Structural requirements of the interleukin-6 signal transducer gp130 for its interaction with Janus kinase 1: the receptor is crucial for kinase activation

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We analysed the interaction of gp130, the common signal-transducing receptor chain of interleukin (IL)-6 type cytokines, with Jak1, the Janus family kinase which is crucial for signal transduction of this group of cytokines. With a truncated chimaeric IL-5R β -gp130 receptor expressed in COS-7 cells, we show that the membrane-proximal 69 amino acids are sufficient to mediate Jak1 binding and activation. Deletion of box2 drastically reduced binding of endogenous, but not of over-expressed, Jak1. Several point mutations in the membrane-proximal region of gp130 (W652A, P671/P672A, F676A, Y683F, where W, A, P, F and Y are tryptophan, alanine, proline, phenylalanine and tyrosine) did not affect Jak1 association. However, stimulation of chimaeric receptors with the mutations P671/P672A and F676A in the interbox1/box2 region resulted in a reduced activation of STAT (signal transducer and activator of transcription) transcription factors. Most importantly, signal-

ling by the receptor with the box1 mutation W652A was totally abrogated. Although this mutation did not affect Jak1 association, stimulation-dependent phosphorylation of Jak1 was prevented. The W652 mutation acts dominantly, since no signalling occurred even when only a single cytoplasmic chain of a gp130 dimer contained the mutation. Our data demonstrate that the mere proximity of Jaks in an activated receptor complex is not sufficient to mediate their activation. Rather, it seems that parts of the receptor, including the box1 region, are involved in positioning Jaks correctly so that ligand-induced receptor dimerization and reorientation can lead to their mutual activation and subsequently to downstream signalling events.

Key words: box1/box2 region, cytokine, Jak association, Jak/STAT, tyrosine kinase.

INTRODUCTION

Interleukin-6 (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 (LIF), oncostatin M (OSM), ciliary neurotrophic factor ('CNTF'), cardiotrophin-1 ('CT-1') and novel neurotrophin-1/B cell-stimulating factor-3 ('NNT-1/BSTF-3'). These IL-6 type cytokines have overlapping functions. They all share gp130 as part of their multi-component signalling receptor complexes. Whereas IL-6 and IL-11 induce homodimerization of gp130 after binding to their respective ligand-specific α -receptors, the others induce the heterodimerization of gp130 with the LIF (LIFR) or OSM (OSMR) receptor [1,3].

Like other cytokine receptors, gp130 transduces signals via the Jak/STAT (Janus kinase/signal transducer and activator of transcription) pathway. Cytoplasmic tyrosine kinases of the Jak family are pre-associated with the receptor. Of the four mammalian Jak homologues (Jak1, Jak2, Jak3 and Tyk2), cytokines utilizing gp130 have been reported to activate Jak1, Jak2 and Tyk2 [4]. Among these, Jak1 is essential for signal transduction, as demonstrated for Jak1-deficient fibrosarcoma cells and for cells derived from Jak1 knock-out mice [5,6]. Upon cytokine-induced receptor aggregation the Jaks become activated. The kinases subsequently phosphorylate tyrosine residues in the cytoplasmic domain of the receptor, thereby providing docking sites for other signalling proteins such as transcription factors of

the STAT family. Upon phosphorylation, STATs translocate into the nucleus, where they bind to specific DNA sequences in the promoter regions of their target genes [7,8].

Jaks consist of a C-terminal kinase domain preceded by a kinase-like domain. The N-terminal half of the Jaks mediates receptor association, which very likely involves a predicted FERM (four-point-one, ezrin, radixin, moesin) domain (amino acids 24–415 in Jak1) [9,10].

Jaks associate with the membrane-proximal region of cytokine receptors comprising the box1 region, a proline-rich motif of eight amino acids, and the box2 region, a cluster of hydrophobic amino acid residues often followed by charged amino acids [1,11]. In certain receptors, areas C-terminally located to box2 have been implicated in Jak binding and activation [12–14]. For gp130 it has been shown that point mutations of amino acid residues in box1 of gp130 led to an impaired binding [15] and thus to an impaired activation [15,16] of Jaks and abolished the proliferative response of stable Ba/F3 pro-B cell transfectants after cytokine stimulation [11]. A receptor truncation lacking box2 was also unable to sustain a proliferative response in Ba/F3 cells [11], although the deletion of box2 and a truncated receptor lacking box2 did not abrogate binding to overexpressed Jaks [15,17]. We recently demonstrated that the region between box1 and box2 also contains a residue [W666 (Trp⁶⁶⁶)] critical for Jak1 binding to gp130 [18].

In the present paper we further delineate the requirements of gp130 for interaction with Jak1. We show that regions C-terminal to box2 are not involved in Jak1 binding or activation and that box2 of gp130, in contrast with other reports [15,17], is essential for Jak association. Among several point mutations, the most striking effect was observed for mutation of the aromatic

Abbreviations used: EMSA, electrophoretic-mobility-shift assay; EPOR, erythropoietin receptor; Jak, Janus kinase; LIF, leukaemia inhibitory factor; OSM (R), oncostatin M (receptor); sIL-6R, soluble IL-6 receptor; STAT, signal transducer and activator of transcription; W652A etc., Trp⁶⁵² → Ala mutant etc.; C/EBP, CCAAT-enhancer-binding protein; ECL[®] (Amersham), enhanced chemiluminescence; FCS, fetal-calf serum; SIE, c-sis-inducible element.

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residue W652 in the box1 region of gp130. Although Jak1 association was unchanged on its replacement by alanine, cytokine stimulation of the mutant receptor complex did not result in phosphorylation of Jak1. Thus the role of box1 in cytokine receptor signalling is not restricted to Jak binding. Our data provide evidence that W652 of gp130 is part of a structure necessary to allow Jak activation on ligand-induced receptor oligomerization.

EXPERIMENTAL

Cell culture and transfection

Monkey kidney cells (COS-7) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal-calf serum (FCS), 100 mg/l streptomycin and 60 mg/l penicillin. Cells were grown at 37 °C in a water-saturated 5% CO₂ atmosphere. COS-7 cells were transiently transfected using the DEAE/chloroquine transfection method with modifications as described [19] or using FuGENE[®] 6 (Roche, Mannheim, Germany) according to the manufacturer's instructions. IL-5 was purchased from Cell Concepts (Umkirch, Germany).

Construction of IL-5R α / β -gp130 mutants

Standard cloning procedures were performed throughout this study. The mutations were introduced by PCR technique. The PCR fragments were introduced into the pSVL-based expression vectors for gp130 [20] using the restriction enzymes *EcoRI*/*BstEII* for the construct W652A, *BstEII*/*Kpn2I* for the constructs Δ box2 and P671A/P672A, and *EcoRI*/*AspI* for the construct F676A. Gp130-Y683F was generated by introducing the *Bg/II*/*SalI*-fragment of pSVL-EPOR-gp130-6F [21] into the pSVL-based expression vector for gp130 (EPOR refers to erythropoietin receptor). The corresponding IL-5R α -gp130- and IL-5R β -gp130-chimaeras were generated by exchange of the *XhoI*/*EcoRI* fragment encoding the extracellular domain of gp130 against the ones encoding the extracellular parts of IL-5R α or IL-5R β respectively [22]. The integrity of all constructs was verified by DNA sequencing using the ABI PRISM 310 Genetic Analyzer (PerkinElmer, Norwalk, CT, U.S.A.). The construct Δ box1 was described previously [18].

Flow cytometry

Transiently transfected COS-7 cells were resuspended in cold PBS supplemented with 5% FCS and 0.1% NaN₃ (PBS/azide). Cells (5×10^5 – 1×10^6) in 100 μ l of PBS/azide were incubated with 1 μ g/ml of either the monoclonal anti-IL-5R α antibody (16-4; kindly provided by Dr J. Tavernier, Flanders Inter-university Institute for Biotechnology, Department of Medical Protein Chemistry, Faculty of Medicine, University of Ghent, Belgium) or the monoclonal anti-IL-5R β antibody (S-16, Santa Cruz Biotechnology, Heidelberg, Germany) for 30 min at 4 °C. Cells were then washed with cold PBS/azide. To visualize the bound antibodies, the cells were subsequently incubated in darkness with a 1:50 dilution of a R-phycoerythrin-conjugated anti-mouse IgG-Fab (Dianova, Hamburg, Germany) for 30 min at 4 °C. Cells were again washed with cold PBS/azide and then resuspended in PBS/azide with 1% formaldehyde. Cells (10^4 /sample) were analysed by flow cytometry using a FACScalibur (Becton Dickinson, Heidelberg, Germany) equipped with a 488 nm argon laser.

Electrophoretic-mobility-shift assays (EMSA)

Cells were stimulated with 10 ng of IL-5/ml for 30 min. EMSAs

were performed as described in [23]. Protein concentrations were measured with the Bio-Rad[®] protein assay. A double-stranded mutated *c-sis*-inducible element (SIE)-oligonucleotide from the *c-fos* promoter (m67SIE: 5'-GAT CCG GGA GGG ATT TAC GGG AAA TGC TG-3') was labelled by filling in 5' protruding ends with the Klenow enzyme, using [α -³²P]dATP (3000 Ci/mmol; 10 mCi/ml). This probe binds STAT1 and STAT3 homo- and hetero-dimers, resulting in retarded bands of different mobility. Nuclear extracts containing 5 μ g of protein were incubated with about 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% glycerol/5 mM dithiothreitol/0.7 mM PMSF/0.1 mg/ml poly(dI-dC)/1 mg/ml BSA] for 10 min at room temperature. For the competition assays non-radiolabelled SIE, zinc-finger transcription factor Egr-1 binding probe (5'-GGAT-CCAGCGGGGCGAGCGGGGGCGA-3'; Santa Cruz Biotechnology) and CCAAT-enhancer-binding protein (C/EBP) binding probe (5'-TGCAGATTGCGCAATCTGCA-3'; Santa Cruz Biotechnology) were incubated for 30 min with the radiolabelled probe and the nuclear extracts as described above. The supershifting STAT1 antibody (E23; Santa Cruz Biotechnology) and the supershifting STAT3 antibody (AS421/2; provided by Dr W. Müller-Esterl, Institute for Biochemistry II, University Hospital Frankfurt, Frankfurt, Germany) were incubated with the nuclear extracts for 45 min at 0 °C before addition of the radiolabelled SIE probe. The protein-DNA complexes were separated on a 4.5%-(w/v)-polyacrylamide gel containing 7.5% glycerol in 0.25-fold TBE (0.16 M Tris/borate (pH 8.0)/1 mM EDTA) at 20 V/cm for 4 h. Gels were fixed in water/methanol/acetic acid (80:10:10, by vol.) for 30 min, dried and autoradiographed.

Cell lysis, immunoprecipitation and Western-blot analysis

Cells were lysed on the dish with 500 μ l of lysis buffer containing 1% Brij 96 or Triton X-100, 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM sodium vanadate, 10 mM PMSF, 1 mM benzamidine, 5 μ g/ml aprotinin, 3 μ g/ml pepstatin, 5 μ g/ml leupeptin and 1 mM EDTA. Lysates were cleared by centrifugation at 12000 *g*. After overnight incubation at 4 °C, the immunoprecipitates were collected with Protein A-Sepharose (1 h, 4 °C), washed three times with washing buffer (0.1% Brij 96 or Triton X-100, 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM sodium vanadate, 10 mM PMSF, 1 mM benzamidine, 5 μ g/ml aprotinin, 3 μ g/ml pepstatin, 5 μ g/ml leupeptin and 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 enhanced chemiluminescence (ECL[®]; Amersham). Anti-IL-5-R β (S16, Santa Cruz Biotechnology) and anti-Jak1 (HR785, Santa Cruz Biotechnology) were used for immunoprecipitation. Anti-phosphotyrosine (PY99; Santa Cruz Biotechnology and 4G10, Upstate), anti-IL-5R β (N20, Santa Cruz Biotechnology), anti-Jak1 polyclonal antiserum (from Dr A. Ziemiecki, The Tiefenau Laboratory, Department of Clinical Research, University of Berne, Berne, Switzerland), anti-phosphotyrosine-705-specific STAT3 antibody (New England Biolabs, Schwalbach, Germany), anti-phosphotyrosine-701-specific STAT1 antibody (New England Biolabs), anti-STAT3 antibody (Transduction Laboratories, San Diego, CA, U.S.A.) and anti-STAT1 antibody (Transduction Laboratories) were used for detection. The horseradish peroxidase-conjugated secondary antibodies were purchased from Dako (Hamburg, Germany).

RESULTS

Exchange of the gp130 amino acids W652, P671/P672, F676 and Y683 does not significantly affect binding of Jak1

The box1/box2-region of gp130 is involved in binding Jaks. To gain further insight into the structural receptor requirements for Jak binding and activation, we mutated a number of conserved hydrophobic amino acids in the box1/box2 region of gp130 (see Figure 1A). Owing to the presence of a second proline-rich motif WSPHTPP (amino acids 666–672) similar to the gp130 box1 region (IWPNVDPDP)[11], we also exchanged the proline residues at positions 671 and 672 to alanine. We introduced the mutations

into chimaeric receptors consisting of the extracellular parts of the IL-5R α and β chains and the transmembrane domain of gp130 in order to be able to analyse the mutated receptors independently of endogenous gp130, which is expressed ubiquitously. Moreover, experimental tools such as antibodies have been well established and are readily available [19,22].

We transiently expressed the IL-5R β -gp130-constructs in COS-7 cells. To monitor Jak1 association, the chimaeric receptors were immunoprecipitated with an IL-5R β -specific antibody and subsequently analysed by SDS/PAGE and Western blotting. Co-precipitated Jak1 was detected using a Jak1-specific antibody (Figure 2). The IL-5R β -gp130 wild-type construct efficiently

B IL-5R α and - β /gp130 chimeric receptors

Figure 1 Schematic representation of the mutations introduced into the receptor sequence

(A) Alignment of the membrane-proximal sequences of the IL-6-type cytokine signal transducers gp130, LIFR and OSMR, the granulocyte-colony-stimulating-factor (GCSF) receptor and EPOR. Overall conserved residues are indicated with an asterisk. Overall conserved positive residues are indicated by '+', and overall conserved hydrophobic residues are indicated by 'h'. The positions of the mutations and the box1 and box2 regions are indicated. (B) Schematic representation of the chimaeric receptors used in the present study. They consist of the extracellular region of IL-5R α or IL-5R β respectively, and the transmembrane and intracellular regions of gp130.

Figure 2 Binding of Jak1 to the IL-5Rβ-gp130 mutant receptors

(A) Mutations W652A, P671/P672A, F676A, Y683F and ΔB do not affect Jak association. (B) Overexpression alters the apparent binding behaviour of Jak1 to gp130 lacking box2. Transiently transfected COS-7 cells expressing IL-5Rβ-gp130 wild-type (WT) or mutant constructs with or without overexpressed Jak1 as indicated were lysed and subjected to immunoprecipitation using an anti-IL-5Rβ monoclonal antibody (S16). The immunoprecipitates and the lysates were further analysed by SDS/PAGE. Western blots were probed with an anti-Jak1 polyclonal antiserum. The signals were detected using the ECL[®] system. The blots were reprobed with a polyclonal anti-IL-5Rβ (N20) antibody.

bound Jak1 (Figure 2A, lanes 1 and 5). Also the C-terminal deletion construct IL-5Rβ-gp130ΔB associated with the kinase, demonstrating that the membrane-proximal 69 amino acids are sufficient for binding (lane 9). Association of the kinase to the Δbox1-construct was greatly impaired (lane 2), as described previously [18]. The constructs with point mutations in the interbox1/box2 region P671A/P672A, F676A and Y683F bound Jak1 as effectively as the wild-type receptor (lanes 4, 6, and 7). Also a single amino acid exchange, W652A, in the box1 region did not affect Jak1 association (lane 3). However, deletion of box2 proved to be detrimental for the binding of Jak1 to gp130 (Figure 2A, lane 8).

Overexpression of Jak1 alters the apparent behaviour of the kinase in its binding to the construct Δbox2

Our finding that deletion of the box2 region abrogated Jak association was unexpected, since in previous studies the association of Jaks to analogous gp130 constructs could be demonstrated [15,17]. However, in these studies Jaks were overexpressed. To investigate whether the expression level of Jak1 influences the apparent binding behaviour to gp130, we co-transfected COS-7 cells with an expression vector for Jak1 along

Figure 3 STAT activation is abrogated by point mutation W652A and by deletion of box2, while P671A/P672A and F676A lead to a modulation of STAT activity

COS-7 cells were transiently co-transfected with IL-5Rα-gp130 and IL-5Rβ-gp130 wild-type or with two α-β mutant expression constructs as depicted in the upper left panel. At 48 h post transfection the cells were starved (0% FCS) for 6 h before they were stimulated with 10 ng/ml IL-5 for 30 min. (A) Nuclear extracts were prepared and analysed by EMSA using the SIE probe. Expression of the chimaeric constructs was monitored by flow-cytometric analysis using a FACScalibur instrument. Histograms resulting from cells stained with the IL-5Rβ-specific antibody are shown in grey; they are overlaid with IL-5Rα-specific histograms (black line). A control histogram (secondary antibody only) is shown on the left side for comparison. (B) A Personal Imager-FX and Quantity-One software (both from Bio-Rad) were used to quantify the signals for wild-type, P671A/P672A and F676A. In the diagram, signal intensities are represented as percentages of the wild-type signals. Shown are the means ± S.D. for four independent experiments. (C) Nuclear extracts of COS-7 cells expressing the wild-type receptor combinations were used for competition studies using a non-radioactively labelled SIE probe and two non-STAT1-binding probes which contain binding sites for Egr-1 and CREB-β, respectively (purchased by Santa Cruz Biotechnology) demonstrating specific binding to the SIE probe. Moreover, supershift experiments were performed with a supershifting STAT1 antibody (E23; Santa Cruz Biotechnology) and a supershifting STAT3 antibody (AS 421/2; provided by Dr W. Müller-Esterl), indicating that the signal in the EMSA is due to binding of STAT1 to the probe.

with the IL-5Rβ-gp130-constructs. Deletion of box1 abrogated co-precipitation with Jak1 irrespective of its expression level (Figure 2B, lanes 3 and 7). However, overexpression of Jak1 resulted in an association of the kinase to the Δbox2 construct similar to that of the wild-type construct (Figure 2B; compare lanes 2 and 4 with lanes 6 and 8). Thus overexpressing Jak1 to high levels affects the binding behaviour of the kinase to the Δbox2 mutant in COS-7 cells, which may explain the divergent results of other studies with regard to the gp130-Δbox2 mutant [15,17].

Point mutation of W652 to alanine in gp130 results in impaired STAT activation

After having investigated Jak1 association to the novel muteins (mutant proteins), we tested their ability to activate STAT transcription factors on cytokine treatment. COS-7 cells were transiently co-transfected with IL-5Rα and IL-5Rβ expression

constructs as depicted in Figure 3. Wild-type and mutant constructs were expressed on the cell surface to a similar extent, as determined by flow cytometry. In double-staining experiments for the different mutant proteins, no skewing of the relative amounts of double-positive cells was observed (results not shown). After stimulation with IL-5, nuclear extracts were analysed by EMSA. Homodimerization of two gp130 wild-type cytoplasmic chains induced a strong STAT1 signal in COS-7 cells (Figures 3A and 3C), as observed previously [18,19]. Exchange of Y683 to phenylalanine had no effect on STAT1 activation (Figure 3A, upper right panel). As expected for constructs that show an impaired Jak1 association, IL-5-induced dimerization of the constructs Δ box1 and Δ box2 respectively did not elicit a STAT1 signal (lower panel). The mutants P671A/P672A and F676A mediated a reproducibly reduced STAT1 DNA-binding activity (Figure 3B). Interestingly, the single amino acid substitution in the box1-region, W652A, totally prevented the activation of STAT1 (Figure 3A), although this mutant bound Jak1 (Figure 2A). Thus the presence of W652 in the cytoplasmic region of gp130 is crucial for cytokine-dependent signal transduction.

Point mutation of W652 to alanine in gp130 leads to an impaired Jak1 activation

The mutant construct W652A did not affect Jak1 binding, but totally abolished the gp130-induced DNA-binding activity of STATs. To determine further at which point signal transduction stalls, we were interested as to whether Jak1 still becomes activated on stimulation of the W652A receptor mutant. In addition to the wild-type construct, we used the Δ B mutant as another control which lacks the C-terminal STAT recruitment sites of gp130. COS-7 cells were transiently co-transfected with IL-5R α and IL-5R β expression vectors. Whereas the wild-type and W652 constructs were expressed to a similar extent on the cell surface of transfected cells, surface expression of Δ B was more pronounced, as observed previously for analogous EPOR chimaeras [24], most likely because these constructs lack the L732-L733 motif crucial for the constitutive internalization of gp130 [25,26] (Figure 4, upper panel). After stimulation with IL-5, lysates were prepared, Jak1 was immunoprecipitated from the cellular lysates and the phosphorylation levels were investigated by Western-blot analysis using an anti-phosphotyrosine antibody (PY99). Interestingly, the single amino acid substitution W652A totally prevented Jak1 phosphorylation (Figure 4, middle panel) and, not surprisingly, phosphorylation of STAT transcription factors (Figure 4, lower panel). The Δ B mutant was able to activate Jak1 but not the STATs (owing to the receptor truncation). Thus the presence of W652 in the cytoplasmic region of gp130 is crucial for Jak1 activation, although its mutation to alanine does not influence association of the kinase (Figure 2A).

Jak association to both chains of a gp130 dimer is essential, but not sufficient, to promote Jak1 activation

The use of heterodimeric receptor chimaeras based on the IL-5R α - and β -chains enabled us to induce the dimerization of the cytoplasmic parts of a gp130 W652A mutant with a wild-type receptor construct. In such a receptor complex, both chains associate with Jaks, which is a prerequisite for signal transduction to occur [18,27]. However, only the wild-type cytoplasmic part of gp130 sustains Jak activation. Would this be sufficient to induce signal transduction? COS-7 cells were transiently co-transfected with IL-5R α and IL-5R β expression constructs as depicted in Figure 5. Wild-type and mutant constructs were expressed on the cell surface to a similar extent, as determined by flow cytometry

Figure 4 Jak1 activation is impaired by the single amino acid exchange W652A

COS-7 cells were transiently co-transfected with expression constructs as indicated. At 36 h post transfection the cells were starved (0% FCS) for 6 h before they were stimulated with 10 ng/ml IL-5 for 10 min. Lysates were prepared and were subjected to immunoprecipitation using an anti-Jak1 antibody (HR785). The immunoprecipitates were analysed by SDS/PAGE. Western blots were probed with an anti-phosphotyrosine monoclonal antibody (PY-99; Santa Cruz Biotechnology). The signals were detected using the ECL[®] system. The blots were re-probed with an anti-Jak1 polyclonal antiserum. STAT3^{Y705} and STAT1^{Y701} phosphorylation was investigated using phosphotyrosine-specific antibodies for detection of the Western blots of the lysates. The blots were re-probed using STAT3- and STAT1-specific antibodies. Surface expression of the transfected IL-5R α -gp130 and IL-5R β -gp130 receptors was monitored as described in the legend to Figure 3.

(results not shown). After stimulation with IL-5, nuclear extracts were analysed by EMSA. Interestingly, IL-5-induced dimerization of one wild-type with a mutant W652A chain leads only to a marginal STAT1 signal. Again this shows that Jak1 binding to both chains of a gp130 dimer is not enough for productive signal transduction. Both receptor chains have to be able to sustain Jak activation. Even a single point mutation that impairs Jak activation in one chain of a gp130 dimer is detrimental to the signalling capacity of such a receptor complex.

Figure 5 Mutation of W652 to alanine acts dominantly to impair STAT activation upon dimerization with a wild-type cytoplasmic tail of gp130

COS-7 cells were transiently transfected with expression constructs as indicated. At 48 h post transfection the cells were starved (0% FCS) for 6 h before they were stimulated with 10 ng/ml IL-5 for 30 min. Nuclear extracts were prepared and analysed by EMSA using the SIE probe.

DISCUSSION

The present study was aimed at further delineating the structural requirements of gp130 necessary for interaction with Jak1. We focused our work on Jak1, which has been found to be most important for signal transduction of IL-6-type cytokines [5,6], although gp130-dependent activation of Jak2 and Tyk2 has also been described [4]. In untransfected COS-7 cells Jak1 is also clearly tyrosine-phosphorylated on stimulation with IL-6-sIL-6R complexes, whereas phosphorylation of Jak2 and Tyk2 is hardly detectable (results not shown). Moreover, our attempts to co-precipitate endogenous Jak2 and Tyk2 with gp130 from COS-7 cells proved unsuccessful.

The membrane-proximal region comprising the box1 and box2 sequences of gp130 mediate Jak binding and activation. The IL-5R β -gp130 Δ B construct bound endogenous Jak1 as efficiently as the wild-type receptor construct (Figure 2). Moreover, IL-5 stimulation of the constructs IL-5R α -gp130 Δ B and IL-5R β -gp130 Δ B induced tyrosine-phosphorylation of Jak1 to a level that was comparable with that promoted by the full-length constructs (Figure 4). Therefore, in the case of gp130, sequences downstream of box2 do not seem to contribute to binding and activation of Jaks to gp130, i.e. 69 membrane-proximal amino acids are sufficient.

The involvement of box1 of gp130 in Jak binding has well been documented [11,15,18]. We have previously shown that the interbox1/box2 region (W666) is also crucial for Jak1 association [18]. The role of box2, however, was less clear: a truncated receptor lacking box2 was unable to mediate proliferation in Ba/F3 cells [11] and activation of Jak2 [16], although the binding of overexpressed Jaks was unaffected [17]. Moreover, a box2 deletion mutant of gp130 did not abrogate the binding to an overexpressed Jak2 construct [15]. In our experiments deletion of box2 abrogated signal transduction, and co-precipitation of endogenous Jak1 with the Δ box2 construct was drastically reduced compared with the wild-type construct (Figure 2). However, when Jak1 was overexpressed, its binding to the Δ box2 construct was essentially similar to the association with the wild-type construct. Overexpressed Jak1 could not be co-precipitated with a construct lacking the box1 region (Figure 2A) or with the inactive W666A construct (results not shown), but overexpression of Jak1 also resulted in co-precipitation with the otherwise non-binding IL-5R β -gp130P656A/P658A receptor which is mutated

within the box1 region (results not shown). Our finding that the expression level of a Jak can drastically influence its apparent binding behaviour to a given receptor demonstrates the possibility of false-positive results, so that caution has to be taken in the interpretation of experiments with overexpressed kinases.

The most important finding of the present study is that binding of Jak1 to gp130 is not sufficient for signal transduction. Residues within the receptor are, in addition, crucial for proper Jak activation and, thus, for eliciting downstream signalling. Box1 of gp130, as of other cytokine receptors, has been known to be involved in association with Jaks: point mutations of two proline residues in box1 of gp130 abrogate binding of Jaks and impair signal transduction [11,15,16,18]. Combined point mutations of the three hydrophobic residues in box1 also abolished the growth response in Ba/F3 transfectants, but the cause for this finding was not further analysed [11]. Here we show that the role of box1 in cytokine receptor signalling cannot be only restricted to mere Jak binding: a receptor mutant with a single amino acid substitution within box1, W652A, displayed a binding behaviour comparable with that of the wild-type receptor, but was unable to mediate STAT1 activation in COS-7 cells. This was due to the inability of the mutated receptor to activate Jak1 (Figure 4). Even when a W652A mutant receptor was dimerized with the cytoplasmic part of wild-type gp130, no signalling occurred, indicating that the mutation acts dominantly. Thus, the simple presence of two bound Jak1 molecules in a gp130 dimer is not sufficient to induce Jak1 activation and further signal-transduction events. Mutation of residues P671-P672 and F676 in the interbox1/box2 region to alanine were found to have no effect on the binding of Jak1, but dampened STAT1 activation mediated by the resultant receptors. However, the effect of these mutations was much less pronounced than the one observed for W652.

Very recently a similar finding has been described for EPOR: the exchange of three hydrophobic amino acid residues in the juxtamembrane region of EPOR similarly resulted in mutant receptors unable to activate Jak2 and to elicit a proliferative response in transfected cells, although Jak2 binding was not impaired [28]. Moreover, it is known that mutation of a tryptophan residue in the interbox1/box2 region of the EPOR also abrogated signal transduction, although binding to Jak2 was maintained [12,27] or, conversely, could not be detected [29].

Thus it seems to be a general phenomenon that the mere proximity of Jaks in ligand-associated receptor complexes is not sufficient for their activation. Rather it is likely that parts of the receptor are involved in positioning Jaks correctly so that ligand-induced receptor dimerization and reorientation can allow their mutual activation. The information of ligand binding to the extracellular region of a receptor is transmitted through the transmembrane region into the cell. There is growing evidence that rigidity of the transmembrane regions, likely due to α -helical structures, can extend into the intracellular region [28,30] as well as into the extracellular region [31]. Thus ligand binding seems to be able to force the intracellular parts of the receptor into a very defined position towards each other [32-34]. The interaction of the Jak with cytokine receptors seems to involve large portions of the kinase {approx. the N-terminal 300 amino acid residues comprising the JH6-7 (Jak homology) domains [35-37]} and the receptor box1/box2 region, comprising about 70 amino acids. It can well be envisaged that the Jaks are bound to the receptor in a complex way that dictates their position in a defined orientation. Residues such as W652 may be necessary for providing the correct positioning of the Jaks that may be a prerequisite for their mutual activation upon ligand-mediated reorientation of the receptors. Alternatively, it is possible that the activation of

the Jaks involves structural reorganization of the Jak/gp130 binding interface, and that residues such as W652 are involved in this dynamic process. Moreover, exchange of W652 to alanine may prevent allosteric interactions essential for Jak1 enzymic activity. However, these possibilities are not mutually exclusive.

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