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Identification of the Domain in the Human Interleukin-11 Receptor that Mediates Ligand Binding

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The interleukin-11 receptor (IL-11R) belongs to the hematopoietic receptor superfamily. The functional receptor complex comprises IL-11, IL-11R and the signal-transducing subunit gp130. The extracellular part of the IL-11R consists of three domains: an N-terminal immunoglobulin-like domain, D1, and two fibronectin-type III-like (FNIII) domains and D2 and D3. The two FNIII domains comprise the cytokine receptor-homology region defined by a set of four conserved cysteine residues in the N-terminal domain (D2) and a WSXWS sequence motif in the C-terminal domain (D3). We investigated the structural and functional role of the third extracellular receptor domain of IL-11R. A molecular model of the human IL-11/IL-11R complex allowed the identification of amino acid residues in IL-11R to be involved in ligand binding. Most of them were located in the third extracellular domain, which therefore should be able to bind with high affinity to IL-11. To prove this prediction, domain D3 of the IL-11R was expressed in *Escherichia coli*, refolded and purified. For structural characterization, circular dichroism, fluorescence and NMR spectroscopy were used. By plasmon resonance experiments, we show that the ligand-binding capacity of this domain is as high as that one for the whole receptor. These results provide a basis for further structural investigations that could be used for the rational design of potential agonists and antagonists essential in human therapy.

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Keywords: Interleukin-11 receptor; cytokine receptors; ligand binding; cytokine receptor-homology region

Introduction

Interleukin-11 is a pleiotropic cytokine playing an important role in hematopoiesis (Neben *et al.*, 1994; Yonemura *et al.*, 1992) and anti-inflammatory activities (Redlich *et al.*, 1996; Trepicchio *et al.*, 1996). Due to its thrombopoietic potential, it has been demonstrated in a phase II clinical trial that recombinant IL-11 reduces chemotherapy-induced thrombocytopenia in breast cancer patients (Tepler *et al.*, 1996). Furthermore, IL-11 was shown to play a critical role in female reproduction: female mice lacking the receptor for IL-11 are infertile due to a failure of trophoblast implantation (Robb *et al.*, 1998).

IL-11 belongs to the so-called IL-6-type family of cytokines, all sharing a four-helix-bundle fold (Bazan, 1990a; Grötzinger *et al.*, 1999). All members of this family, namely interleukin-6 (IL-6), interleukin-11 (IL-11), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM) and cardiotrophin-1 (CT-1) signal *via* a gp130 homodimer (Hibi *et al.*, 1990) or a heterodimer of gp130 and the LIF receptor (Gearing *et al.*, 1991) and/or OSM receptor (Mosley *et al.*, 1996). Whereas IL-11 and IL-6 use a gp130 homodimer (Murakami *et al.*, 1993; Yi

Abbreviations used: HSQC, hetero single quantum correlation; INEPT, insensitive nuclear enhanced polarization transfer; rmsd, root-mean-square differences; ru, resonance units; IL-11R, interleukin-11 receptor; CNTF, ciliary neurotrophic factor; LIF, leukemia inhibitory factor; OSM, oncostatin M; CT-1, cardiotrophin-1; CRH, cytokine receptor homology region; hGH, human growth hormone; hGHR, hGH receptor.

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Introduction

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Abbreviations used: HSQC, hetero single quantum correlation; INEPT, insensitive nuclear enhanced polarization transfer; rmsd, root-mean-square differences; ru, resonance units; IL-11R, interleukin-11 receptor; CNTF, ciliary neurotrophic factor; LIF, leukemia inhibitory factor; OSM, oncostatin M; CT-1, cardiotrophin-1; CRH, cytokine receptor homology region; hGH, human growth hormone; hGHR, hGH receptor.

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CT-1 (Pennica *et al.*, 1995) use a heterodimer of gp130 and LIF receptor for signal transduction. OSM is able to signal *via* a heterodimeric gp130/OSM receptor (Ichihara *et al.*, 1997) or gp130/LIF receptor, respectively. The binding of IL-11, IL-6 and CNTF to their specific non-signaling α-receptors is a prerequisite for their interaction with the signal transducers, whereas OSM and LIF bind directly to their signal-transducing subunits. In the case of IL-11 and IL-6 gp130, homodimerization leads to the activation of the constitutively associated Janus tyrosine kinases (Jaks), which then phosphorylate tyrosine residues of the cytoplasmic part of gp130. These phosphotyrosine residues are specific docking sites for the transcription factors of the STAT family (Gerhartz *et al.*, 1996) which then themselves become phosphorylated. The phosphorylated STATs dimerize and translocate into the nucleus to activate target gene expression (Heinrich *et al.*, 1998; Lütticken *et al.*, 1994).

Two isoforms of the human IL-11 receptor have been identified that differ in their cytoplasmic domains (Cherel *et al.*, 1995). One isoform of the human IL-11 receptor (IL-11Rα1) has a short cytoplasmic domain and is similar to the human IL-6 receptor and the murine IL-11 receptor. The other isoform, similar to the human CNTF receptor, lacks this domain (IL-11Rα2). Lebeau *et al.* (1997) described similar functions and properties of both isoforms when transfected with gp130 in Ba/F3 cells. 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 region (CRH) consisting of two fibronectin type III-like domains (Yamasaki *et al.*, 1988). The N-terminal domain of the CRH contains four conserved cysteine residues, whereas the C-terminal domain is characterized by a tryptophan-serine-X-tryptophan-serine (WSXWS) sequence motif (Bazan, 1990b). The extracellular part of the IL-11R is predicted to consist of an Ig-like domain (D1) and one CRH (D2 and D3) (Cherel *et al.*, 1995). For several members of this receptor family, e.g. the IL-6R, the human growth hormone receptor, the IL-4 receptor, the prolactin receptor and the erythropoietin receptor, it has been shown that the two domains of the CRH are responsible for the interaction with their ligands (Wells & de Vos, 1996). In the case of IL-6R, the Ig-like domain stabilizes the receptor during intracellular trafficking (Vollmer *et al.*, 1999) but is not necessary for the assembly of a functional receptor (Vollmer *et al.*, 1996; Yawata *et al.*, 1993), whereas the third domain is sufficient for ligand binding but not for gp130 association (Özbek *et al.*, 1998).

So far, the three-dimensional structure of the complete CRH of human gp130 as well as its C-terminal domain has been solved by X-ray (Bravo *et al.*, 1998) and NMR spectroscopy (Kernebeck *et al.*, 1999), respectively. The latter consists of seven β-strands constituting a fibronectin type III-like domain. It is structurally related to the homologous domains of the receptors for erythropoietin, growth hormone and prolactin (Kernebeck *et al.*, 1999). Since no structural information is available for the CRH of IL-11R, a molecular model of the IL-11/IL-11R complex has been built using the X-ray structures of the human growth hormone (hGH)/human growth hormone receptor complex (hGHR) (de Vos *et al.*, 1992) and human CNTF, respectively, as a template (McDonald *et al.*, 1995). This model allowed the prediction of amino acid residues within human IL-11 to be involved in the interaction with IL-11R and gp130, which was confirmed by site-directed mutagenesis (Tacken *et al.*, 1999). According to this model, amino acid residues within the IL-11R involved in ligand binding are located mainly in the third domain. To verify these predictions experimentally, this domain was expressed in *Escherichia coli*, refolded and purified. Circular dichroism, fluorescence and NMR experiments were used to characterize the recombinant IL-11R-D3. We demonstrate that the binding capacity of this domain is as high as that for the full-length receptor, confirming the model of the IL-11/IL-11R complex.

Results

Molecular modeling

Figure 1(a) shows the molecular model of the human IL-11/IL-11R complex built as described in Material and Methods. To assess the qualitiy of the molecular model we calculated the amount of residues in the not-allowed regions of a Ramachandran plot (IL-11 model, four residues out of 166; IL-11R model, seven residues out of 202) and the rmsd values for equivalent C^{α} atoms between the final models and the templates (IL-11 model, 0.8 Å; IL-11R model, 1.1 Å). The epitope of IL-11 in contact with the IL-11R (site I) comprises the beginning of helix A, the end of helix D and the end of the AB-loop, and was identified as the IL-11R binding epitope by site-directed mutagenesis (Tacken *et al.*, 1999). The regions in IL-11R that are in contact with site I of IL-11 are located within domains D2 and D3 of IL-11R. Figure 1(b) shows the sequential alignments with homologous cytokines and their receptors of the regions involved in the interaction between the two molecules. Both domains consist of seven β-strands and interact with the ligand *via* their loop regions. These are, in particular, the EF-loop in D2 and the B′C-′ and F′G′-loops in D3, respectively.

To identify the residues participating in the interaction area, the difference of the accessible surfaces (in $Å^2$) of IL-11R was determined in the free and ligand-bound state, respectively. The locations of the affected residues within the model of IL-11R are depicted in Figure 1(c). Residues contributing most to the interaction area are F187 and W188 in D2, and P250, H251, L253, F298, L299, D300 in D3, respectively. One residue (Q213) is located in the hinge region

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connecting the two domains. This analysis revealed that it is mainly the third domain that contributes to the interaction area, suggesting that this domain should be significantly involved in ligand binding.

Expression, refolding and purification of the third domain of IL-11R

Preliminary studies with the recombinant wild-type IL-11R-D3 were hampered by problems due to disulfide-linked dimers that were prone to form aggregates. Dimers could be detected by Western blotting using monoclonal antibodies against IL-11R-D3 (data not shown). To avoid aggregation and dimer formation, the only cysteine residue (C248) in IL-11R-D3 was mutated to an alanine residue. This mutated form of the third domain of IL-11R was used throughout all the following experiments.

After expression in *E. coli* and lysis of cells, the protein was found only in inclusion bodies; no recombinant protein was detectable in the soluble fraction of the lysate. By SDS-PAGE (Figure 2(a)) the expressed protein was visualized as a 14 kDa band. After solubilization of the inclusion bodies in guanidine hydrochloride, the protein was refolded and purified by size-exclusion chromatography (Figure 2(b)).

Circular dichroism spectroscopic characterization of the refolded IL-11R-D3

The circular dichroism spectra of the refolded IL-11R-D3 in both the far and the near-UV are indicative of a protein in a folded state (Figure 3(a) and (b)). The positive peak at 232 nm reflects a positive lobe of a couplet, which can be attributed to interacting tryptophan side-chains (Grishina & Woody, 1994). This band is observed in the far-UV CD spectrum of the third domain of gp130 (Müller-Newen *et al*., 1997) and IL-6R (Özbek *et al*., 1998) as well as in the corresponding domain of the granulocyte-colony-stimulating factor receptor (Anaguchi *et al*., 1995). This positive peak seems to be characteristic for the WSXWS motif present in the C-terminal domain of the CRH in all class I cytokine receptors. Secondary structure analysis (Sreerama & Woody, 1994) of the far-UV CD spectrum reflects the β-sheet character of the protein (IL-11R-D3, α-helix 5 %, β-sheet 49 %, turn 16 %), which is typical for a fibronectin type III-like domain (Müller-Newen *et al*., 1997; Özbek *et al*., 1998). The folded state of the protein is confirmed by the near-UV CD spectrum (Figure 3(b)). The shape of the spectrum shows that several distinct bands attributable to tyrosine and tryptophan side-chains are overlaid. The presence of such bands are indicative for a protein in its folded state (Grishina & Woody, 1994).

Thermal unfolding of the third domain of IL-11R

The thermal stability of the recombinant protein at pH 8.0 was studied by CD spectroscopy in the far-UV range. A series of far-UV CD spectra were recorded at different temperatures. The prominent band at 232 nm, which decreases with increasing temperature, was used to monitor the denaturation of the protein. Figure 4 shows the ellipticity at 232 nm as a percentage of the native state of D3, as a function of temperature. The transition midpoint was estimated to be about 25 °C, which indicates a remarkably low thermal stability of this domain. Above 30 °C, the protein precipitated.

Fluorescence anisotropy decay measurements

The results of the time-resolved fluorescence measurements are presented in Table 1. The anisotropy decay had to be fitted with two rotational correlation times in order to achieve a good fit. The shorter correlation time is in the typical range of side-chain motions (Kouyama *et al*., 1989) while the slower one is probably attributed to the overall motion of the protein. Assuming a spherical particle, the theoretical rotational correlation time $\phi_{theor.}$ can be calculated to 6.7 ns, which is shorter than the measured value of 12.2 ns. The higher value compared to the theoretically calculated one might be explained by the presence of the large unstructured, loose C-terminal part of the molecule and the non-spherical shape of the molecule, which both slow the rotational motion. On the other hand, the presence of high molecular mass oligomers can be neglected, as indicated by the low limiting anisotropy r_∞ (Table 2).

NMR spectroscopy

In order to facilitate the elucidation of the solution structure of the third domain of IL-11R by NMR spectroscopy, we produced ^{15}N-labeled protein. The protein sample was prepared in the presence of 7 mM arginine and concentrated to a final concentration of about 0.2 mM. Figure 5 shows the ^{1}H-^{15}N-HSQC-NMR spectrum of IL-11R-D3. The wide spread of the resonances indicates a folded state of the protein. The spectrum shows 92 reson-

Table 1. Fluorescence intensity decay data (B_i is the fractional amplitude associated with the decay time τ_i; $\langle\tau\rangle$ is the mean lifetime)

B_1 (%)	B_2 (%)	B_3 (%)	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	χ^2	$\langle\tau\rangle$ (ns)
4.8	23.7	71.5	0.56	3.87	7.40	1.27	6.23

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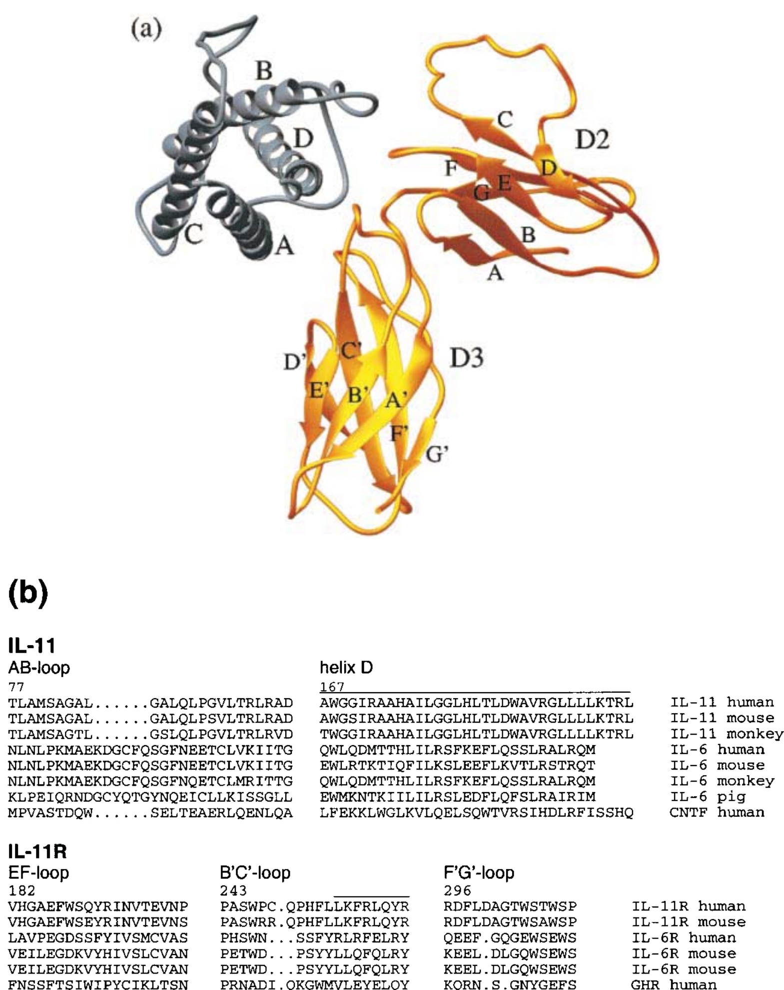

Figure 1 (*legend opposite*)

ances. Since less than 20 amino acid residues are expected to be part of the unstructured C terminus and the 17 proline residues cannot be monitored in this experiment, the number of resonances fits well with the expected number of signals for a protein consisting of 126 amino acid residues.

Surface plasmon resonance studies

Surface plasmon resonance biosensor analysis was used to investigate whether, and to what extent, the IL-11R-D3 domain could contribute to the IL-11R/IL-11 interaction. Similar amounts of IL-11R-IL-2 fusion protein (Blanc *et al*., 2000) and IL-11R-D3 were immobilized on separate sensorchips. As shown in Figure 6(a), the anti-IL-11R mAb E24.2 bound to these two sensorchips with similar kinetics. The binding capacities show that similar numbers of IL-11R and IL-11R-D3 molecules were coupled to the matrices with respect to their molecular masses. Trx-IL-11 fusion protein also bound with similar efficiencies to IL-11R-IL-2 and IL-11R-D3 (Figure 6(b)). Analysis of the sensorgrams (Table 3) indicates that the equilibrium dissociation

Table 2. Fluorescence anisotropy decay data (r_i are the anisotropies; ϕ_i the rotational correlation times)

r_o	r_1	r_2	r_∞	ϕ_1 (ns)	ϕ_2 (ns)	χ^2	$\phi_{theor.}$ (ns)
0.148	0.120	-	0.028	8.5	-	1.81	6.7
0.162	0.028	0.119	0.015	0.8	12.2	1.44	6.7

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