THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 46, Issue of November 17, pp. 36197–36203, 2000

Printed in U.S.A.

Interleukin-11 Signals through the Formation of a Hexameric Receptor Complex*

Received for publication, May 30, 2000, and in revised form, August 15, 2000
Published, JBC Papers in Press, August 17, 2000, DOI 10.1074/jbc.M004648200

Victoria A. Barton‡, Mark A. Hall‡§, Keith R. Hudson¶, and John K. Heath‖

From the Cancer Research Campaign Growth Factor Group, School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom

Interleukin-11 (IL-11) is a member of the gp130 family of cytokines. These cytokines drive the assembly of multisubunit receptor complexes, all of which contain at least one molecule of the transmembrane signaling receptor gp130. IL-11 has been shown to induce gp130-dependent signaling through the formation of a high affinity complex with the IL-11 receptor (IL-11R) and gp130. Site-directed mutagenesis studies have identified three distinct receptor binding sites of IL-11, which enable it to form this high affinity receptor complex. Here we present data from immunoprecipitation experiments, using differentially tagged forms of ligand and soluble receptor components, which show that multiple copies of IL-11, IL-11R, and gp130 are present in the receptor complex. Furthermore, it is demonstrated that sites II and III of IL-11 are independent gp130 binding epitopes and that both are essential for gp130 dimerization. We also show that a stable high affinity complex of IL-11, IL-11R, and gp130 can be resolved by nondenaturing polyacrylamide gel electrophoresis, and its composition verified by second dimension denaturing polyacrylamide gel electrophoresis. Results indicate that the three receptor binding sites of IL-11 and the Ig-like domain of gp130 are all essential for this stable receptor complex to be formed. We therefore propose that IL-11 forms a hexameric receptor complex composed of two molecules each of IL-11, IL-11R, and gp130.

Interleukin-11 (IL-11)[1] is a secreted polypeptide cytokine, which has been shown to exhibit *in vitro* biological effects on a diverse range of cell types including hemopoietic cells, hepatocytes, adipocytes, neurons, and osteoblasts (reviewed in Ref. 1). *In vivo* administration of IL-11 results in the stimulation of megakaryopoeisis and increased platelet counts (2). Recombinant human IL-11 is now used for the treatment of chemotherapy-induced thrombocytopenia (3). IL-11 also has clinical potential for the treatment of several disorders including chemotherapy induced oral mucositis (4), Crohn's disease (5), and rheumatoid arthritis (6). Transgenic deletion of the gene encoding the specific IL-11 receptor (IL-11R) in mice has revealed an important role for IL-11 in embryonic implantation. Female mice deficient in the IL-11R are infertile because of defective decidualization, following implantation of the embryo (7, 8).

IL-11 is a member of the gp130 family of cytokines. These cytokines drive the assembly of multisubunit receptor complexes, which initiates intracellular signal transduction pathways. In all cases, the receptor complexes contain at least one copy of the signal transducer glycoprotein gp130 (9). Other cytokines belonging to this family include interleukin-6 (IL-6), leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1, and a viral homologue of IL-6 encoded by the Kaposi's sarcoma-associated herpesvirus. Each of these cytokines exerts its action by either homo- or heterodimerization of gp130, which leads to the stimulation of signaling cascades via protein kinases belonging to the Janus kinase, mitogen-activated protein kinase, and Src families (10–13).

The gp130 cytokines exhibit both overlapping and unique biological activities *in vitro* and *in vivo* (reviewed in Ref. 14). The signal exerted by a cytokine and therefore the biological response depends on the exact composition of the signaling receptor complex. Signaling specificity of the gp130 cytokines is conferred by the use of ligand-specific receptors. Specific receptors for IL-6 (15), IL-11 (16, 17), and CNTF (18) have been identified. These receptors are not directly involved in cytoplasmic signaling, but their function is to promote the formation of a high affinity complex between the respective ligand and gp130. These ligand specific receptors and gp130 are all members of the hemopoietic family of receptors (reviewed in Ref. 19), characterized by the presence of a cytokine binding homology domain (CHD).

The CHD, of approximately 200 amino acids, comprises two fibronectin type III domains (D1 and D2), with four positionally conserved cysteine residues in the first domain and a WS*X*WS motif (where *X* is any amino acid) in the second domain (20). The crystal structure of the CHD of gp130 revealed that the two fibronectin type III domains exhibit an approximate L-shape (21), and mutagenesis studies have identified residues in the hinge region that are important for ligand binding (22–24). In addition to the CHD, gp130 and all known receptors that bind to the gp130 family of cytokines also contain an amino-terminal domain predicted to adopt a seven-β-stranded immunoglobulin-like conformation in their extracellular region (Ig-

* This work was supported by Cancer Research Campaign grants and a Cancer Research Campaign studentship from 1996 to 1999 (to V. A. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ These authors contributed equally to this work and should both be considered first authors.

§ Current address: Walter and Elisa Hall Inst. for Medical Research and the Rotary Bone Marrow Research Laboratories, Royal Melbourne Hospital, Parkville, Victoria, Australia 3050.

¶ Current address: Genesis Research and Development Corp., Parnell, Auckland, New Zealand.

‖ To whom correspondence should be addressed. Tel.: 44-0-121-414-7533; Fax: 44-0-121-414-3983; E-mail: j.k.heath@bham.ac.uk.

[1] The abbreviations used are: IL-11, interleukin-11; R, receptor; IL-6, interleukin-6; LIF, leukemia inhibitory factor; OSM, oncostatin M; CNTF, ciliary neurotrophic factor; CHD, cytokine binding homology domain; Ig, immunoglobulin; m (prefix), murine; Fc, constant region of human IgG; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; b (prefix), biotinylated; HA

The gp130 cytokines share a common four-α-helix bundle fold. Crystal structures have been determined for LIF (25), CNTF (26), IL-6 (27), and OSM.[2] Detailed structural analysis and mutagenesis studies of the gp130 family of cytokines have revealed clear patterns of receptor engagement (reviewed in Ref. 29). It is now apparent that receptor binding epitopes are conserved among the gp130 cytokines. Three receptor binding sites have been identified for IL-6 (30, 31), IL-11 (32), and CNTF (33, 34) (termed sites I, II, and III). Sites I and II are analogous to the two receptor binding sites identified for human growth hormone (35). In contrast, LIF and OSM have been shown to have two binding sites (sites II and III), which enable them to form trimeric receptor complexes (36).[2] IL-6 is known to form a hexameric receptor complex consisting of two molecules each of IL-6, IL-6R, and gp130 (37, 38).

IL-11 has been shown to have three distinct receptor binding sites analogous in location to sites I, II, and III of IL-6 (32). Site I enables IL-11 to bind to IL-11R, while sites II and III both mediate binding to gp130. Taken together with the finding that IL-11 signaling requires IL-11R and gp130, but not LIF receptor (LIFR) or OSM receptor (OSMR) (16, 39), it is predicted that IL-11 forms a signaling complex in a manner analogous to that of IL-6. However, published work regarding the composition and stoichiometry of the IL-11 receptor complex has, as yet, not been conclusive. Neddermann *et al.* (40) reported a pentameric IL-11 receptor complex consisting of two IL-11, two IL-11R, and one gp130. They suggested that gp130 homodimerization is not involved in IL-11-mediated signaling and that another, as yet unidentified, signaling receptor component is required. Furthermore, Grotzinger *et al.* (41) have suggested that the IL-11 receptor complex may be a tetramer, consisting of one IL-11, one IL-11R, and two gp130 molecules (41). Here we report findings from *in vitro* immunoprecipitation experiments and gel shift assays, which clearly demonstrate that the IL-11 receptor complex is a hexamer, consisting of two molecules each of IL-11, IL-11R, and gp130.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The design and construction of pIG/mIL-11R-Fc, pIG/mgp130-Fc, and pGEX/mIL-11 plasmids (IL-11 wild type, R111A/L115A, and W147A) has been described previously (32, 39). The pIG/mgp130(Ig$^-$)Fc plasmid was derived from pIG/mgp130-Fc (39) by a PCR overlap technique. Two DNA fragments upstream and downstream of the region encoding the NH_2-terminal Ig-like domain of gp130 were generated by PCR, using two external primers and two internal primers with overlapping ends (the sequences of all primers used are available on request). The two DNA fragments were then combined in a subsequent PCR reaction with the two external primers, which amplified the fusion product of the two fragments. This fusion product was then cloned back into pIG/mgp130-Fc, therefore replacing the region encoding the Ig-like domain of gp130.

pIG/mIL-11R-Myc and pIG/mgp130-Myc, which encode IL-11R and gp130 ectodomains with COOH-terminal Myc tags, were derived from pIG/mIL-11R-Fc and pIG/mgp130-Fc (39) by subcloning. For both, the region encoding the Fc region of human IgG1, bounded by *Eco*4711 and *Not*I, was replaced with a 401-base pair *Eco*4711/*Not*I fragment from pIG/mLIFR-poly(Asn)-Myc[3] that encodes six Asn residues followed by the Myc antibody epitope (EEQKLISEEDL).

pGEX/HA-IL-11 was constructed as follows. The sequence encoding the hemagglutinin (HA) tag (YPYDVPDYA) was added to the 5′ end of the IL-11 coding region by PCR, using a 5′ primer encoding a *Bam*HI restriction site and the HA tag, and a 3′ primer encoding an *Eco*RI restriction site. The PCR product was then subcloned as a 571-base pair *Bam*HI/*Eco*RI fragment into pGEX-3C (42).

pCDNA3/IL-11R-HA(3C)Fc was generated by the addition of the HA tag coding sequence at the 3′ end of IL-11R by PCR. The IL-11R-ectodomain coding region was amplified using a 5′ primer encoding an *Eco*RI restriction site, and a 3′ primer encoding the HA tag and a *Bam*HI restriction site. The PCR fragment was then subcloned as a 1137-base pair *Eco*RI/*Bam*HI fragment into pcDNA3–3C-Fc.[4]

Expression and Purification of Proteins—Murine IL-11R-Fc, gp130-Fc, gp130(Ig$^-$)Fc, IL-11R-HA-Fc, IL-11R-Myc, and gp130-Myc were expressed in human embryonic kidney 293T cells (43) by transient transfection, as described previously (39). Conditioned media containing Fc fusion receptors were then subjected to Protein A affinity chromatography and receptor ectodomains were released by on-column cleavage with the human rhinovirus protease 3C (44). Conditioned media containing Myc-tagged proteins were stored at −20 °C until required. IL-11, wild type and mutants, and HA-tagged IL-11 were expressed as glutathione *S*-transferase fusion proteins in *Escherichia coli* (strain JM109). Details of induction, purification using glutathione-Sepharose (Amersham Pharmacia Biotech) and cleavage using human rhinovirus protease 3C, are as described previously for leukemia inhibitory factor (36).

Proteins were analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue R250, or Western blotting and detection with antisera. For Western blotting, proteins were transferred onto polyvinylidene difluoride (Millipore) using a standard protocol (45). Membranes were then blocked overnight in PBS, 3% BSA and subjected to immunodetection using antisera diluted in blocking buffer. Blots were developed using Super Signal West-Pico enhanced chemiluminescence (ECL) (Pierce).

Biotinylation of Proteins—IL-11 was modified by biotinylation on ϵ-amino groups of lysine residues using biotin amidocaproate *N*-hydroxysuccinimide (Sigma) following a published protocol (32). gp130 was biotinylated on oxidized oligosaccharides using biotin-hydrazide (Pierce) as follows; gp130 was first buffer-exchanged into phosphate/EDTA buffer (100 mM sodium phosphate, pH 6.0, 5 mM EDTA) and treated with 20 mM sodium *m*-periodate for 20 min at 4 °C in the dark. gp130 was then buffer exchanged into fresh phosphate/EDTA buffer and reacted with an equimolar quantity of biotin-hydrazide for 16–18 h at 4 °C. Dialysis against PBS was carried out to remove unbound biotin. Biotinylated proteins were examined by SDS-PAGE followed by Western blotting and detection with streptavidin-HRP conjugate (Amersham Pharmacia Biotech) and ECL.

Co-immunoprecipitation of Differentially Tagged Cytokine-Receptor Complexes—Slightly different strategies were adopted for co-immunoprecipitation of the different components of the IL-11 receptor complex. For immunoprecipitation of bIL-11·HA-IL-11 complexes, equimolar concentrations (100 nM) of HA-IL-11, bIL-11, gp130, and IL-11R were mixed together in various combinations, in a total volume of 500 μl of binding buffer (PBS, 1% BSA, 0.05% Tween 20). Mixtures were incubated for 3 h at room temperature at which point, NeutrAvidin-agarose (Pierce) (10 μl) was added and then agitated for 16–18 h at 4 °C.

For immunoprecipitation of complexes containing Myc-tagged components, 5 μl of anti-Myc monoclonal antibody (clone 9E10, BABCo) was first immobilized on 8 μl of Protein G-Sepharose (Amersham Pharmacia Biotech), in a final volume of 500 μl of binding buffer. The resin was then used to immunoprecipitate gp130-Myc from 500 μl of 293T conditioned medium. This "loaded" resin was then added to 500 μl of binding buffer containing equimolar concentrations (100 nM) of IL-11, IL-11R, and bgp130, in various combinations, and incubated for 16–18 h at 4 °C with agitation. Similarly, IL-11R-Myc was immunoprecpitated using resin coated with anti-Myc monoclonal antibody and then added to 500 μl of binding buffer containing equimolar concentrations (100 nM) of IL-11, IL-11R-HA, and gp130 or bgp130, in various combinations, and incubated for 16–18 h at 4 °C with agitation.

Following all immunoprecipitation reactions, complexes were harvested by centrifugation, extensively washed with PBS, 0.1% Tween 20, and resuspended in 20 μl of SDS-PAGE loading buffer (300 mM Tris, pH 6.8, 600 mM dithiothreitol, 12% SDS, 0.6% bromphenol blue, 30% glycerol). The immunoprecipitated proteins were then resolved by SDS-PAGE, Western-blotted, and detected using a mouse anti-HA monoclonal antibody (clone 12CA5, Roche Molecular Biochemicals) or streptavidin-HRP conjugate, followed by ECL.

Nondenaturing PAGE and Second Dimension Denaturing PAGE—Equimolar concentrations of IL-11 and soluble receptor components were mixed together, in various combinations, in a total volume of 16 μl of PBS, 0.05% Tween 20. Complexes were allowed to form for a minimum of 4 h at 18–22 °C. 4 μl of native gel loading buffer (120 mM Tris, pH 6.8, 745 mM glycine, 50% glycerol, 0.5% bromphenol blue) was then added and each sample loaded onto a 4–20% Tris-glycine gel (Novex).

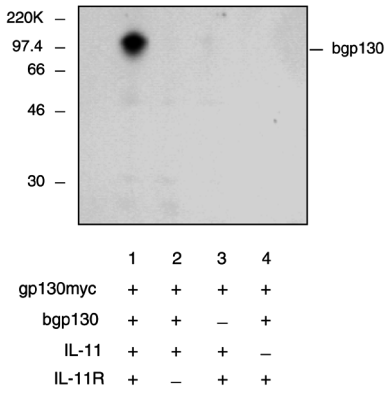

FIG. 1. **Immunoprecipitation of gp130-Myc complexes.** gp130-Myc was immobilized on resin and incubated with combinations of bgp130, IL-11, and IL-11R (100 nM amounts of each). After incubation and washing, bound components were analyzed by SDS-PAGE, followed by Western blotting. Detection was carried out using streptavidin-HRP conjugate (diluted 1 in 4,000) and ECL.

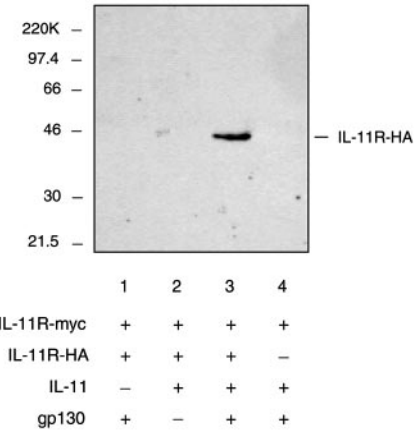

FIG. 2. **Immunoprecipitation of IL-11R-Myc complexes.** IL-11R-Myc was immobilized on resin and incubated with combinations of IL-11R-HA, gp130, and IL-11 (100 nM amounts of each). After incubation and washing, bound components were analyzed by SDS-PAGE followed by Western blotting. Detection was carried out using a biotinylated anti-HA monoclonal antibody (diluted 1 in 5,000) followed by streptavidin-HRP conjugate (diluted 1 in 5,000) and ECL.

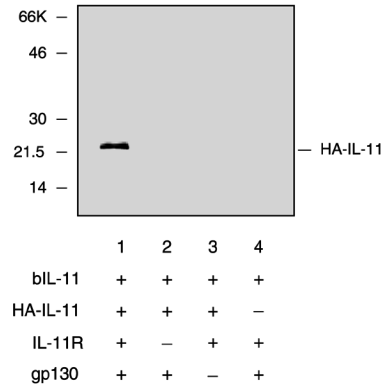

FIG. 3. **Immunoprecipitation of bIL-11 complexes.** Combinations of bIL-11, HA-IL-11, IL-11R, and gp130 were mixed together and incubated (100 nM amounts of each). Complexes were then immunoprecipitated using NeutrAvidin-agarose. After washing, bound components were analyzed by SDS-PAGE followed by Western blotting. Detection was carried out using an anti-HA monoclonal antibody (diluted 1 in 5,000), followed by sheep anti-mouse HRP conjugate (diluted 1 in 5,000) and ECL.

Electrophoresis was then carried out at 15 mA for 2 h in native running buffer (24 mM Tris, 149 mM glycine). Proteins were detected using either Coomassie Brilliant Blue R250 or silver staining (46).

For second dimension SDS-PAGE, Coomassie-stained bands were excised from the gel and soaked in SDS loading buffer (62.5 mM Tris, pH 6.8, 5% 2-mercaptoethanol, 2% SDS, 0.1% bromphenol blue, 10% glycerol) for 5 min. The proteins were then resolved by SDS-PAGE using a 12% polyacrylamide gel and detected by silver staining (46).

RESULTS

Multiple Copies of gp130, IL-11R, and IL-11 Are Present in the IL-11 Receptor Complex—IL-11-mediated signaling has been shown to require both IL-11R and gp130 (16, 32, 39). A specific interaction between IL-11 and the IL-11R has been demonstrated, and a soluble form of IL-11R has been shown to promote the formation of a high affinity complex between IL-11 and gp130 (39). To assess the stoichiometry of this high affinity IL-11 receptor complex immunoprecipitation experiments using differentially tagged receptor components, similar to the assays used by Paonessa *et al.* (38), were carried out. Each component of the receptor complex was labeled with two different tags. Receptor complexes were then immunoprecipitated using one tag and examined by Western blot analysis to determine whether the second tag could be detected. The ability to co-precipitate differentially tagged forms of a protein indicated the presence of two copies of that protein in the receptor complex.

To examine the number of gp130 molecules in the IL-11 receptor complex, Myc-tagged gp130 was first immobilized on Sepharose beads coated with anti-Myc monoclonal antibody. Immunoprecipitations were then carried out using various combinations of IL-11, IL-11R, and biotinylated gp130 (bgp130). Following SDS-PAGE and Western blotting, immunoprecipitated bgp130 was detected using streptavidin-HRP conjugate. The results, as shown in Fig. 1, indicate that bgp130 (which migrates with an approximate molecular mass of 97 kDa) was co-precipitated with gp130-Myc, in the presence of IL-11 and IL-11R (see *lane 1*). In the absence of either IL-11R or IL-11, immunoprecipitated bgp130 could not be detected (see *lanes 2* and *4*). These results show that at least two copies of gp130 are present in the IL-11 receptor complex and that both IL-11 and IL-11R are required for gp130 dimerization.

A similar approach, using Myc-tagged IL-11R and HA-tagged IL-11R, was used to investigate the number of IL-11R molecules present in the IL-11 receptor complex. Following results, as shown in Fig. 2, show that IL-11R-HA (which migrates with an approximate molecular mass of 45 kDa) was co-precipitated with IL-11R-Myc, but only in the presence of both IL-11 and gp130 (see *lane 3*). This indicates that the high affinity IL-11 receptor complex contains at least two copies of IL-11R.

To examine the number of IL-11 molecules in the IL-11 receptor complex, biotinylated IL-11 (bIL-11) was first immobilized on NeutrAvidin-agarose. Immunoprecipitations were then carried out using various combinations of IL-11R, gp130, and HA-tagged IL-11. The presence of HA-IL-11 in the immunoprecipitates was detected using anti-HA antiserum. The results, as shown in Fig. 3, show that HA-IL-11 (which migrates with an approximate molecular mass of 22 kDa) was co-precipitated with bIL-11, but only in the presence of both IL-11R and gp130 (see *lane 1*). This indicates that at least two copies of IL-11 are present in the high affinity IL-11 receptor complex. The complex thus contains multiple copies of IL-11, IL-11R,

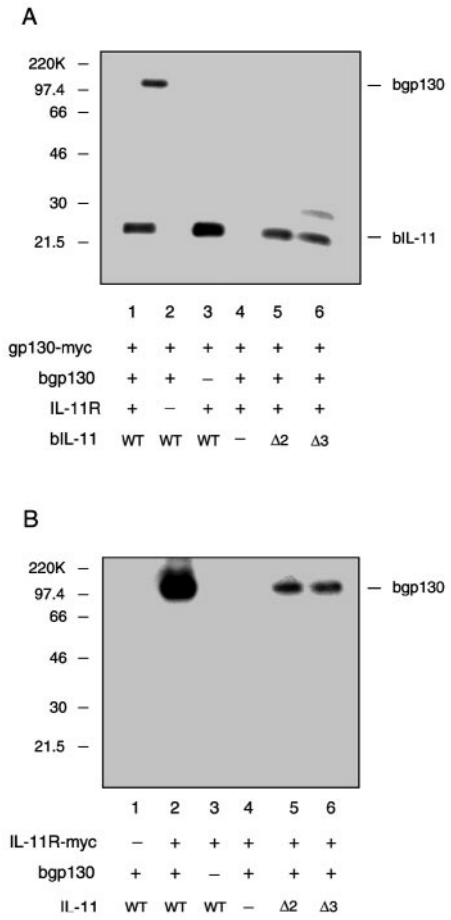

FIG. 4. **Immunoprecipitation of gp130-Myc and IL-11R-Myc complexes formed by IL-11 mutants.** *A*, gp130-Myc was immobilized on resin and incubated with combinations of bgp130, IL-11R, and bIL-11 (100 nM amounts of each). *B*, IL-11R-Myc was immobilized on resin and incubated with combinations of bgp130 and bIL-11 (100 nM amounts of each). *WT* represents wild type, while *Δ2* and *Δ3* represent the site II mutant R111A/L115A and the site III mutant W147A, respectively. After incubation and washing, bound components were analyzed by SDS-PAGE followed by Western blotting. Detection was carried out using streptavidin-HRP conjugate (diluted 1 in 4,000) and ECL.

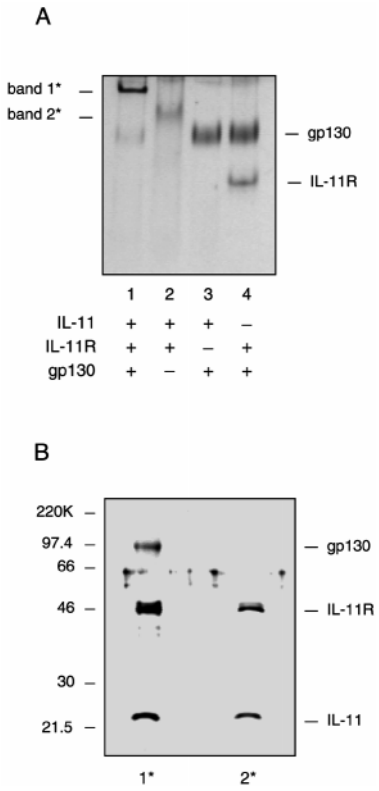

FIG. 5. **Nondenaturing PAGE and second dimension SDS-PAGE of IL-11 receptor complex.** *A*, native PAGE. Equimolar concentrations (1 μM) of IL-11, IL-11R, and gp130 were mixed together in various combinations. After incubation, complexes were subjected to nondenaturing PAGE. Proteins were detected using Coomassie stain. *B*, SDS-PAGE. Bands 1* and 2* (see Fig. 5*A*) were excised from the Coomassie-stained gel, soaked in SDS loading buffer, and subjected to SDS-PAGE. Proteins were detected by silver staining.

scribed above, were also used to examine the ability of IL-11 mutants to form high affinity receptor complexes. It has previously been shown that both the site II mutant, R111A/L115A, and the site III mutant, W147A, exhibit reduced binding to gp130 and hence reduced biological activity, while maintaining normal affinity for IL-11R (32). Immunoprecipitation assays were performed using immobilized gp130-Myc and various combinations of bgp130, IL-11R, and wild type or mutant bIL-11. By using both biotinylated ligand and bgp130, this enabled us to examine the ability of the IL-11 mutants to bind to gp130-Myc in the presence of IL-11R, and also the ability of the mutants to co-precipitate bgp130. The results, as shown in Fig. 4*A*, confirm those described earlier, *i.e.* bgp130 is only co-precipitated with gp130-Myc in the presence of IL-11R and wild type IL-11 (see *lane 1*). Neither the site II mutant, R111A/L115A, nor the site III mutant, W147A, were able to dimerize gp130, as bgp130 was not co-precipitated with gp130-Myc in the presence of IL-11R (Fig. 4*A*, see *lanes 5* and *6*). However, the fact that biotinylated mutant ligand was detected (see *lanes 5* and *6*) indicates that both of the IL-11 mutants were co-precipitated with gp130-Myc, in the presence of IL-11R, even required for the dimerization of gp130. The results suggest that both the site II mutant and the site III mutant, although unable to dimerize gp130, can bind a single molecule of gp130 in the presence of IL-11R. This was confirmed by the co-precipitation of bgp130 with IL-11R-Myc by the two mutants, as shown in Fig. 4*B*. These results indicate that mutation of one gp130 binding site (either site II or site III) does not affect the other gp130 binding site, which remains free and intact to bind a single molecule of gp130. If IL-11 binds to IL-11R with a 1:1 stoichiometry, this indicates that the two mutants formed trimeric complexes, consisting of one molecule each of IL-11, IL-11R, and gp130.

A Stable Complex of IL-11, IL-11R, and gp130 Can Be Resolved by Nondenaturing PAGE—The results described above, together with the fact that a complex of IL-11 and soluble IL-11R can mediate signaling by association with gp130 (39), suggest that interactions between IL-11 and the extracellular regions of gp130 and IL-11R are sufficient for the formation of a high affinity receptor complex. A stable complex formed between IL-11 and soluble forms of IL-11R and gp130 can be resolved by nondenaturing PAGE. Equimolar quantities (1 μM) of IL-11, soluble IL-11R, and soluble gp130 were mixed together in various combinations, incubated to allow complexes to form, and subjected to nondenaturing PAGE. The results, as shown in Fig. 5*A*, show that a complex of IL-11, IL-11R, and gp130 can be resolved as a discrete band (see *lane 1*), which was not be detected if any one of the three components was absent.

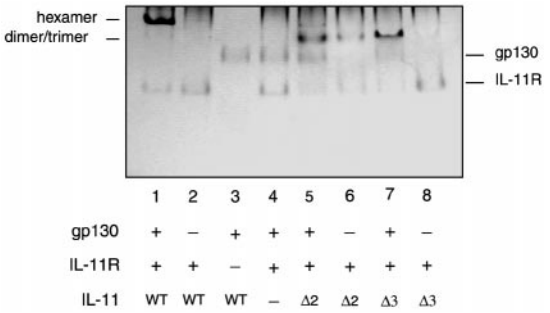

FIG. 6. **Nondenaturing PAGE of receptor complexes formed by IL-11 mutants.** Equimolar concentrations (300 nM) of IL-11, IL-11R, and gp130 were mixed together in various combinations. *WT* represents wild type, while *Δ2* and *Δ3* represent the site II mutant R111A/L115A and the site III mutant W147A, respectively. After incubation, complexes were subjected to nondenaturing PAGE and detection was carried out using silver staining (46).

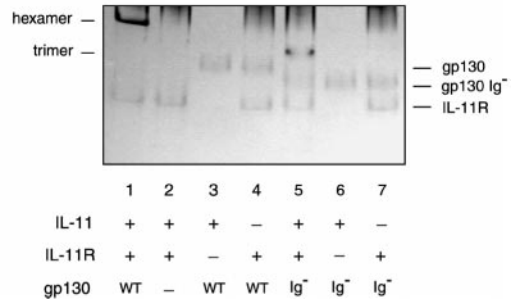

FIG. 7. **Nondenaturing PAGE of IL-11 receptor complexes formed by an Ig deletion mutant of gp130.** Equimolar concentrations (300 nM) of IL-11, IL-11R, and gp130 or gp130(Ig^-) were mixed together in various combinations. After incubation, complexes were subjected to nondenaturing PAGE and detection was carried out using silver staining (46).

value (predicted to be 11.7). A complex of IL-11 and IL-11R was also detected as a faint band, which had migrated further into the gel compared with the IL-11·IL-11R·gp130 complex (see Fig. 5*A*, *lane 2*). However, this IL-11·IL-11R complex was only observed if high concentrations (1 μM) of recombinant protein were used. If lower concentrations (in the nanomolar range) of recombinant protein were used, only the high affinity IL-11·IL-11R·gp130 complex could be detected (see Fig. 6). This is probably because complexes of IL-11 and IL-11R dissociate more easily compared with ternary complexes containing IL-11, IL-11R, and gp130. IL-11 has been previously shown to bind gp130 in the presence of IL-11R with higher affinity compared with binding IL-11R alone (16, 39).

The high affinity complex resolved by nondenaturing PAGE (labeled as *band 1** in Fig. 5*A*) was predicted to contain IL-11, IL-11R, and gp130 because in the absence of either one of the components it could not be detected. The composition of the complex was confirmed by second dimension SDS-PAGE. The results, as shown in Fig. 5*B*, indicate that IL-11, IL-11R, and gp130 were all present in the ternary complex, which resolved as a discrete band during nondenaturing PAGE. Similarly, the composition of the IL-11·IL-11R complex (labeled as *band 2** in Fig. 5*A*) was confirmed using this method (see Fig. 5*B*).

IL-11 Site II and Site III Mutants Are Unable to Form a Stable Ternary Receptor Complex—To further investigate the stoichiometry of the ternary receptor complex, observed as a discrete band following nondenaturing PAGE, the ability of IL-11 mutants to form such a complex was examined. The results described earlier indicate that the site II mutant, R111A/L115A, and the site III mutant, W147A, are both unable to dimerize gp130, although they can bind a single molecule of gp130 in the presence of IL-11R. The ability of these two mutants to form a stable receptor complex was therefore examined using nondenaturing PAGE. Equimolar concentrations (300 nM) of IL-11, IL-11R, and gp130 were mixed together, incubated, and subjected to nondenaturing PAGE. Receptor complexes were then visualized using silver staining (46). The results, as shown in Fig. 6, were consistent with those described above, *i.e.* a complex of IL-11 wild type, IL-11R, and gp130 was observed as a single discrete band (see *lane 1*), which could not be detected if either IL-11R or gp130 were absent. A complex of IL-11 and IL-11R was not detectable using these concentrations of recombinant protein (300 nM).

The results in Fig. 6 also show that both the site II mutant, R111A/L115A, and the site III mutant, W147A, were unable to ing with the ternary complex formed by IL-11 wild type, but also a second complex, which has migrated further into the gel. This second complex appears to co-migrate with a dimer of IL-11 R111A/L115A and IL-11R, observed in *lane 6*. However, the intensity of the band is stronger in the presence of gp130 (compare *lanes 5* and *6*), which, together with the earlier results from immunoprecipitation experiments, indicate that this band is a trimer. The fact that a dimer of IL-11 R111A/L115A and IL-11R was detected, while a dimer of IL-11 wild type and IL-11R was not detectable, correlates with the fact that the site II mutant has a 4-fold increase in affinity for IL-11R compared with IL-11 wild type, as described previously (32). The results in Fig. 6 also show that the site III mutant, W147A, was unable to form a stable receptor complex co-migrating with that of IL-11 wild type during nondenaturing PAGE. Instead, a single band that migrated further into the gel was detected (see *lane 7*). This band was not detected in the absence of gp130 (see *lane 8*), which suggests that it represents a trimeric receptor complex, as opposed to a dimer of IL-11 W147A and IL-11R.

The results described here from nondenaturing PAGE, and the immunoprecipitation experiments described earlier, suggest that the single discrete band formed by IL-11 wild type, IL-11R, and gp130 (as seen in *lanes 1* of Figs. 5*A* and 6) represents a hexamer. Trimeric receptor complexes can also be detected as single bands although they co-migrate with dimers of IL-11·IL-11R and, like the dimers, they appear to be less stable than the hexamer. The fact that the site II mutant exhibited a significant reduction in its ability to form a hexamer, compared with IL-11 wild type (i.e small amounts of hexamer were detected), while the site III mutant was unable to form a hexamer correlates with the observed biological activities of these two mutants. That is, R111A/L115A shows more than a 10-fold reduction in biological activity, compared with IL-11 wild type, while the activity of W147A is completely abolished, as described previously (32).

The Ig-like Domain of gp130 Is Required for a Stable Ternary Complex of IL-11, IL-11R, and gp130 to Be Formed—The ability of a gp130 mutant, lacking the Ig-like domain, to form a stable receptor complex was also examined using nondenaturing PAGE. Various combinations of IL-11 wild type, IL-11R, and either wild type or the Ig deletion mutant of gp130 were mixed together, incubated, and subjected to nondenaturing PAGE. Receptor complexes were then visualized using silver staining (46). The results, as shown in Fig. 7, show that a complex of IL-11, IL-11R, and the Ig deletion mutant of gp130 was detectable as a band, which migrated further into the gel,

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.