Activation of the signal transducer gp130 by interleukin-11 and interleukin-6 is mediated by similar molecular interactions

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The transmembrane glycoprotein gp130 is involved in many cytokine-mediated cellular responses and acts therein as the signal transducing receptor subunit. Interleukin-6 (IL-6) and interleukin-11 (IL-11), in complex with their specific α -receptors, homodimerize gp130 and, as a consequence, activate the Janus kinase (Jak)/signal transducer and activator of transcription (STAT) signalling pathway in their target cells. So far, it is not clear whether gp130 is bound to these cytokines and their specific α -receptor subunits through identical or different epitopes. In order to study the interaction of IL-11 and IL-11R with human

gp130 the soluble form of the recently cloned human IL-11R was expressed in baculovirus-infected insect cells. By a coprecipitation binding-assay it is demonstrated that IL-11 and IL-6 compete for binding to gp130. Using deletion and point mutants of gp130 it is shown that IL-11–IL-11R and IL-6–IL-6R recognize overlapping binding motifs on gp130. Moreover, using well-established Jak-deficient cell lines we demonstrate that STAT activation by IL-11 requires Jak1. Taken together, our data support the concept that IL-6 and IL-11 activate gp130 by very similar molecular mechanisms.

INTRODUCTION

gp130 is the common signal transducing receptor subunit for a group of cytokines, including interleukin-6 (IL-6), interleukin-11 (IL-11), leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M (OSM) and cardiotrophin-1 (CT-1). These so-called IL-6-type cytokines are involved in the regulation of complex biological processes such as haematopoiesis, immune responses and inflammation [1]. gp130 is a transmembrane glycoprotein of 896 amino acids [2] and was first described as the signal transducing subunit of the IL-6 receptor complex [3]. The extracellular part of gp130 consists of six fibronectin-type III-like domains, the second and third of which constitute the cytokine receptor homology module (CRHM). This module is characterized by a set of four conserved cysteine residues in its N-terminal domain and a WSXWS motif in the C-terminal domain [4]. The redundancy of the biological effects exerted by IL-6-type cytokines can in part be explained by the similarity of their active receptor complexes. IL-6, after binding to its specific α -receptor subunit (IL-6R), induces the homodimerization of gp130 [5]. LIF, CNTF, CT-1 and OSM induce a heterodimerization of gp130 and the LIF receptor (LIFR) [6–8]. Alternatively, OSM can form a heterodimer of gp130 and the recently cloned OSM receptor (OSMR) [9]. For IL-11 it is not clear whether this cytokine, after binding to its specific α -receptor subunit (IL-11R), induces solely the homodimerization of gp130 [10,11] or is able to recruit an additional, still unknown, receptor subunit to the complex [12]. All cells expressing gp130 but lacking membrane-bound IL-6R can be stimulated by a combination of IL-6 and soluble IL-6R (sIL-6R), since the cytoplasmic and transmembrane parts of IL-6R are not required for signal transduction [3,13]. Similarly, in the absence of

membrane-bound IL-11R, cells can be stimulated with IL-11 and sIL-11R [14,15], suggesting that the expression of gp130 is sufficient for cells to become IL-11–sIL-11R responsive and that dimerization of gp130 is therefore at least one possible mechanism by which the IL-11 signal is transduced into cells.

The molecular mechanisms of the interaction of gp130 with the IL-6-type cytokines and their specific α -receptor subunits are not understood in detail. So far, most investigators have concentrated on the interactions of human gp130 with IL-6 and IL-6R. Using soluble receptor proteins, lacking the transmembrane and cytoplasmic parts, it was demonstrated that the ternary complex is a hexamer consisting of two molecules of each of IL-6, sIL-6R and gp130 [16,17]. In a study using deletion mutants of gp130 it was shown that the complex of IL-6 and IL-6R binds to the membrane-distal half of gp130, including the CRHM [18]. More recently, by molecular modelling guided mutagenesis, Val-252 in the predicted BC-loop of domain 3 of gp130 was identified to be important for the interaction with IL-6–IL-6R complexes [19]. Domain 3 of gp130 was expressed in *Escherichia coli* and prepared for NMR structural analysis [20]. Preliminary structural data from NMR studies on domain 3 of gp130 confirm that Val-252 is indeed located in the BC-loop and is accessible for molecular interactions (J. Grötzinger, personal communication). Whether the interactions of gp130 with other members of the family of IL-6-type cytokines and their receptors is mediated by identical or different binding epitopes has not been elucidated.

Ligand-induced activation of cytokine receptors leads to tyrosine phosphorylation of cytosolic substrates, although these receptors lack a cytoplasmic tyrosine kinase (Tyk) domain. The four members of the family of Janus kinases (Jaks) have been found to be constitutively associated with the cytoplasmic part of cytokine receptors [21]. Whereas most cytokine receptors show a

Abbreviations used: CT-1, cardiotrophin-1; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; EMSA, electrophoretic mobility-shift assay; IL, interleukin; R, receptor; sIL-6R, soluble IL-6 receptor; CNTF, ciliary neurotrophic factor; LIF, leukaemia inhibitory factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; CRHM, cytokine receptor homology module; OSM, oncostatin M; STAT, signal transducer and activator of transcription; Jak, Janus kinase; Tyk, tyrosine kinase; Trx, thioredoxin; mAb, monoclonal antibody.

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specific recruitment of only one Jak, it has been shown that gp130 interacts with Jak1 and Jak2 as well as with Tyk2 [22,23]. After cytokine-induced dimerization of gp130 the Jaks are activated and, as a consequence, phosphorylate the cytoplasmic tyrosine residues of gp130. The four membrane-distal phosphotyrosine residues serve as docking sites for signal transducer and activator of transcription 3 (STAT3), whereas the two C-terminal phosphotyrosine residues are also able to recruit STAT1 [24,25]. The specificity of STAT binding to different phosphotyrosine motifs is determined by their SH2-domains [26,27]. After binding to gp130 the STATs also become tyrosine phosphorylated, which leads to their dimerization and release from the receptor complex. After an additional serine phosphorylation [28] and translocation into the nucleus the activated STAT dimers induce the transcription of target genes by binding to specific response elements [29,30]. A series of cell lines have been established which are deficient in individual Jaks. These cells have been used successfully to elucidate the role of distinct Jaks in the induction of the Jak/STAT pathway by interferons and cytokines [31–34].

The recent cloning of the cDNA encoding the human IL-11R [35] has made it possible to study the interaction of human gp130 with IL-11 and its specific α -receptor subunit. As molecular tools for our studies we expressed soluble human IL-11R in baculovirus-infected insect cells and human IL-11 as a thioredoxin (Trx) fusion protein in *E. coli*. In the present paper, using deletion and point mutants of gp130, we could show that IL-11 and IL-6 use overlapping binding epitopes of the common signal transducer gp130. Moreover, we found that the signal transduction of both cytokines via the Jak/STAT pathway, leading to the activation of STAT3 and STAT1, requires Jak1.

EXPERIMENTAL

Materials

All enzymes used for DNA modification and amplification were obtained from Boehringer Mannheim (Mannheim, Germany). Protein A–Sepharose and CNBr–Sepharose were from Pharmacia (Freiburg, Germany). Dulbecco's modified Eagle's medium (DMEM), Iscove's modified DMEM and Sf900 medium were purchased from Life Technologies Inc. (Eggenstein, Germany), TC100 medium was obtained from Biomedicals (Costa Mesa, CA, U.S.A.) and fetal calf serum (FCS) was purchased from Seromed (Berlin, Germany). Recombinant IL-6 was prepared as described by Arcone et al. [36]. Soluble human IL-6R (sIL-6R) was produced in insect cells as described previously [37]. The monoclonal antibodies (mAbs) directed against Trx, the flag epitope and human IgG were obtained from Invitrogen (Leek, The Netherlands), Kodak (Integra Bioscience, Fernwald, Germany) and Dako (Copenhagen, Denmark) respectively. The mAb recognizing sIL-6R (mAb7) has been characterized previously [37]. Recombinant human IL-11, at a concentration of 11.2 mg/ml, was a gift from the Genetics Institute (Cambridge, MA, U.S.A.). Oligonucleotides were synthesized by MWG Biotech (Ebersberg, Germany). Construction of the expression vectors pSVL-sgp130–IgG (encoding a fusion protein of soluble gp130 and the constant region of human IgG1) and the derived deletion constructs sgp130–IgG(D1-3) and sgp130–IgG(D4-6) has been described previously [18].

Cell culture

COS-7 cells (American Type Culture Collection, CRL 1651), 2fTGH fibrosarcoma cells and the related Jak-deficient cell lines (U4A, γ 2a and U1A) were grown in DMEM; Ba/F3 cells and Ba/F3 transfectants were cultured in DMEM containing 5%

(v/v) conditioned medium from X63Ag8-653 BPV-mIL-3 myeloma cells (as a source of IL-3) at 5% CO₂ in a water-saturated atmosphere. These media were supplemented with 10% (v/v) FCS, streptomycin (100 μ g/ml) and penicillin (60 μ g/ml). B9 cells were grown in Iscove's modified DMEM containing 5% (v/v) FCS, 50 μ M 2-mercaptoethanol, streptomycin (100 μ g/ml), penicillin (60 μ g/ml) and 100 pg IL-6/ml. SF158 insect cells were cultured in serum-free SF-900 medium without antibiotics. SF21 cells were maintained in TC100 medium with 10% (v/v) FCS.

Expression of Trx–IL-11

Human IL-11 cDNA was amplified by standard PCR technology with the oligonucleotides 5'-ATC ACGGTACCT GGG CCA CCA CCT GGC-3' and 5'-TAG CTTCTAGAG GGT CAC AGC CGA GTC TTC A-3' (introduced restriction sites are underlined) using pBluescriptSK + hIL-11 as a template. The amplicon was subcloned into the *Kpn*I and *Xba*I sites of the plasmid pTrxFus (Invitrogen BV, Leek, The Netherlands). The resulting expression vector encodes Trx followed by an enterokinase recognition site and the mature form of human IL-11 [38,39]. Expression of Trx–IL-11 in *E. coli* was performed as described by LaVallie et al. [40]. Supernatants of osmotically shocked *E. coli* containing highly enriched Trx–IL-11 were used for B9 cell proliferation assays and coprecipitation experiments. The protein concentration of the supernatant was determined (Bio-Rad protein assay, Bio-Rad Laboratories, Hercules, CA, U.S.A.) and the Trx–IL-11 portion (usually 30% of total protein) was estimated from a Coomassie-stained SDS/polyacrylamide gel.

Construction of a transfer vector encoding soluble human IL-11R-flag

Annealed oligonucleotides coding for a flag epitope and providing the respective 5'-overhang sequences were ligated into the *Eco*RI–*Bam*HI-digested baculovirus transfer vector pVL1392, yielding the vector pVL1392-flag. The sequences of the flag sense and antisense oligonucleotides were 5'-AAT TCC CGG GTG ACT ACA AAG ACG ATG ACG ATA AAT AGA ATT CG-3' and 5'-GAT CCG AAT TCT ATT TAT CGT CAT CGT CTT TGT AGT CAC CCG GG-3' respectively. A part of the IL-11R cDNA encoding the signal sequence and the complete ectodomain of the receptor was amplified using standard PCR technology. A plasmid harbouring the human IL-11R cDNA (pcDNA3-hIL-11R) served as a template. The amplicon was first subcloned into pSVL (Pharmacia) and then transferred into pVL1392-flag. The integrity of the resulting plasmid pVL1392-sIL-11R-flag was verified by DNA sequencing. It encodes amino acids 1–362 of the human IL-11R followed by the sequence GMHVEGDYKDDDDK. The underlined sequence constitutes the flag epitope.

Isolation of recombinant baculoviruses and expression of sIL-11R-flag

Recombinant baculoviruses were generated as described in the BaculoGold transfection kit manual (Pharmingen, San Diego, CA, U.S.A.). Briefly, Sf21 cells were cotransfected with 0.5 μ g of BaculoGold®-DNA and 2 μ g of transfer vector pVL1392-sIL-11R-flag. From the supernatant a single recombinant virus was isolated and amplified by infection of Sf158 cells. For the preparation of supernatants containing sIL-11R-flag, 2×10^7 Sf158 cells were infected with an equal amount of recombinant viruses and incubated for 3 days at 27 °C. The supernatant was

centrifuged (130 g) to remove the cells, sterile-filtered (0.2 µm) and used for further experiments. Since no ELISA is available for exact quantification of sIL-11R the amount of the recombinant protein was estimated from band intensities in silver-stained SDS/polyacrylamide gels and immunoblots. The expression level of sIL-11R-flag (20 µg/ml) was comparable with that of sIL-6R [37]. Supernatants from non-infected insect cells were prepared the same way and served as a control.

Precipitation of sIL-11R with Trx-IL-11 covalently linked to Sepharose

Trx-IL-11 from supernatants from osmotically shocked bacteria was coupled to BrCN-activated Sepharose as described in the manual of the supplier (Pharmacia). Trx-IL-11-Sepharose and Sepharose 4B as a control were incubated for 2 h at 4 °C with 0.5 ml of insect cell supernatant containing sIL-11R-flag. After three washing steps with PBS containing 0.05 % (v/v) Tween-20, bound proteins were released from the Sepharose by the addition of Laemmli sample buffer [41] and subsequent incubation at 95 °C for 5 min. After separation by SDS/PAGE, bound proteins were analysed by immunoblotting using a flag antibody.

Formation of the soluble ternary complexes

Supernatants of COS-7 cells transiently transfected with expression vectors encoding sgp130-IgG fusion protein and the respective deletion mutants were precipitated with 0.5 mg/ml of Protein A-Sepharose at 4 °C for 12 h via the IgG part of the fusion proteins. The Protein A-Sepharose-bound sgp130-IgG constructs in 650 µl TNET buffer [20 mM Tris/HCl (pH 7.5)/140 mM NaCl/5 mM Na₂EDTA/1 % (w/v) Triton X-100/2 mM methionine/1.5 mM Na₂S₂O₃] were then incubated with 200 µl of sIL-11R-flag containing supernatants of baculovirus-infected insect cells (about 4 µg of sIL-11R-flag) and 150 µl of Trx-IL-11-containing supernatants of osmotically shocked *E. coli* (about 3 µg Trx-IL-11) which were previously equilibrated with PBS. Additional experiments were performed in the presence of increasing amounts of IL-6 (0.02, 0.2, 1 or 2 µg) and a constant amount of sIL-6R (4 µg) (see Figure 2C). After overnight incubation at 4 °C the complexes formed were sedimented via Protein A-Sepharose. The bound proteins were separated by SDS/PAGE and visualized by immunoblotting.

Cell proliferation assays

B9 cells (5000 cells/well) or Ba/F3 cells (20000 cells/well) and the respective transfectants (Ba/F3-gp130, Ba/F3-gp130-V252D and Ba/F3-gp130-Y218K) were plated on 96-well plates and stimulated with various amounts of IL-6 and IL-11 in the presence of 1 µg/ml of the respective soluble receptor. After 60 h of incubation viable and metabolically active cells were quantified using a colorimetric assay based on the Cell Proliferation Kit II (XTT) (Boehringer Mannheim, Germany).

Immunoblotting

Proteins were separated by SDS/PAGE [41] and transferred to PVDF membrane by a semi-dry blotting procedure [42]. The membranes were incubated with the antibody mixtures as indicated in the Figures and were processed for chemiluminescence detection as described in the enhanced chemiluminescence (ECL) manual (Amersham, U.K.).

Electrophoretic mobility-shift assay (EMSA)

2fTGH cells, Jak-deficient cells (U4A, γ2a and U1A) and Jak1-deficient cells stably transfected with Jak1 (U4A/Jak1) [33] were incubated at 37 °C for 15 min or for the periods of time indicated in Figure 5 in the presence of various amounts of IL-11, sIL-11R-flag, IL-6 and sIL-6R, as indicated in Figures 5 and 6. Preparation of nuclear extracts and EMSAs were performed as described [43]. A mutated double-stranded oligonucleotide corresponding to the c-fos promoter (m67SIE: 5'-GAT CCG GGA GGG ATT TAC GGG GAA ATG CTG-3' [44]), which provides STAT3 and STAT1 binding sites, was used as a ³²P-labelled probe. Protein-DNA complexes were separated on a 4.5 % polyacrylamide gel containing 7.5 % glycerol in 23 mM Tris/23 mM boric acid (pH 8.0)/0.5 mM EDTA at 20 V/cm for 4 h. Gels were fixed in 10 % (v/v) methanol, 10 % (v/v) acetic acid and 80 % (v/v) water for 1 h, dried and analysed by autoradiography.

RESULTS

Expression and characterization of Trx-IL-11 fusion protein and sIL-11R-flag

The human IL-11 cDNA encodes a preprotein consisting of 199 amino acids which in mammalian cells is N-terminally processed to its biologically active mature form of 177 amino acids. Several trials to express mature human IL-11 in *E. coli* did not lead to any detectable amounts of recombinant protein (results not shown). Therefore a functional equivalent fusion protein of Trx and IL-11 (Trx-IL-11) was produced in *E. coli* as described by LaVallie et al. [40]. The fusion protein can be easily detected by a monoclonal Trx antibody (see Figure 2). Furthermore, soluble human IL-11 receptor tagged with a short C-terminal flag epitope (DYKDDDDK) was expressed in baculovirus-infected insect cells (sIL-11R-flag). Soluble IL-11R-flag could be precipitated from conditioned media by Trx-IL-11 covalently linked to Sepharose and was detected by immunoblotting with a flag antibody as a protein with an apparent molecular mass of about 45 kDa (Figure 1A, lane 2). The deviation from the calculated molecular mass of 37 kDa is probably due to glycosylation, since the ectodomain of the human IL-11R contains two N-glycosylation sites [45]. Sepharose alone did not lead to any detectable precipitation of sIL-11R-flag (Figure 1A, lane 1), indicating that sIL-11R-flag specifically interacts with Trx-IL-11.

To confirm that the bioactivity of IL-11 is not impaired by the fusion of Trx to its N-terminus, the proliferative response of B9 (B-cell hybridoma) cells [46] induced by either IL-11-sIL-11R-flag or Trx-IL-11-sIL-11R-flag was compared (Figure 1B). Neither IL-11 (open circles) nor Trx-IL-11 (open triangles) alone led to a stimulation of B9 cell growth, probably due to the lack of membrane-bound IL-11R [47]. In the presence of sIL-11R-flag, B9 cells proliferated in response to both IL-11 (filled circles) and Trx-IL-11 (filled triangles) with a similar dose dependence. B9 cells did not respond to Trx in the presence of sIL-11R-flag (results not shown). These experiments show that (a) Trx-IL-11 and sIL-11R-flag form bioactive complexes and (b) the bioactivity of Trx-IL-11 is comparable with that of IL-11.

IL-11-sIL-11R complexes compete with IL-6-sIL-6R complexes for gp130 binding

In order to study ternary complex formation of IL-11, IL-11R and gp130 a coprecipitation assay with soluble receptor proteins was established (Figure 2A). The ectodomain of gp130 (sgp130) was expressed in COS-7 cells as an IgG fusion protein (sgp130-IgG) as described [18]. The C-terminal human IgG part allows

Figure 1 Bioactivity of the recombinant proteins Trx-IL-11 and sIL-11R-flag

(A) Supernatants of baculovirus-infected insect cells expressing sIL-11R-flag were precipitated with Sepharose alone (lane 1) or Trx-IL-11 covalently linked to Sepharose (lane 2). Bound proteins were separated by SDS/PAGE and coprecipitated sIL-11R-flag was detected by immunoblotting with the flag antibody M2. (B) B9 cells were incubated with increasing amounts of IL-11 and Trx-IL-11 in the presence or absence of about 1 μ g/ml of sIL-11R-flag. Cell proliferation was determined by a colorimetric XTT-assay as described in the Experimental section.

direct precipitation of sgp130-IgG from cell culture supernatants via Protein A-Sepharose. After addition of Trx-IL-11 and sIL-11R-flag the coprecipitation of Trx-IL-11 could be detected by immunoblot analysis (Figure 2B, lane 1), indicating ternary complex formation. No coprecipitation of Trx-IL-11 was observed in the absence of sIL-11R-flag or sgp130-IgG (lanes 2 and 4). Also, the use of Trx instead of Trx-IL-11 did not lead to any detectable precipitation of Trx (lane 3). These findings show that Trx-IL-11 binds to gp130 only in the presence of sIL-11R-flag and that binding is not due to unspecific interaction of the Trx fusion part of the protein. The detection of coprecipitated sIL-11R-flag was not possible because the protein was masked by a band of unknown origin with identical mobility observed in all precipitation experiments (Figures 2 and 3).

When complex formation with Trx-IL-11-sIL-11R-flag was performed in the presence of increasing amounts of IL-6-sIL-6R

Figure 2 Competition of Trx-IL-11-sIL-11R-flag and IL-6-sIL-6R for sgp130 binding

(A) Schematic representation of the coprecipitation assay. (B) sgp130-IgG was secreted from transfected COS-7 cells, precipitated with Protein A-Sepharose and subsequently incubated with Trx-IL-11 and sIL-11R-flag (lane 1) or Trx-IL-11 alone (lane 2) or Trx and sIL-11R-flag (lane 3). Lane 4 shows a precipitation from supernatants of COS-7 cells mock-transfected with empty vector. Precipitated proteins were separated by SDS/PAGE and analysed by immunoblotting using antibodies to Trx and human IgG simultaneously. (C) A coprecipitation experiment, as described in Figure 2(B) lane 1, was performed in the presence of increasing amounts of IL-6-sIL-6R complexes. The numbers at the bottom indicate the molar ratios of Trx-IL-11-IL-6 in the presence of constant amounts of sIL-11R-flag and sIL-6R (4 μ g/ml each). Precipitated proteins were separated by SDS/PAGE and analysed by immunoblotting using antibodies to Trx, sIL-6R and human IgG simultaneously.

complexes the amounts of coprecipitated Trx-IL-11 decreased (Figure 2C). Instead, sIL-6R could be detected in the precipitate, which is indicative of IL-6-sIL-6R-gp130 ternary complex formation (lanes 1-4). Already at a molar ratio of 1:2 between IL-6 and Trx-IL-11, IL-6-sIL-6R complexes completely displaced Trx-IL-11-sIL-11R-flag complexes from their gp130 binding site (lane 2). These observations suggest that IL-11-sIL-11R and IL-6-sIL-6R recognize at least overlapping binding epitopes on gp130 and that IL-6-sIL-6R complexes bind with higher affinity than Trx-IL-11-sIL-11R-flag complexes to gp130.

The membrane-distal part of gp130 is sufficient for IL-11-sIL-11R binding

To define the binding epitope of gp130 for IL-11-sIL-11R complexes, coprecipitation assays were performed with sgp130 deletion mutants expressed as IgG fusion proteins (Δ sgp130-

Figure 3 Binding of Trx-IL-11-sIL-11R-flag to sgp130-IgG deletion mutants

The complete ectodomain of gp130 (lane 1) and two deletion mutants comprising the membrane-proximal three domains (lanes 2 and 3) and the membrane-distal three domains (lanes 4 and 5) expressed as IgG fusion proteins were precipitated with Protein A-Sepharose from supernatants of transfected COS-7 cells. Subsequently the immobilized gp130-IgG was incubated with Trx-IL-11-sIL-11R-flag (lanes 1, 2 and 4) or, as controls, with Trx-IL-11 alone (lanes 3 and 5). The precipitated proteins were separated by SDS/PAGE and analysed by immunoblotting using antibodies against Trx and human IgG simultaneously.

IgG). The membrane-proximal half (domains 4–6) or the membrane-distal half (domains 1–3) of the gp130 extracellular part were secreted by transfected COS-7 cells as IgG fusion proteins and were precipitated from cell culture supernatants with Protein A-Sepharose (Figure 3, lanes 2–5). Only with the membrane-distal part of the gp130 ectodomain containing the cytokine receptor homology module (domains 2 and 3) was coprecipitation of Trx-IL-11 in the presence of sIL-11R-flag observed (Figure 3, lane 4), indicating that domains 1–3 of gp130 are required and sufficient for IL-11-IL-11R binding. Using a similar experimental setup, binding of IL-6-sIL-6R to gp130 was mapped to the same region of the signal transducer gp130 [18].

Similar epitopes of gp130 are involved in its activation by IL-11-sIL-11R and IL-6-sIL-6R complexes

In a previous study [19], several amino acids in the ectodomain of gp130 have been shown to be involved in the interaction with IL-6-IL-6R complexes. These residues, located in domain 3 and the hinge region between domains 2 and 3 (the CRHM of gp130), were mutated. gp130 wild type and two of these mutants, gp130-V252D and gp130-Y218K, were stably expressed in the factor-dependent murine pre-B-cell line Ba/F3. Parental Ba/F3 cells proliferate in response to IL-3, but not in response to IL-6-sIL-6R complexes, due to the lack of endogenous gp130. After stable transfection with gp130 these cells are able to grow in response to IL-6-sIL-6R complexes. In the study by Horsten et al. [19] it was demonstrated that the mutation of Y218 of gp130 leads to only a moderate disturbance of the interaction of gp130 with IL-

Figure 4 Effect of IL-11-sIL-11R-flag or IL-6-sIL-6R complexes on the proliferation of Ba/F3 cells stably transfected with wild-type gp130 or the mutants gp130-V252D or gp130-Y218K

Ba/F3, Ba/F3-gp130, Ba/F3-gp130-V252K and Ba/F3-gp130-Y218K cells were incubated with increasing amounts of IL-11 (A) or IL-6 (B) in the presence of constant amounts (about 1 μ g/ml) of sIL-11R-flag or sIL-6R respectively. Cell proliferation was determined by a colorimetric XTT assay and compared with the IL-3-induced maximal proliferation of Ba/F3 cells.

6-sIL-6R complexes, whereas the mutation of V252 of gp130 rendered the cells unresponsive to IL-6-sIL-6R complexes.

To characterize the IL-11-IL-11R binding epitope of gp130 in more detail, the above mentioned Ba/F3 transfectants were stimulated with IL-11-sIL-11R-flag (Figure 4A) and the responses were compared with those elicited by IL-6-sIL-6R complexes. As shown in Figure 4(A), parental Ba/F3 cells did not grow in response to IL-11-sIL-11R-flag (open circles), whereas stable transfection of gp130 wild type rendered the cells IL-11-sIL-11R-responsive (closed circles). Ba/F3-gp130 cells did not proliferate upon incubation with IL-11 alone, indicating that those cells do not express membrane-bound IL-11R (results not shown). The response of the Ba/F3-Y218K subclone (closed triangles) was slightly weaker compared with wild-type gp130. However, Ba/F3 cells expressing the gp130-V252D mutant (closed squares) were unresponsive to IL-11-sIL-11R-flag even in the presence of high cytokine doses (up to 0.1 μ g/ml). Comparison of these dose-response curves with the responses of the same cells to IL-6-sIL-6R (Figure 4B) revealed that the point mutations affect the interaction of gp130 with both cytokines and their soluble receptors in a similar manner. Furthermore, Figure 4 shows that Ba/F3-gp130 cells respond much more sensitively to IL-6-sIL-6R than to IL-11-sIL-11R-flag complexes, indicative of a lower affinity of IL-11-sIL-11R-flag

Figure 5 Dose and time dependence of STAT3 and STAT1 activation induced by complexes of IL-11-sIL-11R-flag or IL-6-sIL-6R in 2fTGH cells

(A) Fibrosarcoma cells (2fTGH) were left unstimulated (lane 1) or were stimulated for 15 min with either IL-6 (20 ng/ml) and sIL-6R (1 μg/ml, lane 2) or varying amounts of IL-11 and sIL-11R-flag (lanes 3–7). (B) 2fTGH cells were left unstimulated (lane 1) or were stimulated with either IL-6 and sIL-6R (10 ng/ml and 0.5 μg/ml respectively; lanes 2–5) or IL-11 and sIL-11R-flag (200 ng/ml and 1 μg/ml respectively; lanes 6–9) for different periods of time. Subsequently, nuclear extracts were prepared and incubated with a ³²P-labelled DNA fragment (m67SIE-probe). Protein–DNA complexes were separated from free DNA by PAGE and visualized by autoradiography.

complexes for gp130. This is in agreement with the observed displacement of Trx-IL-11-sIL-11R-flag from the ternary complex with gp130 by IL-6-sIL-6R (Figure 2).

STAT activation induced by IL-11-sIL-11R complexes in 2fTGH cells is dose- and time-dependent

The data presented so far show that IL-11-IL-11R and IL-6-IL-6R complexes bind to gp130 at overlapping binding sites. As a consequence, the downstream signalling events elicited by IL-11 and IL-6 would be expected to be similar. The EMSA presented in Figure 5(A) shows that in 2fTGH fibrosarcoma cells, IL-11-sIL-11R-flag complexes induce STAT activation in a concentration-dependent manner (lanes 5, 6 and 7), whereas neither stimulation with IL-11 alone (lane 3) nor with sIL-11R-flag alone (lane 4) leads to any detectable STAT activation. Again, it is observed that cells are much more sensitive to IL-6-sIL-6R (20 ng/ml and 1 μg/ml respectively; lane 2) than to IL-11-sIL-11R-flag complexes (e.g. lane 6). The time-course of STAT activation by IL-11-sIL-11R-flag or IL-6-sIL-6R in 2fTGH cells is identical (Figure 5B). In both cases a faint band of STAT3 homodimers (lanes 2 and 6) can be detected as early as 3 min after stimulation. A maximal response is observed at about 30 min (lanes 4 and 8), declining during the next 30 min (lanes 5 and 9).

Jak1 is required for STAT activation by IL-11-sIL-11R complexes

In a previous study using well-established mutant human fibrosarcoma cell lines lacking individual Jaks, it was shown that Jak1 plays a major role in the activation of STAT3 and STAT1 after

Figure 6 Comparison of STAT3 and STAT1 activation induced by complexes of IL-11-sIL-11R-flag or IL-6-sIL-6R in fibrosarcoma cells deficient in Jak1, Jak2 or Tyk2

Fibrosarcoma cells (A) (lanes 1–3) deficient in Jak1 (U4A cells), (B) deficient in Jak2 (γ2A cells), and (C) deficient in Tyk2 (U1A cells), and U4A cells retransfected with Jak1 (A, lanes 4–6) were stimulated for 15 min either with IL-11 and sIL-11R-flag (200 ng/ml and 1 μg/ml respectively) or IL-6 and sIL-6R (10 ng/ml and 0.5 μg/ml respectively). Nuclear extracts were prepared and incubated with a ³²P-labelled DNA fragment (m67SIE-probe). Protein–DNA complexes were analysed by EMSA.

IL-6 stimulation [33]. In order to define the Jak requirement for STAT activation by IL-11 these cells were stimulated with IL-11-sIL-11R-flag or IL-6-sIL-6R and STAT activation was analysed by EMSA. In the Jak1-deficient U4A cells, neither IL-11-sIL-11R-flag (Figure 6A, lane 3) nor IL-6-sIL-6R complexes (lane 2) led to a prominent activation of STAT3 or STAT1. When Jak1 was retransfected into U4A cells the response was completely restored (Figure 6A, lanes 6 and 5), suggesting that the lack of Jak1 is the cause for the impaired STAT activation in U4A cells. In Jak2-deficient γ2A cells (Figure 6B), as well as in Tyk2-deficient U1A cells (Figure 6C), STAT activation was observed in response to both IL-11-sIL-11R-flag and IL-6-sIL-6R stimulation, indicating that there is no absolute requirement for these kinases for STAT activation by IL-11 or IL-6.

DISCUSSION

gp130 is the common signal transducing receptor subunit for the so-called IL-6-type cytokines. Whereas LIF, CNTF, OSM and CT-1 induce the heterodimerization of gp130 with a second signal-transducing receptor subunit, namely LIFR or OSMR, IL-6 and IL-11 are able to signal by homodimerization of gp130. The studies presented here were performed to solve the question whether (a) gp130 is recruited by both cytokines in a similar manner and, as a consequence, induces identical signal transduction events, or if (b) different surface epitopes of gp130 are involved in the recognition of IL-11-sIL-11R and IL-6-sIL-6R complexes leading to gp130 homodimers with diverging con-

formations. In the latter case the postulated different gp130 homodimers could induce the Jak-STAT pathway in a different way, e.g. by preferential activation of distinct Jaks or by altering the accessibility of phosphotyrosine motifs in the cytoplasmic part of gp130 which are required for the activation of STAT3 and STAT1.

First of all, human IL-11 was expressed as a Trx fusion protein and it was shown that its biological activity is indistinguishable from IL-11 itself. LaVallie et al. [40] published identical results for a Trx-IL-11 fusion protein which differs from our construct by one N-terminal amino acid of IL-11. Trx is fused to the N-terminus of mature IL-11, a region which, by analogy with other IL-6-type cytokines, is proposed not to be involved in receptor binding [48]. Therefore it is reasonable that an N-terminal extension of IL-11 does not interfere with bioactivity and receptor binding. Soluble human IL-11R was expressed in baculovirus-infected insect cells and the yields were comparable to the ones found in the same cells for the expression of sIL-6R [37]. Since no antibodies are available for the native human IL-11R, the recombinant sIL-11R was equipped with a short C-terminal flag epitope which allows the detection of the protein by a monoclonal flag antibody. The recombinant human sIL-11R showed an apparent molecular mass of 45 kDa and turned out to be biologically active in binding as well as in proliferation assays.

The competition of IL-11-sIL-11R and IL-6-sIL-6R for binding to gp130 indicates that both cytokines, bound to their specific α -subunit, recognize at least overlapping binding epitopes of gp130. The displacement of Trx-IL-11-sIL-11R-flag by IL-6-sIL-6R complexes at relatively low concentrations suggests that Trx-IL-11-sIL-11R-flag binds with lower affinity to gp130. Nandurkar et al. [49] have shown that IL-11 binds with a dissociation constant of 250 pM to Ba/F3 cells cotransfected with membrane-bound human IL-11 receptor and gp130. On hepatoma cells expressing IL-6R and gp130 a dissociation constant for IL-6 binding of 60 pM was found [50]. This difference in dissociation constants is in agreement with the above observation. Coprecipitation experiments with gp130 deletion mutants showed that the part of the molecule which contains the CRHM is sufficient for binding to IL-11-sIL-11R as well as IL-6-sIL-6R complexes [18]. For other cytokine receptors it has also been shown that the CRHM is involved in cytokine binding. The closely related granulocyte colony-stimulating factor receptor (G-CSFR) utilizes the N-terminal IgG-like domain in addition to the CRHM for G-CSF binding [51]. Whether the N-terminal domain of gp130 is involved in binding of the IL-6-type cytokines remains to be shown.

The binding epitope for IL-11-sIL-11R was further narrowed down using gp130 point mutants stably expressed in Ba/F3 cells which were previously used to define the IL-6-sIL-6R binding epitope [19]. Val-252 in the BC-loop of the C-terminal domain of the gp130-CRHM turned out to be a crucial residue for the binding of IL-11-sIL-11R as well as IL-6-sIL-6R complexes. Similar results were found in studies on the common β -chain (β_c) which also belongs to the cytokine receptor superfamily. This transmembrane protein is a common signal transducing receptor subunit for the cytokines IL-3, IL-5 and granulocyte/macrophage CSF (GM-CSF). Here, Tyr-251 in the membrane-proximal CRHM of β_c has been identified to be necessary for the binding of all three ligands [52]. Further investigations on this receptor chain revealed that the side-chains Tyr-365, His-367 and Ile-368 are required for GM-CSF and IL-5, but not IL-3, high-affinity binding, indicating that the binding epitope for IL-3 is overlapping but not identical with the one for GM-CSF and IL-5. Studies with mAbs directed against the ectodomain of gp130

suggest that the binding epitopes for IL-6-sIL-6R and IL-11-sIL-11R are not identical, since an mAb has been identified which selectively inhibits IL-11, but not IL-6 [53], although this selectivity depends on the cell type used [45]. Identification of the amino acid side-chains of gp130 that are required for selective cytokine binding deserves further investigation.

From the above observations it can be concluded that the ternary complexes of IL-11-IL-11R-gp130 and IL-6-IL-6R-gp130 have a very similar overall geometry. As outlined in the beginning of this discussion, as a consequence, the signalling events exerted by IL-11 and IL-6 would be expected to be equivalent. To test this hypothesis, as a first approach 2fTGH cells and cells deficient for individual Jaks were stimulated with IL-11-sIL-11R and IL-6-sIL-6R and subsequently analysed for STAT activation. 2fTGH cells do not respond to IL-11 alone, which is probably caused by the lack of membrane-bound IL-11R. In response to both cytokines and their soluble receptors, STAT3 is predominantly activated. When cells were stimulated for a prolonged period of time, or with increasing amounts of cytokine and soluble receptor, STAT3/1 heterodimers and STAT1 homodimers were also observed. The time-course of STAT activation by IL-11 and IL-6 is identical, peaking around 30 min after stimulation. Again, larger amounts of IL-11-sIL-11R are required to achieve responses comparable with those elicited by IL-6-sIL-6R stimulation. There is, however, no hint of a qualitative differential activation of STAT3 and STAT1 after stimulation with IL-11 or IL-6. Accordingly, as for IL-6 [33], STAT activation by IL-11 requires Jak1.

These findings may explain the observed extreme redundancy of the cellular responses elicited by IL-11 and IL-6 [54,55]. Differential responses of a given cell type (e.g. myeloma cells [56]) to stimulation by IL-11 or IL-6 can therefore be explained by differential expression of the specific α -receptor subunits. From our studies it cannot be excluded that, depending on the cell type, IL-11-IL-11R and IL-6-IL-6R recruit additional components to the receptor complex, possibly in a different manner [12]. Furthermore, differences in the kinetics of complex formation by IL-11 and IL-6 may have consequences for the quality of the cytoplasmic signal transduction cascade and cellular response. Thus in some circumstances IL-11 and IL-6 may exert different biological responses which cannot be explained solely by differential α -subunit expression, although no such case has been reported so far. Taken together, our results support the view that IL-11 and IL-6, in the presence of their specific α -receptor subunits, bind gp130 at overlapping epitopes, leading to ternary complexes of similar geometry. As a consequence, the downstream signalling events, as far as they have been investigated in this study, are indistinguishable, leading to the observed identical cellular responses.

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