Crystal Structure of Interleukin-21 Receptor (IL-21R) Bound to IL-21 Reveals That Sugar Chain Interacting with WSXWS Motif Is Integral Part of IL-21R*†

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Received for publication, October 7, 2011, and in revised form, December 23, 2011. Published, JBC Papers in Press, January 10, 2012, DOI 10.1074/jbc.M111.311084

IL-21 is a class I cytokine that exerts pleiotropic effects on both innate and adaptive immune responses. It signals through a heterodimeric receptor complex consisting of the IL-21 receptor (IL-21R) and the common γ-chain. A hallmark of the class I cytokine receptors is the class I cytokine receptor signature motif (WSXWS). The exact role of this motif has not been determined yet; however, it has been implicated in diverse functions, including ligand binding, receptor internalization, proper folding, and export, as well as signal transduction. Furthermore, the WXXW motif is known to be a consensus sequence for C-mannosylation. Here, we present the crystal structure of IL-21 bound to IL-21R and reveal that the WSXWS motif of IL-21R is C-mannosylated at the first tryptophan. We furthermore demonstrated that a sugar chain bridges the two fibronectin domains that constitute the extracellular domain of IL-21R and anchors at the WSXWS motif through an extensive hydrogen bonding network, including mannoseylation. The glycan thus transforms the V-shaped receptor into an A-frame. This finding offers a novel structural explanation of the role of the class I cytokine receptor signature motif.

Background: The class I cytokine IL-21 exerts pleiotropic effects on innate and adaptive immunity.

Results: We obtained the crystal structure of the partially glycosylated IL-21 receptor (IL-21R) bound to IL-21.

Conclusion: A sugar chain is an integral part of IL-21R.

Significance: This structure offers an insight into the putative role of the class I cytokine receptor signature motif.

IL-21 is a class I cytokine with a four-helix bundle structure arranged in an up-down-down-up topology typical for the class I cytokines (1). It exerts pleiotropic effects on both innate and adaptive immune responses. IL-21 is secreted by activated CD4+ T cells, in particular Th17 and T follicular helper cells, as well as natural killer cells (2). Not only do both Th17 and T follicular helper cells produce IL-21, but this cytokine also plays an important role in promoting the development of Th17 and T follicular helper cells by a feed-forward mechanism (3–8). Furthermore, IL-21 cooperates with other cytokines to increase the cytotoxicity of CD8+ T cells and promotes proliferation of CD8+ cells in the presence of antigens (9). IL-21 also influences antibody production by B cells (10). Recent studies demonstrated that IL-21 produced by CD4+ cells is critical for the ability of CD8+ T cells to control viral infection (11–13). The ability of IL-21 to augment immunity has spurred substantial interest in the therapeutic use of IL-21, and it is currently being evaluated in a number of clinical trials against, for example, metastatic melanoma and renal cancer (14).

IL-21 signals through a heterodimeric receptor complex consisting of the private chain IL-21 receptor (IL-21R)2 and the common γ-chain (γC), the latter being shared by IL-2, IL-4, IL-7, IL-9, and IL-15 (15). Upon binding of IL-21 to the receptor complex and subsequent receptor activation, signaling occurs through the Jak-STAT signaling pathway (16). The IL-21R chain binds IL-21 with high affinity and provides the majority of the binding energy (17). However, interaction with γC is required for signaling (16), and IL-21 mutants that bind IL-21R but fail to interact properly with γC act as potent antagonists of IL-21 signaling (1).

Several structures of γC class cytokines bound to one or more of their receptor chains have been solved (18–22). The receptors generally adopt very similar structures, with two type III fibronectin domains separated by a short linker. Each receptor chain adopts a V-shape, with the ligand binding at the tip of the V. The fibronectin domains each contain seven β-strands forming a sandwich-like structure. Binding of the ligand to the receptor engages residues present within the loops of both fibronectin domains.

Receptors of the γC cytokines are known to be universally N-glycosylated (20). This glycosylation is not required, however, for formation of the ligand-receptor complexes, as structures using receptors purified from Escherichia coli have been

* This work was supported by a research fellowship from the Danish Cancer Society (to O. J. H.) and by grants from the Danish Cancer Society, the Danish Council for Independent Research Natural Science, and the Novo Nordisk Foundation (to R. H.).
† This article contains supplemental Tables S1–S3.
The atomic coordinates and structure factors (code 3TGX) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
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The abbreviations used are: IL-21R, IL-21 receptor; γC, γ-chain; ECD, extracellular domain.
solved (20, 22). Still, the binding affinity between IL-7 and IL-7R has been demonstrated to be increased when IL-7R is glycosylated (20). Whether this is also the case for other members of the family remains to be determined.

The class I cytokine receptor family is characterized by the presence of the so-called class I cytokine receptor signature motif in the second fibronectin domain (24). The consensus sequence of the motif is WSXWS (present in 28 of 36 investigated sequences (25)). Several possible functional roles of the WSXWS sequence have been suggested, including involvement in ligand binding, receptor internalization, proper folding, export, and signal transduction (25–28). Although numerous studies have addressed the functional role of this highly conserved receptor motif, no clear picture has emerged. However, the first structures of class I cytokine receptors have clearly demonstrated that the motif is not directly involved in ligand binding (18–22, 29). Furthermore, despite the highly conserved nature of this motif, considerable sequence diversity is tolerated as demonstrated by several mutagenesis studies (25–27). The most extreme example is the human growth hormone receptor, in which the motif has the sequence YTEFS rather than WS (30).

Interestingly, WXXW is known to be a consensus sequence for C-mannosylation, where a mannose is attached to the first tryptophan (31), and this kind of modification has indeed been found in the WSXWS motif of the class I cytokine receptors IL-12B and EPOR (32, 33). The structures of class I cytokine receptors solved so far have not included this modification, possibly because the proteins have been produced in either insect cells or E. coli, where this modification is not made. The mechanism and potential function of this modification are currently unknown.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—IL-21 was expressed and purified from E. coli as described previously (34). The extracellular domain (ECD) of IL-21R was expressed in HEK293 cells and purified as described (1).

**Complex Formation**—The complex of IL-21 and IL-21R was formed by mixing IL-21 and IL-21R at room temperature at a ratio of 1:5:1. Elevated levels of IL-21 were used, as IL-21 is most readily available. Furthermore, as IL-21 (15–16 kDa) is much smaller than IL-21R (28 kDa), it is more easily separated from the complex (43 kDa) after gel filtration. The complex was loaded onto a HiLoad 16/60 Superdex 75 column (GE Healthcare) and eluted with PBS (10 mM phosphate and 150 mM NaCl, pH 7.4). The fractions containing the complex were concentrated to 5 mg/ml using an Amicon Ultra-4 centrifugal filter device with a 10,000 Mₐ cutoff.

**Crystallization**—All crystals were grown at 18 °C as sitting drops with a reservoir solution containing 500 μl of 1.8–1.9 M diammonium sulfate and 0.1 M sodium acetate at pH 5.5. 1 μl of reservoir solution and 1 μl of protein solution were mixed in the reservoir solution and 1 μl of protein solution were mixed in the reservoir solution and 1 μl of protein solution were mixed in the reservoir solution and 1 μl of protein solution were mixed in the reservoir solution. From this, initial NCS operators were calculated, and density modification was repeated using this 8-fold NCS. A polyalanine model of IL-2RB was created and divided into its two constituent fibronectin domains. These were placed in the regions of the map containing density for IL-21R. Using Resolve (38), one NCS group with eight operators was made for IL-21, as well as for each of the two fibronectin domains of IL-2RB. Upon refinement of the NCS operators, a final round of density modification and phase extension to 3.5 Å was performed in Resolve. The resulting map was of excellent quality at the resolution, and the IL-21R structure was built de novo by repeated cycles of building and refinement in Coot (39) and Phenix, respectively. The final model contains eight molecules of IL-21R and IL-21, forming the IL-21R-IL-21 complex refined to a resolution of 2.8 Å. Evaluation of the Ramachandran plot in Coot showed 94.25% and 5.75% in preferred and allowed regions, respectively.

**RESULTS**

**Overall Structure**—We have determined the structure of IL-21 in complex with the ECD of its private receptor chain IL-21R at a resolution of 2.8 Å (Fig. 1A and Table 1). The unit cell contains eight IL-21R-IL-21 complexes. The core structures...
of the eight IL-21R-IL-21 complexes are highly similar. The main difference observed is in the flexible loop connecting helices C and D in IL-21. The interaction surface between IL-21R and IL-21 is identical, however, in the eight structures. The following description represents molecules A (IL-21R) and B (IL-21) in the Protein Data Bank file (code 3TGX).

**IL-21R Structure**—IL-21R contains two fibronectin III domains that form a V-shaped structure, with the binding site for the cytokine positioned at the tip of the V (Fig. 1B). The two fibronectin domains are connected by a short linker and bent at an angle of ~90°. The D1 domain contains three disulfide bridges: one connecting the N terminus to β-strand 7 (Cys1–Cys90), one connecting β-strands 1 and 2 (Cys6–Cys16), and one connecting β-strands 4 and 5 (Cys46–Cys62). The membrane-proximal domain (D2) does not contain any disulfides but includes a WSXWS motif in the F’G’ loop, which is character-
istic for class I cytokine receptors. IL-21 exhibits the typical type I cytokine structure composed of a four-helix bundle with an up-up-down-down topology. The finished structure consists of residues 1–208 of IL-21R and residues 2–81 and 89–123 of IL-21, with small variations in the number of built residues between the eight NCS-related molecules. The missing part of IL-21 is the loop connecting helices C and D. This part of the loop is close to neither the IL-21R-binding site nor the predicted γC-binding site.

**IL-21R-IL-21 Interface**—The interaction between IL-21 and IL-21R is mediated by residues present in helices A and C and by a small part of the CD loop immediately following helix C of IL-21 (Fig. 1, A and B, and supplemental Table S1). The total binding surface contributed by both IL-21 and IL-21R is 990 Å² (calculated using PDBe PISA (40)) and includes extensive sets of polar and apolar interactions (supplemental Table S1). Ten different residues of IL-21 participate in polar interactions with 11 residues of IL-21R. There are 14 residues of IL-21 forming van der Waals contacts with 16 residues of IL-21R. In IL-21, Arg⁵, Arg⁹, and Gln¹² of helix A and Arg⁷⁶ and Lys⁷³ of helix C are the major contributors to the binding surface. All five residues form extensive polar and van der Waals interactions with residues of IL-21R (supplemental Table S1). Arg⁷⁹, Gln¹², Arg⁷⁶, and Lys⁷³ along with Ile¹⁶ form a pocket for Met⁷⁰ of IL-21R, the main contributor to the binding surface in IL-21R. It is interesting to note that the large hydrophobic side chain of Met⁷⁰ fits into a pocket of mainly hydrophilic residues. Met⁷⁰ seems to play a crucial role in positioning Arg⁶, Gln¹², Arg⁷⁶, and Lys⁷³ correctly in relation to their other interaction partners. Thus, Met⁷⁰ positions Arg⁶ and Arg⁷⁶ of IL-21 for interaction with Asp⁷² and Asp⁷³ of IL-21R, respectively (Fig. 2, A and B). The IL-21-binding residues of IL-21R are located in the loops connecting the β-strands. The AB, CD, EF, B'C', and F'G' loops and the linker all contain residues involved in binding. In IL-21R, Tyr³⁶ in the CD loop, Met⁷⁰ and Asp⁷² in the EF loop, and Tyr¹²⁹ in the B'C' loop contribute the most to the binding surface. The most important loop is the EF loop, which supplies 7 of the 20 amino acids of IL-21R that are involved in binding IL-21 (Fig. 1C and supplemental Table S1).

**Comparison of Free and Receptor-bound IL-21**—The structure of free IL-21 has been previously solved by NMR (34). In contrast to IL-21R-bound IL-21, the structure of the free form contains the N- and C-terminal parts, as well as the CD loop. These are highly disordered, however, explaining why they are absent in the IL-21R-IL-21 structure determined by crystallography. This difference is thus a result of the differences between the techniques of NMR and x-ray crystallography rather than a consequence of ligand binding. In solution, IL-21 exists in equilibrium between two distinct structures, in which helix C is either partially unfolded or formed. The crystal structure reported here indicates that helix C of IL-21 is stabilized upon binding to the receptor. The root mean square deviation using 106 backbone Ca atoms of IL-21 is 1.6 Å (calculated using SSM superimpose in Coot (41)), with the main differences seen in the first part of the CD loop and in the beginning of helix A. In the receptor-bound structure, the N-terminal part of helix A is slightly elongated compared with the free form. This is likely a result of receptor binding, as Arg⁶ of IL-21 is present in this region and interacts with IL-21R (supplemental Table S1). The most noticeable effect of IL-21R binding is observed in the first part of the CD loop of IL-21 (Fig. 3). The CD loop is positioned toward the receptor, allowing Lys⁷⁷, Pro⁷⁹, and Ser⁸⁰ to form a binding pocket for Tyr³⁶ of the receptor. Previously, a homology model of the IL-21R γC-IL-21 complex was built using the NMR model of IL-21 and the structures of IL-2 in complex with IL-2RA, IL-2RB, and γC, as well as IL-4 in complex with IL-4RA and γC (34). The position of the IL-21R chain predicted in this work is in good agreement with the IL-21R-IL-21 crystal structure presented here.

**Sugar Chain Is Integral Part of IL-21R**—The ECD of IL-21R contains five potential N-linked glycosylation sites (Asn⁵⁴, Asn⁷⁸, Asn⁸⁵, Asn¹⁰⁶, and Asn¹¹⁶), and the purified ECD of IL-21R recombinantly expressed in HEK293 cells includes ~10-kDa glycans. To establish which sites might be required for receptor integrity, all potential Asn-linked glycosylation sites were individually mutated to Gln, and the effect was evaluated by expression in HEK293 cells. Only the Asn⁵⁴-to-Gln mutation had a profound effect, as it led to an almost complete loss of secreted protein (Fig. 4A). To avoid interference from nonessential sugar residues in both crystallization and biochemical tests, the glycansubstituted ECD of IL-21R (N⁷⁸Q, N⁸⁵Q, N¹⁰⁶D, and N¹¹⁶Q)
was expressed in HEK293 cells. This was the protein used for crystallization.

Two Domains of IL-21R Are Bridged by Sugar Chain That Could Potentially Stabilize Receptor—To our surprise, we found that Asn54 is connected to the highly conserved WSXWS motif by an N-linked glycan bridge, which forms a cross-bar, turning the flexible V-shape, formed by the two fibronectin domains, into a seemingly rigidified A-frame (Figs. 1A and 4B). Digestion with peptide:N-glucosidase F to deglycosylate the protein failed (data not shown), suggesting that the sugar-based cross-link is an integral part of IL-21R and therefore might be protected from digestion. The composition of the sugar chain attached to Asn54 was investigated by LC-MS, revealing that the glycosylation is rather heterogeneous (supplemental Table S2). However, a common core consisting of two N-acetylglucosamines and four hexoses, most likely mannose, was observed (Fig. 4D and supplemental Table S2). In most of the sugar chains, a fucose is also present. The IL-21R structure presented here has been built to represent this most prevalent glycan chain, i.e. with two N-acetylglucosamines, four mannoses, and one fucose residue (Figs. 1A and 4B). The electron density and the LC-MS analysis indicate that additional sugar residues are present in the receptor chain (Fig. 4, B and D, and supplemental Table S2). However, these cannot be placed with confidence due to the heterogeneity of the glycosylation and the possible flexibility of the sugars, which do not engage in the hydrogen bonding network described above. The position of the additional glycosylation is indicated in Fig. 4D.

Mannosylation at First Tryptophan in WSXWS Motif Forms Extensive Hydrogen Bonding Network with Sugar Chain Originating from Asn54—The hydrogen bonding network between the sugar chain and the WSXWS motif offers a unique insight into how glycans can be integrated into protein structures (Fig. 4C and Table 2). Arg182 is held tightly in place between the two tryptophans of the WSXWS motif, forming the W-R-W zipper described in the text. D, the glycan structure is heterogeneous. The extra sugar residues might exist at this position, which cannot be built in the electron density. The core structure depicted here is found in the large majority of the glycans. The full glycan structure was determined by mass spectrometry (supplemental Table S2).
additional density was observed around Trp\textsuperscript{195}, suggesting that this residue was modified. Indeed, protease digestion followed by LC-MS analysis confirmed that Trp\textsuperscript{195} is C-mannosylated at C1 of the indole ring (data not shown). Thus, the carbohydrate leading to formation of a pocket providing binding of Tyr\textsuperscript{36} of structure of IL-21 in solution, with the most prominent differences between the D1 and D2 domains, making contact with the WSXWS motif (23). This helix thus occupies the same position as the glycan bridge in IL-21R. It is tempting to speculate that the sugar bridge has an influence on IL-21R activity (34). Helix C in IL-4 is more ordered than its equivalent part of IL-21, 29, 43). These structures can thus not help elucidate the case for other members of the family remains to be determined. Biochemical studies of EPOR have shown that mutations within the WSXWS motif impair export of EPOR to the cell surface and lead to accumulation in the Golgi (25, 45). In particular, the first Trp of the WSXWS showed no tolerance for substitution. Interestingly, EPOR has an N-terminal helix that takes up the space between the D1 and D2 domains, making contact with the WSXWS motif (23). This helix thus occupies the same position as the glycan bridge in IL-21R. It is tempting to speculate that the sugar bridge has an influence on IL-21R signaling, possible via a stabilizing effect on the ECD of IL-21R. However, we have been unable to address this important question properly, as mutations that eliminate the sugar chain originating from Asn\textsuperscript{54} also impair cell surface expression of the receptor.

Several members of the class I cytokine receptor family have potential N-linked glycosylation sites in the D1 domain facing the D2 domain (e.g. IL-2RB has Asn\textsuperscript{17}, \gammaC has Asn\textsuperscript{49}, and IL-7R has Asn\textsuperscript{29}) (17, 20). Most of the previous structures of type I cytokine complexes use receptors expressed in insect cells, which show less complex glycosylation patterns than mammalian cells and do not mannosylate the WSXWS sequence (19, 21, 29, 43). These structures can thus not help elucidate whether the N-linked glycan bridge observed in the IL-21R structure, is a specific feature of IL-21R or a more general feature of the class I cytokine receptor family.

Acknowledgments—We thank V. Olieric (Swiss Light Source) for assistance with synchrotron data collection. Beamline access was supported in part by the Dmascott Consortium.

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