A single amino acid substitution (Trp$^{666} \rightarrow$ Ala) in the interbox1/2 region of the interleukin-6 signal transducer gp130 abrogates binding of JAK1, and dominantly impairs signal transduction

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INTRODUCTION

Interleukin (IL)-6 is a pleiotropic cytokine that plays a central role in host defence, e.g. it induces the acute-phase response and mediates proliferation and differentiation of B- and T-cells. Overexpression has been implicated in the pathology of a number of diseases, such as rheumatoid arthritis, Castleman’s disease and multiple myeloma [1,2].

IL-6 belongs to a family of cytokines including IL-11, leukaemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, cardiotrophin-1 and novel neurotrophin-1 receptors [1,3]. These IL-6-type cytokines have overlapping functions, which might be explained by the fact that they all share gp130 as part of their multicomponent signalling receptor complexes. Whereas IL-6 and IL-11 induce homodimerization of gp130 after binding to their respective ligand-specific receptors, the others induce the heterodimerization of gp130 with the leukaemia inhibitory factor- or oncostatin M-receptor [1,3].

Like other cytokine receptors, gp130 transduces signals via the JAK/STAT (Janus kinase/signal transducers and activators of transcription) pathway. JAKs, which are cytoplasmic tyrosine kinases of the Janus family, are preassociated with the receptor. Upon cytokine-induced receptor aggregation, the JAKs are thought to transphosphorylate, and thereby autoactivate themselves. The kinases subsequently phosphorylate tyrosine residues in the receptor, providing docking sites for SH2-domain-containing signalling proteins. These include transcription factors of the STAT family. Upon phosphorylation, STATs translocate to the nucleus, where they bind to specific DNA sequences in the promoter regions of their target genes. In addition, phosphatases (SHP-1, SHP-2) and adaptor proteins, such as Shc, are recruited to certain receptors, thereby creating a link to the Ras/Raf/mitogen-activated protein kinase pathway [4,5].

The family of JAKs comprises four mammalian homologues: JAK1, JAK2, JAK3 and Tyk2. Cytokines utilizing gp130 have been reported to activate JAK1, JAK2 and Tyk2. Among these, JAK1 is essential for signal transduction, as demonstrated for JAK1-deficient fibrosarcoma cells and for cells derived from JAK1 knock-out mice [6,7]. The N-terminal half (≈ 600 amino acids) of the JAKs is involved in receptor binding, whereas the C-terminal region contains the kinase and the pseudokinase domains [8].

The membrane-proximal part of gp130 contains sequences that are conserved among various cytokine receptors and are crucial for JAK activation: box1, a proline-rich motif of eight amino acids, and box2, a cluster of hydrophobic residues followed by positively charged amino acids. Point mutations of amino acid residues in box1 of gp130 led to impaired binding [9] and activation [9,10] of JAKs, and abolished the proliferative response of stable Ba/F3 pro-B-cell transfectants after cytokine stimulation [11]. Although deletion of box2 did not abrogate binding to an overexpressed JAK2 construct [9], a truncated receptor lacking box2 was unable to sustain a proliferative response in Ba/F3 cells [11]. Apart from box1 and box2, regions N-terminally located to box1, between box1 and box2, or C-terminally located to box2 have been implicated in JAK binding and activation in certain receptors [12–19].

In the present paper, we show that an aromatic residue, Trp$^{666}$, in the interbox1/2 region is crucial for JAK binding to gp130. Receptor constructs with a single point mutation at this position are no longer able to associate with JAK1. Moreover, using heterodimeric receptor chimaeras, we found that Trp$^{666}$ has to be present in both chains of a functional gp130 dimer to allow signalling to occur.

MATERIALS AND METHODS

Cell culture and transfection

Simian monkey kidney cells (COS-7 cells) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal-calf serum (FCS), 100 mg/l streptomycin and 60 mg/l penicillin. Cells were grown at 37 °C in a water-saturated atmosphere in air/CO$_2$ (19:1). COS-7 cells were transiently transfected using the DEAE/chloroquine transfection method, with modifications as described previously [20].

Abbreviations used: DMEM, Dulbecco’s modified Eagle’s medium; EMSA, electrophoretic mobility-shift assay; EPOR, erythropoietin receptor; FCS, fetal-calf serum; JAK, Janus kinase; IL, interleukin; sIL-6R, soluble IL-6 receptor; SIE, cis-inducible element of the c-fos promoter; STAT, signal transducer and activator of transcription; WT, wild-type.

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Ba/F3 cells were transfected by electroporation of 28 μg of either pSVLgp130 or the mutant constructs (see Figure 1), and 2 μg of pSV2neo using 3.5 × 10^6 cells in 0.8 ml of medium at 200 V and 70 ms. Selection with G418 (1 mg/ml) in IL-3-conditioned medium was initiated 24 h after transfection. Selected Ba/F3 clones were screened by flow cytometry for cell-surface expression of gp130. Recombinant IL-6 was prepared as described previously [21]. The specific activity was 2 × 10^6 B-cell stimulatory factor-2 units/mg protein. Soluble IL-6 receptor (sIL-6R) was prepared in insect cells, as described previously [22].

**Cell proliferation assay**

Ba/F3 cells (2 × 10^5) expressing the receptor mutants were cultured in DMEM containing the indicated concentrations of IL-6 and 1 μg/ml sIL-6Rα, or conditioned medium from X63Ag-653 BPV-IL-3 myeloma cells as a source of IL-3. After 72 h of incubation, viable and metabolically active cells were quantified using the colorimetric cell proliferation kit, XTT (Boehringer Mannheim, Mannheim, Germany), as described by the manufacturer.

**Construction of gp130 and IL-5Rα/β-gp130 mutants**

Standard cloning procedures were performed throughout the present study. The mutations were introduced by the technique of PCR. The primers used were 5'-ATTGTCCCTTGTGACTA-3' (sense) and 5'-CTGGGCAAATACCTAC-3' (antisense). The primers used for introducing the mutations were as follows: for the mutation Δbox1, 5'-AAAAAACACTCAAGGATGC-ATAATTGCCC-3' (sense) and 5'-ACTCTTTGAGTTTTTATAATTAGGCTCCG-3' (antisense); and for Trp^66^Ala, 5'-TTGCCAGGGGTCACTCCACCTACTCC-3' (sense) and 5'-TGAGTGACCCTGGGCAAATAGCCTTGGT-3' (antisense). The nucleotides that are underlined indicate the position where the mutation was introduced.

The restriction enzymes used for cloning the Trp^66^Ala PCR-amplified fragment into the pSVL-based expression vector for gp130 [23] were EcoRI and Asp718. For insertion of the PCR fragments into the expression vector for IL-5Rβ/gp130 [24], EcoRI–BstEII (Δbox1) and EcoRI–Asp718 (Trp^66^Ala) restriction sites were used. The corresponding IL-5Rx/gp130 chiamaeras were generated by exchange of the Xhol–EcoRI fragment encoding the extracellular domain of IL-5Rβ with the one encoding the extracellular part of IL-5Rx. The gp130AMP mutant was generated by exchanging the region encoding the ectodomain of the construct IL-5Rx/gp130ABB23 [25] with the region encoding the extracellular part of gp130 using Xhol and EcoRI restriction sites. The integrity of all constructs was verified by DNA sequencing.

**Flow cytometry**

Transiently transfected COS-7 cells or stably transfected Ba/F3-gp130 cells were resuspended in cold PBS supplemented with 5% (v/v) FCS and 0.1% (w/v) sodium azide (PBS/azide). Cells (5 × 10^6 to 1 × 10^7) in 100 ml of PBS/azide were incubated with 1 μg/ml of the corresponding monoclonal antibodies, anti-gp130 antibody BP8 (kindly provided by J. Wijdenes, Diaclone, Besançon, France), anti-IL-5Rβ antibody 1b-4 (kindly provided by J. Tavernier, University of Gent, Ghent, Belgium) or anti-IL-5Rβ antibody S16 (Santa Cruz, Heidelberg, Germany) for 30 min at 4 °C. Cells were then washed with cold PBS/azide. To detect the antibodies bound to the full-length gp130, the cells were subsequently incubated in the dark with a 1:50 dilution of an R-phycocerythrin-conjugated anti-mouse IgG Fab fragment (Dianova, Hamburg, Germany) for 30 min at 4 °C. Cells were again washed with cold PBS/azide, and then were resuspended in PBS/azide with 1% (v/v) formaldehyde. From each sample, 10^4 cells were analysed by flow cytometry using a FACScan (Beckton–Dickinson, Heidelberg, Germany) equipped with a 488-nm argon laser.

**Electrophoretic mobility-shift assays (EMSAs)**

Cells were stimulated with 20 ng IL-6/ml and 1 μg sIL-6R/ml for 15 min, or with 10 ng IL-5/5/ml (Cell Concepts, Umkirch, Germany) for 30 min. EMSAs were performed as described previously [26]. Protein concentrations were measured with the BioRad™ protein assay. A double-stranded mutated sis-inducible element (SIE)–oligonucleotide from the c-fos promoter (m67SIE: 5’-GATCCGGGAGGTATTCGAGAAATGC-TG-3’) was labelled by filling-in 5‘-protruding ends with the Klenow enzyme, using [α-32P]dATP (3000 Ci/ml). This probe binds STAT1 and STAT3 homo- and hetero-dimers, resulting in retarded bands of different mobilities. Nuclear extracts containing 5 μg of protein were incubated with approx. 10 fmol (10000 c.p.m.) of probe in gel-shift incubation buffer [10 mM Hepes, pH 7.8/1 mM EDTA/5 mM MgCl₂/10% (v/v) glycerol/5 mM dithiothreitol/0.7 mM PMSF/0.1 mg/ml poly(dI-C)/1 mg/ml BSA] for 10 min at room temperature. The protein–DNA complexes were separated on a 4.5% (w/v) polyacrylamide gel containing 7.5% (v/v) glycerol in 0.25 × TBE (where 1 × TBE is 45 mM Tris HCl, 1 mM EDTA and 10 mM NaCl) for 1200 g. A monoclonal antibody raised against human IL-5–Rβ (S16; Santa Cruz) was used for immunoprecipitation. After incubation overnight at 4 °C, the immunoprecipitates were collected by chromatography with protein A–Sepharose (1 h, 4 °C), washed three times with washing buffer (0.1% Brij 96/20 mM Tris/Cl, pH 7.5/150 mM NaCl/10 mM NaF/1 mM benzamidin, 5 μg/ml aprotinin, 3 μg/ml pepstatin, 5 μg/ml leupeptin and 1 mM EDTA). Lysates were cleared by centrifugation at 12000 g. A monoclonal antibody raised against human IL-5–Rγ (S16; Santa Cruz) was used for immunoprecipitation. After incubation overnight at 4 °C, the immunoprecipitates were collected by chromatography with protein A–Sepharose (1 h, 4 °C), washed three times with washing buffer (0.1% Brij 96/20 mM Tris/Cl, pH 7.5/150 mM NaCl/10 mM NaF/1 mM benzamidin, 5 μg/ml aprotinin, 3 μg/ml pepstatin, 5 μg/ml leupeptin/1 mM EDTA) and analysed further by SDS/PAGE. The proteins were transferred to a PVDF membrane (Amersham, Braunschweig, Germany), probed with the respective antibodies and detected for signals using the ECL® system (Amersham). Anti-IL-5Rβ (N20, Santa Cruz) and anti-JAK1 polyclonal antiserum (kindly provided by A. Ziemicki, University of Bern, Switzerland) were used for detection. The horseradish-peroxidase-conjugated secondary antibodies were purchased from Dako (Copenhagen, Denmark).

**RESULTS**

A single amino acid substitution, Trp^66^Ala, in the interbox1/2 region of gp130 leads to total ablation of the proliferative response of stably transfected Ba/F3 cells

The box1 region of gp130, comprising a number of conserved hydrophobic residues, has been shown to be involved in JAK activation and mediation of proliferative responses in Ba/F3 transfecants [10,11]. In the present work, we mutated a bulky

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Trp666Ala-mutated gp130 prevents interleukin-6 signalling

**Figure 1 Schematic representation of the receptors used in the present study**

The receptors consist of the extracellular region of gp130, IL-5Rα or IL-5Rβ, and the transmembrane and intracellular regions of gp130. Deletions and point mutations were introduced into the membrane-proximal part of gp130, as indicated.

Hydrophobic amino acid, Trp666, in the inter-box1/2 region of gp130 to alanine (Figure 1), since protein–protein interactions are often mediated by hydrophobic residues. As a negative control, we constructed gp130ΔMP with a deletion of the membrane-proximal amino acids 643–666, including box1. We generated stable Ba/F3 transfectants with a similar surface expression of gp130, or the mutants thereof (Figure 2A). The gp130 wild-type (WT) and mutant (ΔMP and Trp666Ala) Ba/F3 transfectants were cultured for 72 h with increasing amounts of IL-6 in the presence of the sIL-6R, which is known to act agonistically [27,28]. Subsequently, the metabolically active cells were quantified (Figure 2B). The cells expressing gp130WT proliferated at concentrations of IL-6 as low as 3.3 ng/ml, whereas the transfectants expressing the mutant receptors ΔMP and Trp666Ala did not proliferate even at the highest IL-6 concentrations tested. However, all Ba/F3 clones expressing gp130WT or mutant receptors proliferated IL-3-dependently, demonstrating that their cell-growth machinery was functional. Thus the presence of Trp666 in the cytoplasmic region of gp130 is crucial for the proliferative response of Ba/F3-gp130 transfectants upon stimulation with IL-6/sIL-6R.

**Mutation of Trp666 to alanine prevents the activation of STAT3 factors in stably transfected Ba/F3 cells**

After having demonstrated the inability of the mutant gp130 Trp666Ala to mediate proliferation of stably transfected Ba/F3 cells, we investigated its capability to activate STAT transcription factors upon cytokine treatment. STAT activation is known to be necessary for gp130-mediated proliferation of Ba/F3 transfectants [29,30]. Three independent cell clones (Trp666Ala/3, Trp666Ala/6 and Trp666Ala/7; Figure 3) were stimulated with 20 ng/ml IL-6 and 1 μg/ml sIL-6Rα for 30 min, nuclear extracts were prepared and analysed by EMSA with the radiolabelled SIE probe (Figure 3). In contrast with gp130WT, mutant gp130 Trp666Ala was not able to induce STAT3 activation upon stimulation with IL-6/sIL-6Rα.

**Figure 2. The mutations ΔMP and Trp666Ala abrogate the gp130-mediated proliferative response in Ba/F3 transfectants**

(A) Surface expression of gp130 on stable Ba/F3 clones. Parental cells and transfectants were incubated with the anti-gp130 monoclonal antibody BP-8, and subsequently with phycoerythrin-conjugated anti-mouse IgG(ab)2 (filled-in traces). The cells treated with secondary antibody alone are represented by dotted-line traces. (B) Proliferation of Ba/F3 transfectants. Cells were cultured for 4 days with increasing amounts of IL-6 in the presence of 1 μg/ml sIL-6Rα. Cell growth was assessed by an XTT test, and is represented as the percentage of the values obtained with IL-3 (set to 100%).

**Mutation of Trp666 to alanine impairs JAK1 binding as potently as deletion of the box1 region**

Since JAKs have been shown previously [4] to bind to the membrane-proximal region of cytokine receptors, the failure of the Trp666Ala mutant to induce STAT3 activation might reflect its inability to bind JAKs. Because of the low expression levels of gp130WT and mutant proteins in Ba/F3 cells, co-precipitation experiments did not yield satisfactory results. Therefore we...
Figure 3 A single point mutation in gp130 (Trp666Ala) abrogates STAT3 activation

Stable Ba/F3 transfectants expressing gp130 or the gp130Trp666Ala mutant (three independent clones: Trp666Ala/3, Trp666Ala/6 and Trp666Ala/7) were stimulated for 30 min with 20 ng IL-6/ml + 1 μg/ml sIL-6R. Nuclear extracts were prepared and analysed by EMSA using the SIE probe.

Figure 4 Gp130 Dbox1 and gp130Trp666Ala mutants do not associate with JAK1

Transiently transfected COS-7 cells expressing IL-5Rβ/gp130WT or mutants, as indicated, were lysed and subjected to immunoprecipitation using an anti-(IL-5Rβ) monoclonal antibody (S16), as described in the Materials and methods section.

switched to COS-7 cells, which can be efficiently transfected and yield high levels of heterologously expressed proteins. However, since COS-7 cells contain endogenous gp130, we transiently expressed chimaeric receptors consisting of the extracellular part of the IL-5Rβ chain and the transmembrane and intracellular parts of gp130WT, gp130Δbox1 or gp130 Trp666Ala respectively. The chimaeric receptors were immunoprecipitated with an IL-5Rβ-specific antibody. Immunoprecipitates were analysed further by SDS/PAGE and Western blotting. Co-precipitation of JAK1 was detected using a JAK1-specific antibody. Whereas the gp130WT construct bound JAK1 efficiently, association of the kinase with the Dbox1 and Trp666Ala mutants was greatly impaired (Figure 4). Therefore the inability of the Trp666Ala mutant to associate with JAK1 is likely to account for the lack of receptor function.

Both chains of a functional gp130 dimer have to associate with JAKs

The use of heterodimeric receptor chimaeras on the basis of IL-5Rα- and β-chains enabled us to induce the dimerization of the cytoplasmic parts of a gp130 mutant with a WT receptor construct. Thus we could investigate whether both chains of a gp130 dimer have to associate with JAKs to activate STATs.

COS-7 cells were transiently co-transfected with IL-5Rα and IL-5Rβ expression constructs, as depicted in Figure 5. WT and mutant constructs were expressed on the cell surface to a similar level, as determined by flow cytometry (results not shown). After stimulation with IL-5, nuclear extracts were analysed by EMSA. Homodimerization of two gp130WT cytoplasmic chains induced a strong STAT signal (Figure 5). Interestingly, IL-5-induced dimerization of one WT with one mutant chain that was unable to associate with JAK1 (Trp666Ala or Dbox1) did not elicit a STAT signal. This shows that even a single point mutation that abrogates JAK association in one chain of a gp130 dimer is detrimental to the signalling capacity of such a receptor complex.

DISCUSSION

This is the first report showing that exchange of a single amino acid residue (Trp666) in the cytoplasmic region of gp130 results in a receptor that is functionally inactive. Moreover, we have shown that a residue outside the conserved box1 and box2 regions of gp130 is important for coupling with JAKs. Finally, we provide evidence that the Trp666Ala mutation even prevents receptor function when dimerized with the corresponding gp130WT cytoplasmic region.

To date, it was known that point mutations of two proline residues in box1 of gp130 led to impaired binding [9] and activation [9,10] of JAKs, and abolished the proliferative response in stable Ba/F3 pro-B-cell transfectants after cytokine stimulation [11]. In addition, combined point mutations of an isoleucine, a tryptophan and a valine residue within box1, or of five positively charged amino acid residues in the region between box1 and box2, abolished the growth response in Ba/F3 transfectants, but the cause for this was not analysed further [11]. A truncated receptor lacking box2 was also unable to mediate proliferation in Ba/F3 cells [11], although deletion of box2 did not abrogate binding to an overexpressed JAK2 construct [9].
Our data provide evidence that the gp130 region between box1 and box2 is also important for the interaction of gp130 and JAK1. The interaction of JAKs with cytokine receptors seems to be complex, and with large N-terminal portions of the kinases implicated in the mechanism. Thus it is not surprising that receptor regions in addition to box1 are necessary for JAK1 association. Hydrophobic interactions between gp130 and JAK1 might well involve other residues in the membrane-proximal region, which is currently under investigation.

We cannot rule out, however, the possibility that Trp<sup>668</sup> plays a structural role (rather than being a direct binding partner for JAKs), so that its substitution by alanine would destroy the structural interface of the receptor necessary for JAK association. The three-dimensional structure of the cytoplasmic region of gp130 (or other members of the cytokine receptor family) still awaits elucidation.

The importance of tryptophan residues in the interbox1/2 regions has been studied for other receptors: replacement of Trp<sup>659</sup> with arginine for the human granulocyte colony stimulating factor receptor, or replacement of Trp<sup>659</sup> of the erythropoietin receptor (EPOR) with arginine resulted in non-functional proteins. Science, 264, 1415–1421


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