Direct Determination of the Interleukin-6 Binding Epitope of the Interleukin-6 Receptor by NMR Spectroscopy*

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All cytokines belonging to the interleukin-6 (IL-6)-type family of cytokines utilize receptors that have a modular build of several immunoglobulin-like and fibronectin type III-like domains. Characteristic of these receptors is a cytokine receptor homology region consisting of two such fibronectin domains defined by a set of four conserved cysteines and a tryptophan-serine-X-trypophan-serine-X-trypophan-serine sequence motif. On target cells, interleukin-6 first binds to its specific receptor and subsequently to a homodimer of the signal transducer protein gp130. The interleukin-6 receptor consists of three extracellular domains. The N-terminal immunoglobulin-like domain is not involved in ligand binding, whereas the third membrane proximal fibronectin-like domain accounts for more than 90% of the binding energy to IL-6. Here, the key residues of this fibronectin-like domain involved in the interaction with IL-6 are described. Chemical shift mapping data with 15N-labeled IL-6R-D3 and unlabeled IL-6 coupled with recent structural data clearly reveal the epitope within the IL-6R-D3 responsible for mediating the high affinity interaction with its cognate cytokine.

Cytokines are key mediators in the regulation and coordination of immune responses and hematopoiesis (1). Such molecules act on their target cells by binding to specific cell surface receptors and thereby induce receptor oligomerization (2). Cytokine receptors are type 1 transmembrane proteins. The receptors ectodomains consist of a number of fibronectin (FN)1 type III-like and immunoglobulin-like (Ig) domains (3). The cytoplasmic region of these receptors lack intrinsic protein kinase activity but are constitutively associated with tyrosine kinases of the Janus kinase family (2, 4). Ligand binding enforces homo- or heterodimerization of receptor molecules leading to activation of the associated kinases and initiation of cytoplasmic signaling cascades (2, 3).

Interleukin-6 (IL-6) is a pleiotropic cytokine involved in hematopoiesis, regulation of immune responses, and the acute phase reaction (2, 5). This cytokine belongs to the family of interleukin-6-type cytokines that includes interleukin-11 (IL-11), cardiotoxin-1 (CT-1), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), and cardiotrophin like factor. All of these cytokines share the characteristic four-helix bundle protein fold (6). This family of proteins signals via receptor complexes, which contain at least one molecule of gp130, the common signal transducing protein of the IL-6-type family of cytokines (3). IL-6 and IL-11 act via a homodimer of gp130, whereas CNTF, CT-1, LIF, and OSM induce a signaling event via the heterodimerization of gp130 and LIFR. Alternatively, OSM recruits gp130 and the OSM receptor. On target cells, IL-6, IL-11, and CNTF first bind to their specific α-receptor subunits, IL-6R, IL-11R, and CNTFR, respectively, which are not involved in the intracellular signal transduction cascade (3). These membrane-bound α-receptors can be functionally replaced by their soluble forms, which lack transmembrane and cytoplasmic parts (7). Interestingly, it has been shown recently that CNTF not only interacts with CNTFR but also with the IL-6R (8).

All receptors of the IL-6-type cytokines belong to the cytokine receptor class I family characterized by the presence of at least one cytokine receptor homology (CRH) region consisting of two FN domains (6). The conserved CRH plays a central role in cytokine recognition by the receptor. The N-terminal FN domain of the CRH contains four conserved cysteine residues, whereas the C-terminal FN domain is characterized by a tryptophan-serine-X-trypophan-serine (WSXWS) sequence motif. The extracellular part of the IL-6R consists of an Ig domain (D1) and one CRH (D2 and D3). It is the C-terminal domain of IL-6R and IL-11R of the corresponding CRH that are primarily responsible for ligand binding, since these domains have been shown to account for more than 90% of the binding energy (9, 10). However, the IL-6/IL-6R-D3 complex is not able to associate with the signal transducer gp130 and as such is not able to elicit a signaling cascade (9). The individual structures of IL-6 (11, 12) as well as IL-6R (13) have been solved by x-ray crystallography or NMR spectroscopy. In addition, the crystal structure (3.65 Å resolution) of the hexameric IL-6/IL-6Rgp130 complex has recently been published (14). A number of amino acid residues of IL-6 and IL-6R involved at the interaction interface have been defined by mutational studies (15) from molecular modeling (16) and with limitations from the recently published crystal structure of the hexameric IL-6/IL-6Rgp130 complex (14). Using chemical shift mapping in combination with activation studies, the structural basis of the interaction was elucidated, allowing for the design of novel antagonists or agonists.
with the current structural data the amino acid residues of IL-6R that are key in the interaction with its cognate cytokine are detailed. The results confirm the conclusions drawn from previous mutagenesis studies and clearly indicate that structural rearrangement of the IL-6R-D3 is required to induce the correct interaction between this domain and its cognate cytokine. In contrast, the NMR results presented do not entirely translate to the recent crystal structure of the complex and indicate that such structural rearrangements are not fully accounted for in the current complex model.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Unlabeled interleukin-6 was expressed and purified as described previously (17). The third domain of IL-6R was expressed using the BL21(DE3)pLysS Escherichia coli strain, refolded, and purified as described previously (9). 15N-Labeled and 15N/13C double-labeled IL-6R-D3 was prepared by growing the bacteria in M9 minimal medium using 15NH4Cl (1 g/liter) and 13C6-labeled glucose (2 g/liter) as the sole nitrogen and carbon sources, respectively.

NMR Sample Preparation—After purification protein concentrations were determined by UV spectroscopy. Unlabeled IL-6 was mixed with the 15N-labeled IL-6R-D3 in a molar ratio of 1:1. This sample was concentrated using an Amicon stirring cell (Millipore, Eschborn, Germany) to a final concentration of about 0.5 mM in 20 mM phosphate buffer at pH 5.0 containing 95% H2O/5% D2O and used to record two-dimensional 1H-15N HSQC spectra.

NMR Spectroscopy—The NMR sample of the complex consisted of a 0.5 mM 1:1 complex of 15N-enriched IL-6R-D3/IL-6, 20 mM sodium phosphate in 260 ml of 95% H2O/5% D2O, pH 5.0. The NMR sample of 15N-enriched IL-6R-D3 was prepared in identical buffer conditions. All NMR data were acquired at 20 °C on a Varian Inova Unity 600 MHz NMR spectrometer equipped with a z axis pulsed-field gradient 1H/15N/13C probe optimized for 1H detection. Two-dimensional 1H-15N HSQC experiments were recorded with the 1H carrier positioned in the center of the amide proton frequency (7.52 ppm) and the 15N carrier at 118.4 ppm. 15N decoupling was applied during data acquisition. Sequence-specific backbone assignments of IL-6R-D3 were achieved2 from a series of three-dimensional triple res-

FIG. 1. Sequential assignment of the third extracellular domain of the interleukin-6 receptor. A, two-dimensional 1H-15N HSQC spectrum of the third extracellular domain of IL-6R acquired at 20 °C. Sequential assignments obtained in this study are indicated. The inset (top left) shows an enlargement of the most crowded region (boxed) of the spectrum. Side-chain amide resonances of Asn and Gln residues and the protons of the indole side chain of tryptophan residues are not labeled. Due to the low peak intensities, the resonances of Gly-266 and Gln-313 are not contoured and marked by rectangles. B, secondary structure prediction of IL-6R-D3 based on chemical shift data. Differences of more than ±0.1 ppm with respect to random coil values for the \( ^1\)H\( _\alpha \)/\( ^1\)HN, \( ^13\)C\( _\alpha \)/\( ^1\)HN, and \( ^13\)C\( _\beta \)/\( ^1\)HN chemical shifts received an index value of +1 or −1. For the \( ^1\)H\( _\alpha \) chemical shifts, three consecutive positive (downfield shift) or four consecutive negative (upfield shift) indices designate \( \beta \)-strand or \( \alpha \)-helical structures, respectively. \( ^13\)C\( _\beta \) chemical shifts can only be used to identify stretches of \( \beta \)-strands, and a grouping of four or more positive indices denotes such a secondary structure (20, 21). For the \( ^1\)HN and \( ^13\)C\( _\beta \) chemical shift values, three consecutive negative index values indicate that those residues form a \( \beta \)-strand, whereas four positive indices imply an \( \alpha \)-helix (20, 21). All other regions are designated as random coil or flexible regions of the protein. Residue numbers correspond to the full-length IL-6R.

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FIG. 1—continued

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B

$C_{\alpha}$

$C_{\beta}$

$H_{\alpha}$

$H_N$

Consensus

| A | B | C | D | E | F |
|---|---|---|---|---|---|
| 1 | 1 | 1 | 1 | 1 | 1 |

residues
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The two-dimensional $^1$H-$^{15}$N HSQC spectrum of the IL-6R-D3 is presented in Fig. 1A. The sequence-specific $^1$H-$^{15}$N assignment of IL-6R-D3 was derived from a series of three-dimensional triple resonance experiments (see “Experimental Procedures”) and resonances are labeled with assignment information. The chemical shift index (CSI) is a simple and reliable statistical method to predict secondary structure elements from recorded chemical shifts by comparison with their corresponding random coil values (20, 21). From the calculated CSI values derived from the $^1$H, $^1$H, $^{13}$C$_o$, and $^{13}$C$_p$ chemical shifts a consensus CSI value can be used to identify the occurrence of $\alpha$-helical, $\beta$-strand, or coiled structures by indices of $-1$, $+1$, or 0, respectively (20, 21). The third domain of the extracellular part of IL-6R has been predicted to share a fibronectin type III-like fold consisting of seven $\beta$-strands that has been recently confirmed by the x-ray structure of the extracellular part of the IL-6R (13). The chemical shifts of $^1$H$_N$, $^1$H$_O$, $^{15}$N, $^{13}$C$_o$, and $^{13}$C$_p$ nuclei of the IL-6R-D3 were derived from the following set of experiments: two-dimensional $^1$H-$^{15}$N HSQC, three-dimensional HBHA/CBCACO/NH, three-dimensional CBCA(CO)NH, three-dimensional CBCANH, and three-dimensional HBHA(CBCACO)NH spectra. Chemical shift indexing was performed with the $^1$H$_N$, $^1$H$_O$, $^{13}$C$_o$, and $^{13}$C$_p$ chemical shifts to obtain an estimate of the secondary structure of the IL-6R-D3 (Fig. 1B). Of the expected seven $\beta$-strands observed in the crystal structure, six $\beta$-strands were estimated from the CSI values. Only the short $\beta$-strand G was not clearly identified. The CSI results agree with the lengths of the $\beta$-strands determined in the x-ray structure (13) with the exception of $\beta$-strand C that extends from Leu-232 to Ala-240 in the x-ray structure and from Leu-236 to Ala-240 in the IL-6R-D3. Apparently, the shorter $\beta$-strand C is due to the absence of assignment data for the $^1$H$_O$ and $^{13}$C$_p$ of Glu-235 due to spectral overlap.

For a quantitative analysis of these data the normalized chemical shift differences ($\Delta(\delta^1$H, $^{15}$N) = $(\Delta^1$H$^2 + \Delta^{15}$N$^2)^{1/2}$) of $^1$H and $^{15}$N resonances of the free IL-6R-D3 and the IL-6R-D3 bound to IL-6. Asterisks indicate residues for which the chemical shift differences could not be calculated, since the resonances arising from these residues were not identified in the spectrum of the protein complex. Residues not assigned are marked with hash marks. Arrows mark the $\beta$-strands (arrow A, Ala-204 to Thr-208; arrow B, Leu-215 to Gln-220; arrow C, Leu-236 to Ala-240; arrow D, Thr-248 to Met-250; arrow E, Cys-258 to Ile-260; arrow F, His-270 to Glu-275). The $\beta$-strands A–F have been derived from the consensus chemical shift index, whereas $\beta$-strand G has been taken from the structure of the IL-6R-D3 (11). Amino acid residues in loop regions involved in binding to IL-6 are: Trp-225 to Arg-231 (B/C loop), Leu-254 to His-256 (D/E loop), Gly-280 to Glu-283 (F/G loop). Ps-276 was not visible in both spectra due to chemical exchange.
calculated for some of the IL-6R-D3 residues, since their corresponding resonances in the 1H-15N HSQC spectra of the protein complex could not be unambiguously assigned. Although chemical shift differences were not calculated for these resonances, the residues were considered to be involved in the binding interface with IL-6. For some resonances (e.g. Asp-253 and Gln-272), nearby or overlapping peaks obscured accurate chemical shift measurements and were excluded from this analysis.

Using the crystal structure of the IL-6R (13), the measured changes in the 1H and 15N chemical shifts of the amide groups were projected onto the structure of the third extracellular domain of this receptor with regions colored red indicating the largest changes observed (i.e. > 0.03 ppm). As can be seen in Fig. 3A, changes in IL-6R-D3 conformation upon interacting with IL-6 occur predominately in the B/C, D/E, and F/G loops and the adjacent stretches in the corresponding β-strands. Interestingly, some significant chemical shift changes were observed on the opposite side of this domain in the E/F loop. Since a contact with the ligand can be excluded, this may indicate that upon binding IL-6 a slight structural displacement of one or both of the β-strands E and F results in a subsequent movement of the E/F loop to accommodate the displacement. The involvement of the B/C, D/E, and F/G loops in the binding interface with IL-6 has been predicted from model building and site-directed mutagenesis studies (15, 16, 22). These predictions are confirmed by the NMR data.

Recently, the medium-resolution x-ray data of the IL-6-IL-6R-gp130 complex (14) coupled with high resolution structures of the single components, IL-6 (11), IL-6R (13), and the three membrane-distal domains of gp130 (23) were used to create a model of the protein complex. Using this model, the authors suggested several residues in IL-6R to be involved in the interaction with IL-6. In Fig. 3B the crystal structure of the IL-6-IL-6R (D2 and D3) complex is shown. The crucial role of the residues Phe-229 and Tyr-230 in the B/C loop and Glu-278 and Phe-279 in the F/G loop for ligand binding has been demonstrated by site-directed mutagenesis (15) and was confirmed by the structure of the IL-6-IL-6R complex (14). According to this model Phe-229 (B/C loop) and Phe-279 (F/G loop) contribute 28 and 20%, respectively, to the total binding area (14).

Besides Phe-229, an apparent conformational change of the IL-6R-D3 was also observed for the adjacent residues in the B/C loop upon binding to its cognate cytokine. Chemical shift differences greater than 0.03 ppm were observed between residues Trp-225 (0.040 ppm) and Arg-231 (0.037 ppm). The resonances arising from the amide groups of Asn-226 to Tyr-230 either broadened due to intermediate chemical exchange or disappeared and reappeared due to slow exchange in the spectrum and were consequently not assigned in the 1H-15N HSQC spectrum of the complex. Such changes clearly showed that these residues undergo conformational changes upon binding to IL-6. One of the significant differences between the crystal structure and our NMR data concerned Arg-233 (start of β-strand C). Arg-233 and the adjacent residues display chemical shift differences suggesting their involvement in IL-6 binding. In accordance with this observation are site-directed mutagenesis results that showed Arg-233 plays an important role in binding to IL-6 (15). In the crystal structure, this residue apparently does not participate in the interaction with IL-6 but has intramolecular contacts with main-chain atoms of residue Phe-297, which is one of the signature residues in the IL-6/IL-6R interface (14). Therefore, the observed chemical shift difference of Arg-233 reflects a conformational change at the beginning of strand C rather than a direct involvement in ligand binding.

The chemical shift mapping data revealed residues located in the D/E loop (Fig. 3A) to be influenced by ligand binding. The amino acid residues in this loop, especially Leu-254, Gln-255, His-256, and in addition Cys-258 (beginning of β-strand E),
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cysteine receptors these conserved glycines precede the canonical WSXWS sequence motif that is the typical signature of the C-terminal domain of the CRH. It has been suggested that this motif plays a structural role (26–28). Our data and other data suggest that this motif serves as an anchor for the adjacent mobile F/G loop that is crucial for the ligand receptor interaction.

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Exhibit pronounced chemical shift differences (0.050, 0.048, 0.077, and 0.042, respectively), which are among the largest differences observed in this study. These observations are in excellent agreement with data derived from a site-directed mutagenesis studies (22). Yawata et al. (22) reported that a double mutant Gln-255-Leu and His-256-Gln resulted in a decreased binding capacity of 39% compared with the wild type protein. These data are somewhat contradictory to what can be deduced from the crystal structure of the complex (14). In this model, the D/E loop is far from the bound IL-6 with the shortest Co-Co distance of 11.9 Å (IL-6, Lys-27; IL-6R-D3, Asp-253). Furthermore, the side chains of the corresponding residues of IL-6-D3 D/E loop are not geometrically positioned in the crystal structure to be making opposite contacts with the bound IL-6. Like in the case of the B/C loop, the pronounced chemical shift differences observed in the D/E loop seem to indicate a more general conformational change rather than a direct involvement in the interaction with IL-6.

Promounced chemical shift differences were measured for resonances arising from residues located in the F/G loop (Fig. 3, A and B). This loop and the adjacent β-strands comprise the residues Gln-276 to Glu-283. This region contains Phe-279, one (Glu-278), no resonances could be unambiguously identified in the 1H,15N HSQC spectra. The missing assignment data of Glu-278 and Phe-279 of IL-6R-D3 are indicative of fast chemical exchange (i.e. conformational flexibility) in the unbound form. Both residues have been shown to be crucial for IL-6 binding (15, 22). This loop also contains two glycines, Gly-280 and Gly-282. Mutagenesis of Gly-282 abolished binding to IL-6 completely (22), reflecting the importance of this residue in the F/G loop. Since glycines are known to impart flexibility to loop regions, these two residues might also be responsible for the apparent flexibility of other residues in this loop. The measured chemical shift differences for the resonances corresponding to these two glycines are the largest measured (Fig. 2), indicating a distinct change in their chemical environment. These results imply an induced fit mechanism, in which the apparently flexible F/G loop adopts a specific rigid conformation upon IL-6 binding. Many cytokine receptors exhibit glycines at identical positions in the F/G loop (16) and the residues crucial for binding (hot spots) are also located in this loop (22, 24–26). Intriguingly, in all type I