The structure of the unliganded extracellular domain of the interleukin-6 signal transducer gp130 in solution

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Keywords:
Interleukin-6
gp130
Trans-signalling
Structure
Small-angle X-ray scattering
Rigid-body modelling

Article history:
Received 1 September 2010
Received in revised form 27 September 2010
Accepted 27 September 2010

Introduction

The family of the IL-6 type cytokines includes, besides IL-6, leukemia inhibitory factor (LIF), IL-11, IL-27, IL-31, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-like cytokine (CLC), neuropoietin (NPN) and cardiotophin-1 (CT-1) (Scheller et al., 2006). The trans-membrane protein gp130 is the common β-receptor subunit and signal-transducer of the whole family, except IL-31 (Hibi et al., 1990). Together with specific α-receptors (IL-6R, IL-11R, CNTFR, CLF-1) and other β-receptors (LIFR, OSMR, GPL, Wsx1), the use of gp130 as a signal transducer is responsible for the overlapping and in part distinct cellular functions described for IL-6 type cytokines (Scheller et al., 2006). Gp130 belongs to the class of tall cytokine receptors which consists of three fibronectin-type III like domains between their ligand-binding domains and trans-membrane domains (Sprang and Bazan, 1993). Nine of the eleven potential N-glycosylation sites of gp130 are glycosylated (Moritz et al., 2001), and the N-linked glycans are essential for the stability but not the signalling function of gp130 (Waetzig et al., 2010).

IL-6 receptor assembly is sequential: neither IL-6 nor the (s)IL-6R alone are able to bind to gp130 (Taga et al., 1989). First, IL-6 has to bind to (s)IL-6R, and the resulting complex is then able to recruit two gp130 molecules, which can be present as preformed non-covalently linked dimers (Tenhumberg et al., 2006). Only this tripartite complex leads to signal transduction resulting in the transcription of target genes, such as acute phase response genes in hepatocytes (Heinrich et al., 2003). The cellular expression of the IL-6R is limited mainly to hepatocytes and some leukocytes (Rose-John et al., 2006). Cells lacking the IL-6R are therefore not responsive to IL-6. The soluble form of the IL-6R (sIL-6R), however, can bind IL-6 with the same affinity as the membrane-bound form, and the complex of IL-6 and sIL-6R

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induces signalling in a process called trans-signalling (Peters et al., 1998; Rose-John and Heinrich, 1994). Since the IL-6R is only sparingly expressed, IL-6 trans-signalling dramatically increases the number of potential IL-6 target cells (Rose-John et al., 2006). Nature\ncalling soluble forms of gp130 (sgp130) selectively inhibit IL-6 responses mediated by sIL-6R without affecting responses via membrane-bound IL-6R (Atreya et al., 2000; Becker et al., 2004; Jostock et al., 2001; Matsumoto et al., 2010; Nowell et al., 2009; Richards et al., 2006). A fusion protein consisting of the extracellular part of gp130 and the Fc part of a human IgG1 antibody (sgp130Fc) selectively inhibits IL-6 trans-signalling without affecting responses via the membrane-bound IL-6R (Jostock et al., 2001). sgp130Fc mimics the natural preformed gp130 dimers on the cell surface (Giese et al., 2005; Tenhumberg et al., 2006). Its affinity to the IL-6/IL-6R complex could further be enhanced by inclusion of a β-sheet of D6 (so-called optimised sgp130Fc) as well as by structure-guided site-directed mutagenesis (Tenhumberg et al., 2008).

The three membrane-distal domains of gp130 (D1–D3) are needed for ligand binding, whereas the three membrane-proximal domains (D4–D6) are indispensable for signal-transduction (Grötzinger et al., 1999; Pfannz et al., 2000). The structure of gp130 domains D1–D3 in complex with IL-6 and the extracellular part of the IL-6R (domains 2 and 3) has been determined by X-ray crystallography (Boulanger et al., 2003; Chow et al., 2004). Recently, the structure of the full-length extracellular part of gp130 (D1–D6) has been determined as a monomer (Xu et al., 2010). We used an sgp130Fc fusion protein – consisting of two extracellular gp130 regions (D1–D6) dimerised by an IgG1-Fc part – to study the shape and spatial organisation of the domains in solution by small-angle X-ray scattering (SAXS). Comparing these data with cryo-electron microscopy studies of the hexameric IL-62/IL-6R2/gp1302 complex (Skiniotis et al., 2005) and the X-ray structure of the monomeric gp130 (Xu et al., 2010) reveals a remarkable reorientation of the three membrane-distal domains D1–D3 relative to the membrane-proximal domains D4–D6 induced by ligand binding. Single domain deletion constructs (ΔD4, ΔD5, ΔD6) have been shown to be unable for signal transduction, and only ΔD5 was still able to bind the ligand (Kurth et al., 2000). The low resolution structure of sgp130Fc presented here explains these puzzling effects.

Materials and methods

Production and purification of optimised sgp130Fc

The production, purification and characterisation of optimised sgp130Fc have been previously described (Schroers et al., 2005; Tenhumberg et al., 2008). The sgp130Fc used in the present study corresponded to wild type gp130 D1–D6 without point mutations.

Small angle X-ray scattering experiments and data processing

All SAXS measurements were done at the bending magnet beamline X33 (EMBL/DESY) using an X-ray wavelength of 0.15 nm and an image plate detector positioned at a distance of 2.7 m downstream of the sample (Roessle et al., 2007). It provides the range of momentum transfer 0.08 < s < 5 nm\(^{-1}\) (s = 4π sinθ/λ, where 2θ is the scattering angle and λ is the wavelength). The protein sample was kept in a cell with polycarbonate windows thermostatically stabilised at 10 degrees, and the exposure time was 180 s. The programme PRIMUS (Konarev et al., 2003) was used for the data reduction and for the calculation of the radius of gyration \(R_g\) of the molecule using the Guinier approximation. The molecular weight was calculated using the forward scattering intensity \(I(0)\) of the standard calibrant BSA (Bovine Serum Albumin, 66 kDa).

The maximum size \(D_{max}\) and the pair distribution function \(p(r)\) of the particle were calculated using the indirect Fourier transform algorithm implemented in the programme GNOM (Semenyuk and Svergun, 1991).

Shape determination

The low-resolution shape was generated ab initio using the programme DAMMIF (Franke and Svergun, 2009). From the modelling in terms of simple three-parameter bodies performed by PRIMUS, the molecule is best represented as a cylinder of 75 Å radius and 250 Å height. This cylinder was thus used as the search volume and a P2 symmetry was applied during the reconstructions. The cylinder was filled with about 10,000 densely packed beads of 5.4 Å radius and the shape of the molecule was represented as an assembly of beads inside the cylinder. A compact and interconnected model was constructed by using simulated annealing to minimise the target function which takes into account the discrepancy between the experimental data and the calculated scattering of the model, as well as penalties related to inter-connectivity and compactness.

A number of high resolution structures for the fragments of gp130 are available. The atomic structures for the membrane-proximal fibronectin type-III like (FNIII) domains (D4–D6) are available at 1.9 and 3.04 Å in 3L51 and 3L5J respectively (Xu et al., 2010). The crystal structure for the domains D1–D3 is known in the hexameric interleukin-6/IL-6 receptor/gp130 complex at 3.65 Å (PDB code: 1P9M) (Boulanger et al., 2003). The crystal structure of the entire ectodomain of gp130 (D1–D6) is also available at 3.60 Å (PDB code: 3L5H) (Xu et al., 2010). The crystal structure of the single dimeric IgG1-Fc part of sgp130Fc was solved in our laboratory (data not shown). The overall structure is in good agreement with the Fc part of an intact IgG antibody (Harris et al., 1992). Given the known structures of the subunits, various approaches were used in order to extract maximum information from the SAXS data. A rigid-body modelling was performed with the programme SASREF (Petrokhov and Svergun, 2005), which uses a simulated annealing protocol to search for a configuration of the rigid bodies fitting the experimental data. In order to obtain models of biological significance contacts between specified residues in different subunits can be defined. As sgp130Fc was expected to be a homodimer, all SASREF modelling was done in P2 symmetry and the anchor (Fc) domain was fixed during the rigid-body refinement. In the simplest case two rigid subunits were used in the asymmetric unit, i.e. the monomeric structures from the crystal dimers of the ectodomain D1–D6 and the Fc domain, assuming that the crystal structure of D1–D6 preserves in the full-length gp130 in solution. A constraint was enforced by keeping the C-terminus of the ectodomain within a distance of 1 nm from the N-terminus of the Fc domain in order to obtain an inter-connected configuration. In another approach, the ectodomain D1–D6 was divided into two rigid subunits, i.e. D1–D3 and the FNIII domains D4–D6. Again the distance between the last residue of the subunit D1–D3 and the first residue of the subunit D4–D6 was defined to be less than 1 nm during the refinement. Finally, bias towards the structure for the extracellular domains D1–D6 was removed by modelling them as six individual rigid subunits and keeping the distance of the N-terminus and C-terminus between the neighbouring domains less than 1.5 nm.

Independently, the SAXS data was analysed using the Ensemble Optimisation Method (EOM) (Bernado et al., 2007). In this method, a pool consisting of 10,000 models was generated by randomly arranging the five rigid subunits in the asymmetric unit (i.e., D1–D3, D4, D5, D6 and Fc (monomer)) and applying P2 symmetry. Subsequently, an ensemble of 20 models was selected using the genetic algorithm so that the averaged X-ray scattering of the models in the ensemble fitted the experimental data and the optimisation was repeated 200 times. Comparison of the \(R_g\) distributions for the ran-
dom pool and for the optimised ensembles provides an assessment of the flexibility of sgp130Fc in solution.

Results and discussion

Small-angle X-ray experiments

SAXS experiments have been performed at different concentrations (at 3.75, 7.5 and 15.7 mg/ml) at pH 7.4 to exclude concentration-dependent effects such as unspecific aggregation. The theoretically calculated iso-electric point of sgp130Fc is 6.7. The reduced scattered intensity at $s < 0.25$ nm$^{-1}$ (Fig. 1A) with increasing concentrations is indicative for repulsive interactions between the sgp130Fc molecules, demonstrating that no concentration-dependent aggregation occurred. As a result of the repulsive interactions, the radius of gyration ($R_g$) of 7.8 nm is smaller at higher concentrations in contrast to $R_g = 8.3$ nm for the sample at 3.75 mg/ml, where the repulsive interactions become negligible. The molecular mass of the solute ($233 \pm 5$ kDa) compares well with that of sgp130Fc (196 kDa), taking into account that the 18 potential N-linked glycosylation sites were glycosylated (Moritz et al., 2001; Waetzig et al., 2010). We conclude from these data that the sgp130Fc sample is monomeric in solution.

The pair distance distribution function $p(r)$ has been calculated from the experimental data and is shown in Fig. 1B. The probability of the pair-distance rises steeply to a shoulder at $\sim 4$ nm, peaks just below 10 nm and decays slowly to 0 at the maximum dimension of the particle, 27(±1) nm. A schematic view of the domain structure of sgp130Fc and the estimated dimensions are depicted in Fig. 1C. The maximum dimension ($D_{max}$) of 27(±1) nm is in good agreement with an elongated shape of the sgp130Fc, whereas the shoulder at 4 nm corresponds to the length of a single domain.

Low-resolution shape of sgp130Fc

The shape reconstruction was performed in a cylindrical search volume as described in “Materials and Methods”. A typical shape (about 5 nm resolution) reconstructed ab initio in P2 symmetry fits the experimental data with discrepancy $\chi = 1.6$ (Fig. 2). The shape corresponds to a disk-like block with two twisted arms with uniform cross-sections.

The crystal structure of the Fc domain (see “Materials and Methods”) can be well accommodated into the disk-shaped block at the bottom of the model. Accordingly, the bent arms correspond to the extracellular domains (D1–D6) of gp130. The twisted region agrees well with the crystal structure of the membrane-distal domains D1–D3 of the gp130 (Fig. 2).

Flexibility of sgp130Fc

The flexibility of sgp130Fc was assessed using EOM (see “Materials and Methods”). The distributions of $R_g$ and $D_{max}$ within the initial pool and selected ensemble are depicted in Fig. 3A and B, respectively. Both values, $R_g$ and $D_{max}$ reveal a broad distribution within the pool. The majority of the models give $R_g$ values between 4 and 9 nm and sizes from 13 to 35 nm. The Full Width at Half Maximum (FWHM) of the $R_g$ and size distributions are 3 and 12 nm, respectively. In contrast, the distributions for the ensembles are much narrower with FWHM values of 1.7 ($R_g$) and 6 nm ($D_{max}$). These results demonstrate the rigidity of sgp130Fc allowing for further rigid-body modelling.

The domain arrangement in sgp130Fc

The rigid-body model shown in Fig. 4A was obtained using P2 symmetry and two rigid subunits in the asymmetric unit, i.e., the crystal structures of the ectodomain D1–D6 and the Fc domain. The structure of D1–D6 in the crystal (PDB code: 3L5H) has a compact “C” shape. Therefore, this model for sgp130Fc has a much smaller overall shape compared to the ab initio model. As shown in Fig. 5, the calculated scattering of this model is also inconsistent with the experimental data (discrepancy $\chi = 9.1$).
Fig. 2. Low resolution SAXS models of sgp130Fc. Ab initio model obtained by using P2 symmetry and a cylindrical search volume. The Fc domain and the membrane-distal domain D1–D3 are fitted into the ab initio shape.

Fig. 3. Flexibility of sgp130Fc in solution. The figure shows the \( R_g \) (A) and \( D_{\text{max}} \) (B) distributions for the pool consisting of randomly generated models (black) and for the optimised ensemble (red), which is in agreement with the averaged scattering of the experimental data.

The rigid-body model given in Fig. 4B uses three subunits in the asymmetric unit, and the calculated scattering of this model is in very good agreement with experimental data (\( \chi = 1.7 \)) depicted in Fig. 5. Compared to the ab initio model, two major features are evident. First, this model results in a similar overall size by stretching out the domains D1–D6. Secondly, the twisted arms are represented by the sharp angle at the interface of the domains D2 and D3 as well as the bending between D4 and D5.

Finally, a rigid-body model was generated using seven individual rigid subunits corresponding to the Fc domain and six single extracellular domains in the asymmetric unit (Fig. 4C). In comparison to the three-subunit model, the agreement with the experimental data for this model is only slightly improved (\( \chi = 1.3 \)) (Fig. 5). With respect to the overall size and the general shape of the domains D1–D6, it is consistent with the rigid-body model using three rigid subunits and the ab initio model. Given the low

Fig. 4. Rigid-body models obtained using different subunits in the asymmetric unit: (A) the ectodomain D1–D6 and the Fc domain; (B) the membrane-distal domain D1–D3, the membrane-proximal domain D4–D6 and the Fc domain; (C) six individual extracellular domains (D1, D2, D3, D4, D5 and D6) and the Fc domain. The Fc domain is at the bottom of the model. Upper part: front view; lower part: front view rotated by 90°.

Fig. 5. Comparison of the experimental X-ray scattering pattern (dots) with the calculated scattering pattern of the ab initio model and the various rigid-body models, which are obtained using (A) two (D1–D6 and Fc), (B) three (D1–D3, D4–D6 and Fc) or (C) seven (D1, D2, D3, D4, D5, D6 and Fc) subunits in the asymmetric unit. Upper part: front view; lower part: front view rotated by 90°.
resolution of the SAXS data, variations in the orientations of the individual domain are observed between different runs of the rigid-body refinement.

However, the overall shape and the twisted features in the domains D1–D6 are consistently reconstructed.

The modelling using three rigid subunits was repeated by randomly adding virtual glycan molecules to the glycosylation sites, and the obtained models do not change significantly (data not shown).

Functional implications

The analysis of the SAXS data in terms of three-dimensional models is inherently ambiguous and care must be taken not to overinterpret the experimental data. We employed several independent modelling approaches to cross-validate the results. Importantly, the EOM analysis indicated that the monomeric construct is rigid in solution, which enable us to construct rigid-body models employing the available high resolution structures.

The crystal structure of the extracellular domains (D1–D6) revealed that domains D1–D3 and D4–D6 form two rigid segments in the molecule (Xu et al., 2010). The use of the entire crystallographic monomer in the rigid-body analysis does not provide adequate fits to the SAXS data. This indicates that the orientation of the two rigid segments (D1–D3 and D4–D6) observed in the crystal might be imposed by crystallisation packing forces. The missing interaction between D3 and D4 observed in the crystal structure may lead to a flexibility of these two segments relative to each other in solution. This observation is in very good agreement with the fact that the measured scattering data can be best fitted by the three-rigid-body model (D1–D3, D4–D6 and Fc). This model (Fig. 4B) is also in good agreement with the ab initio shape of sgp130Fc. Note that in this model the two cytokine-binding segments (D1–D3) are not in the orientation as observed in the hexameric complex of (IL-6/IL-6R/gp130)2. Due to the rotational freedom between D1–D3 and D4–D6, these segments can be easily positioned as observed in the ligand-bound complex (not shown). The rigid body model in Fig. 4C constructed from seven subunits in the asymmetric part, although revealing similar overall features, could already be an overinterpretation of the scattering data, having too many free parameters in the modelling while yielding only marginally better fit to the data.

Cell-bound single domain deletion constructs (∆D4, ∆D5, ∆D6) of gp130 have been described previously (Kurth et al., 2000). Interestingly, none of these constructs was able to induce signal transduction. Whereas the ∆D4 and ∆D6 constructs showed a strongly reduced ability to bind the ligand, ∆D5 behaved like the wild-type molecule in terms of ligand binding (Kurth et al., 2000). These experiments demonstrated the importance of the three membrane-proximal domains for signal transduction. In order to understand the effect of these deletion mutants on ligand binding, we constructed models of these molecules using the sgp130Fc structure in Fig. 4B as a template. For instance, to construct the ∆D6 structure, we moved the D1–D5 segment in such a position that the D5 domain was best superimposed onto D6 of the template. A comparison of the template structure with these generated models is depicted in two different views in Figs. 6 and 7.

As shown in Fig. 6, the ligand-binding domains D1–D3 of the template structure and the ∆D5 model point into the same direction and can be superimposed by a rotation around the connection between D3 and D4 (Fig. 7). In the case of the ∆D4 model, the ligand-binding domains D1–D3 protrude from the rest of the molecule, leading to a strongly elongated structure. A rotation around the linker between D3 and D5 will never lead to an orientation observed in the template. In the case of the ∆D6 model, this becomes even more obvious as shown in Fig. 7D. The ligand-binding

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domains D1–D3 are pointing in opposite directions, thereby preventing any ligand binding. It should be noted that in case of the ΔD5 construct, an additional flexibility is introduced by the non-native interface of D4 and D6, which gives an additional degree of freedom to reorient the ligand-binding domains D1–D3 to enable ligand binding. Although sgp130Fcs is a rigid molecule, the single gp130 extracellular regions are not in direct close contact. The space between the single chains might be occupied by the carbohydrate chains attached, like it has been observed for the Fc part of antibodies (Harris et al., 1992). This would explain the instability and aggregation tendency of unglycosylated gp130 (Waetzig et al., 2010).

The low affinity dimerisation of membrane-bound gp130 argues against a protein/protein interaction and might also reflect the involvement of carbohydrates. The role of the three membrane-proximal domains of gp130 in the transmission of the signal still remains unclear until the structure of sgp130Fc or the full-length extracellular part of gp130 in the presence of its ligand complex have been solved.

Acknowledgements

The authors would like to thank Christin Hollande and Nadja Karl for excellent technical assistance. This work was supported by the German Excellence Initiative (Excellence Cluster “Inflammation at Interfaces”) and by a grant of the Deutsche Forschungsgemeinschaft (Collaborative Research Center SFB 415). RH thanks the Fonds der Chemischen Industrie for continuous support.

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G Model