor complex. *Nat. Neurosci.* **3**, 867–872.
- Horsten, U., Schmitz-Van de Leur, H., Müllberg, J., Heinrich, P. C. and Rose-John, S. (1995) The membrane distal half of gp130 is responsible for the formation of a ternary complex with IL-6 and the IL-6 receptor. *FEBS Lett.* **360**, 43–46.
- Dahmen, H., Horsten, U., Küster, A., Jacques, Y., Minvielle, S., Kerr, I. M., Ciliberto, G., Paonessa, G., Heinrich, P. C. and Müller-Newen, G. (1998) Activation of the signal transducer gp130 by interleukin-11 and interleukin-6 is mediated by similar molecular interactions. *Biochem. J.* **331**, 695–702.
- Kurth, I., Horsten, U., Pflanz, S., Dahmen, H., Küster, A., Grötzinger, J., Heinrich, P. C. and Müller-Newen, G. (1999) Activation of the signal transducer gp130 by both interleukin-6 and interleukin-11 requires two distinct binding epitopes. *J. Immunol.* **162**, 1480–1487.
- Kurth, I., Horsten, U., Pflanz, S., Timmermann, A., Küster, A., Dahmen, H., Tacke, I., Heinrich, P. C. and Müller-Newen, G. (2000) Importance of the membrane-proximal extracellular domains for activation of the signal transducer gp130. *J. Immunol.* **164**, 273–282.
- Hammacher, A., Wijdenes, J., Hilton, D. J., Nicola, N. A., Simpson, R. J. and Layton, J. E. (2000) Ligand-specific utilization of the extracellular membrane-proximal region of the gp130-related signalling receptors. *Biochem. J.* **345**, 25–32.
- Müller-Newen, G., Küster, A., Hemmann, U., Keul, R., Horsten, U., Martens, A., Graeve, L., Wijdenes, J. and Heinrich, P. C. (1998) Soluble interleukin-6 receptor potentiates the antagonistic activity of soluble gp130 on interleukin-6 responses. *J. Immunol.* **161**, 6347–6355.
- Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendorff, J. W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**, 60–89.
- Müller-Newen, G., Pflanz, S., Hassiepen, U., Stahl, J., Wollmer, A., Heinrich, P. C. and Grötzinger, J. (1997) The signal transducer gp130. Bacterial expression, refolding and properties of the carboxy-terminal domain of the cytokine-binding module. *Eur. J. Biochem.* **247**, 425–431.
- Chen, G. C. and Yang, J. T. (1977) Two-point calibration of circular dichrometer with d-10-camphorsulfonic acid. *Anal. Lett.* **10**, 1195–1207.
- Waxman, E., Rusinova, E., Hasselbacher, C. A., Schwartz, G. P., Laws, W. R. and Ross, J. B. (1993) Determination of the tryptophan:tyrosine ratio in proteins. *Anal. Biochem.* **210**, 425–428.
- Horsten, U., Müller-Newen, G., Gerhart, C., Wollmer, A., Wijdenes, J., Heinrich, P. C. and Grötzinger, J. (1997) Molecular modeling-guided mutagenesis of the extracellular part of gp130 leads to the identification of contact sites in the interleukin-6 (IL-6)/IL-6 receptor/gp130 complex. *J. Biol. Chem.* **272**, 23748–23757.
- Sreerama, N. and Woody, R. W. (1994) Protein secondary structure from circular dichroism spectroscopy. Combining variable selection principle and cluster analysis with neural network, ridge regression and self-consistent methods. *J. Mol. Biol.* **242**, 497–507.
- Wijdenes, J., Heinrich, P. C., Müller-Newen, G., Roche, C., Zong-Jiang, G., Clement, C. and Klein, B. (1995) Interleukin-6 signal transducer gp130 has specific binding sites for different cytokines as determined by antagonistic and agonistic anti-gp130 monoclonal antibodies. *Eur. J. Immunol.* **25**, 3474–3481.
- Lebeau, B., Montero-Julian, F. A., Wijdenes, J., Müller-Newen, G., Dahmen, H., Chérel, M., Heinrich, P. C., Brailly, H., Hallet, M.-M., Godard, A. et al. (1997) Reconstitution of two isoforms of the human interleukin-11 receptor and comparison of their functional properties. *FEBS Lett.* **407**, 141–147.
- Bravo, J. and Heath, J. K. (2000) Receptor recognition by gp130 cytokines. *EMBO J.* **19**, 2399–2411.
- Lokker, N. A., O'Hare, J. P., Barsoumian, A., Tomlinson, J. E., Ramakrishnan, V., Fretto, L. J. and Giese, N. A. (1997) Functional importance of platelet-derived growth factor (PDGF) receptor extracellular immunoglobulin-like domains. Identification of PDGF binding site and neutralizing monoclonal antibodies. *J. Biol. Chem.* **272**, 33037–33044.
- Omura, T., Heldin, C. H. and Ostman, A. (1997) Immunoglobulin-like domain 4-mediated receptor–receptor interactions contribute to platelet-derived growth factor-induced receptor dimerization. *J. Biol. Chem.* **272**, 12676–12682.
- Chan, F. K., Chun, H. J., Zheng, L., Siegel, R. M., Bui, K. L. and Lenardo, M. J. (2000) A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science* **288**, 2351–2354.