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

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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) 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 megakaryopoiesis 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 WSXWS 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-like domain).
IL-11 Forms a Hexameric Receptor Complex

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. 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). 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), 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 pGm/mIL-11R-Fc, pGm/mgp130-Fc, and pGEX/mIL-11 plasmids (IL-11 wild type, R111A/L115A, and W147A) has been described previously (32, 39). The pG/mgp130 Ig Fc plasmid was derived from pG/mgp130-Fc (39) by a PCR overlap technique. Two DNA fragments upstream and downstream of the region encoding the NH2-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 into pG/mgp130-Fc, therefore replacing the region encoding the Ig-like domain of gp130.

pG/mIL-11R-Myc and pGm/mgp130-Myc, which encode IL-11R and gp130 ectodomains with COOH-terminal Myc tags, were derived from pG/mIL-11R-Fc and pGm/mgp130-Fc (39) by subcloning. For both, the region encoding the Fc region of human IgG1, bounded by Eco4711 and NotI, was replaced with a 401-base pair Eco4711/NotI fragment from pG/mILFRR-polyn(A)-Myc2 that encodes six Asn residues followed by the Myc antibody epitope (EQQKLISEELD). pGEXHA-IL-11 was constructed as follows. The sequence encoding the hemagglutinin (HA) tag (YPYDDVPDYA) was added to the 5′ end of the IL-11 coding region by PCR, using a 5′ primer encoding a BamHI restriction site and the HA tag, and a 3′ primer encoding an EcoRI restriction site. The PCR product was then subcloned as a 571-base pair BamHI/EcoRI fragment into pGEX-3C (42).

pCDNA3/IL-11-HA/3C/Fc was generated by the addition of the HA tag coding sequence to the 3′ end of IL-11 by PCR. The IL-11 ectodomain coding region was amplified using a 5′ primer encoding an EcoRI restriction site, and a 3′ primer encoding the HA tag and a BamHI restriction site. The PCR fragment was then subcloned as a 1337-base pair EcoRI/BamHI fragment into pcDNA3–3C-Fc.4 Expression and Purification of Proteins—Murine IL-11-Fc, gp130-Fc, gp130-lo g, -Fce, IL-11R-HA-Fc, IL-11R-Myc, and gp130-Myc were expressed in human embryonic kidney 293T cells (strain 293T cells) 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 the 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 antibodies. 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 (Pierce).

Biotinylation of Proteins—IL-11 was modified by biotinylation on ε-amino groups of lysine residues using biotin amidoacproate 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 nm 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) or 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, NeutAvidin-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, BABCco) 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 immunoprecipitated 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, 6 M dithiothreitol, 12% SDS, 0.6% bromophenol 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 46 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% bromophenol blue) was then added and each sample loaded onto a 4–20% Tris-glycine gel (Novex).
IL-11 Forms a Hexameric Receptor Complex

**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 immunoprecipitations, using immobilized IL-11R-Myc and various combinations of IL-11, gp130, and IL-11R-HA, the presence of the latter was detected using anti-HA antiserum. The 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, and gp130.

**IL-11 Site II and Site III Mutants Are Unable to Dimerize gp130—**Immunoprecipitation experiments, similar to those de-
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 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. 4A, 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 bgp130, as bgp130 was not co-precipitated with gp130-Myc in the presence of IL-11R (Fig. 4A, 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 though a second molecule of gp130 was not detected.

These results provide evidence that sites II and III of IL-11 are independent gp130 binding sites and that both sites are 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. 4B. 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 trim  

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, IL-11R, and gp130 were mixed together in various combinations, incubated to allow complexes to form, and subjected to nondenaturing PAGE. Proteins were detected using Coomassie stain. B, SDS-PAGE. Bands 1* and 2* (see Fig. 5A) were excised from the Coomassie-stained gel, soaked in SDS loading buffer, and subjected to SDS-PAGE. Proteins were detected by silver staining.

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. 5A) were excised from the Coomassie-stained gel, soaked in SDS loading buffer, and subjected to SDS-PAGE. Proteins were detected by silver staining.
IL-11 forms a Hexameric Receptor Complex

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. 5A, 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 non-denaturing PAGE (labeled as band 1* in Fig. 5A) 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. 5B, indicate that IL-11, IL-11R, and gp130 were all present in the ternary complex, which resolved as a discrete band during non-denaturing PAGE. Similarly, the composition of the IL-11-IL-11R complex (labeled as band 2* in Fig. 5A) was confirmed using this method (see Fig. 5B).

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 non-denaturing 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 non-denaturing PAGE. Equimolar concentrations (300 nM) of IL-11, IL-11R, and gp130 were mixed together, incubated, and subjected to non-denaturing 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 efficiently form a stable receptor complex co-migrating with that of IL-11 wild type. The receptor complexes formed by the site II mutant (see lane 5) include a very faint band co-migrating 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 non-denaturing 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 non-denaturing 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. 5A 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 non-denaturing 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 non-denaturing 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, compared with the hexamer formed by IL-11, IL-11R, and wild type gp130. This gp130(Ig-) receptor complex co-migrates with the receptor complex formed by IL-11 W147A, IL-11R, and
gp130 (results not shown) and is therefore predicted to be a trimeric receptor complex.

**DISCUSSION**

Cytokines mediate their biological effects through the formation of oligomeric receptor complexes, which initiates intracellular signaling. The nature of this signal, and the resulting biological response, depends on the specificity and affinity of the interactions between cytokines and their receptors. Understanding how cytokines recognize and engage receptors is therefore an important issue in understanding biological function. Information regarding the mechanism by which a cytokine exerts a response can also aid the manipulation of cytokine signaling; for example, in the generation of agonists and/or antagonists which could have therapeutic value. Both the cytokines and the receptors of the gp130 family share common structural features, and general patterns of receptor engagement are now emerging. This study contributes to our understanding of how this family of cytokines form multisubunit receptor complexes.

Current evidence suggests that the gp130 family of cytokines initiate intracellular signaling through either homo- or heterodimerization of gp130. IL-11 has previously been shown to specifically interact with the IL-11R and gp130 (39). Furthermore, cells expressing these two receptors are responsive to IL-11 (32). It has also been shown that IL-11 can mediate a biological response through interaction with a soluble form of the IL-11R and full-length gp130, a response which does not require LIFR or OSMR (16, 39). These results suggest that IL-11 mediates signal transduction through gp130 homodimerization, but published data regarding the composition and stoichiometry of the ternary IL-11 receptor complex has as yet not been conclusive. From immunoprecipitation experiments, similar to those reported here, Neddermann et al. (40) described a pentameric receptor complex consisting of two IL-11, two IL-11R, and one gp130. IL-6, on the other hand, has been shown to form a hexameric receptor complex, consisting of two molecules each of IL-6, IL-6R, and gp130 (37, 38).

The results described here from immunoprecipitation experiments show that multiple copies of gp130 are present in the IL-11 receptor complex. Homodimerization of gp130 was demonstrated to require both IL-11 and IL-11R. Furthermore, it was shown that multiple copies of both IL-11 and IL-11R are also present in the IL-11 receptor complex. The high affinity IL-11 receptor complex therefore contains at least two copies each of IL-11, IL-11R, and gp130 and since ligand-mediated homodimerization of gp130 is clearly sufficient for signal transduction to be initiated, as demonstrated for IL-6, we propose that the IL-11 receptor complex is a hexamer (depicted in Fig. 8), although we cannot formally rule out the possibility of larger complexes. The fact that wild type IL-11 was shown to induce gp130 homodimerization, in the presence of IL-11R, contradicts previous published results (40). The formation of a ternary receptor complex containing at least two molecules of gp130, observed here, excludes the requirement for a novel signaling receptor component as proposed by Neddermann et al. (40).

Further evidence supporting the hexameric model was obtained using two mutant forms of IL-11, R111A/L115A and W147A, the activities of which have previously been reported (32). Results from immunoprecipitation experiments revealed that neither the site II mutant, R111A/L115A, nor the site III mutant, W147A, were able to dimerize gp130. The fact that both of these mutants were able to bind a single molecule of gp130 in the presence of soluble IL-11R shows that sites II and III of IL-11 are independent gp130 binding sites. IL-11 has been shown to interact with the IL-11R through site I (32), and assuming IL-11 binds to the IL-11R with a 1:1 stoichiometry, this suggests that the mutants formed trimeric complexes consisting of one molecule each of IL-11, IL-11R, and gp130. The results presented here from non-denaturing PAGE analysis confirm that interactions between IL-11 and the extracellular domains of IL-11R and gp130 are sufficient to form a stable ternary complex. A complex of IL-11, IL-11R, and gp130 was resolved by PAGE under non-denaturing conditions and its composition verified by second dimension denaturing PAGE. A complex of IL-11 and IL-11R was also detected, although it appeared to be less stable than the ternary IL-11 receptor complex. Similarly, the predicted IL-11-IL-11R-gp130 trimers formed by both the IL-11 site II and site III mutants appeared to be much less stable than the ternary IL-11 receptor complex. This, together with the observation that IL-11 wild type did not form a stable complex with gp130 in the absence of IL-11R and that fact that IL-11 binds the IL-11R through site I (32), shows that all three binding sites of IL-11 are required to drive the formation of a stable ternary receptor complex.

It has recently been proposed that the Ig-like domain of gp130 is involved in the interaction of gp130 with ligands that induce homodimerization of gp130, namely IL-6 and IL-11 (47, 48). The evidence presented here clearly shows that the Ig-like domain of gp130 is essential for IL-11 to form a high affinity hexameric complex with IL-11R and gp130. It is predicted that the Ig-like domain of gp130 interacts with site III of IL-11, as shown in Fig. 8. Results from experiments examining a series
of human/murine LIFR chimeras show that the recognition site for OSM and LIF lies in the Ig-like domain of the LIFR (49).5 This suggests that the Ig-like domain of signaling receptors, belonging to the gp130 family, represents a common motif for binding to site III of the ligand. There is strong evidence, from mutagenesis studies, to suggest that the gp130 recognition epitope for site II of the ligands lies in the hinge region of the CHD (22, 28, 48). The existence of two ligand binding epitopes of gp130, and the evidence presented here that shows IL-11 has two independent gp130 binding sites, strongly supports the hexameric model depicted in Fig. 8.

The experimental data reported here clearly shows that IL-11, like IL-6 (50), forms a stable hexameric receptor complex in vitro, via trimer intermediates. Recently, Grotzinger et al. (41) have proposed that a tetrameric IL-6 receptor complex, not the hexameric model depicted in Fig. 8.

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