The high resolution three-dimensional structure of human interleukin (hIL)-21 has been resolved by heteronuclear NMR spectroscopy. Overall, the hIL-21 structure is dominated by a well defined central four-helical bundle, arranged in an up-up-down-down topology, as observed for other cytokines. A segment of the hIL-21 molecule that includes the third helical segment, helix C, is observed to exist in two distinct and interchangeable states. In one conformer, the helix C segment is presented in a regular, α-helical conformation, whereas in the other conformer, this segment is largely disordered. A structure-based sequence alignment of hIL-21 with receptor complexes of the related cytokines, interleukin-2 and -4, implies that this particular segment is involved in receptor binding. An hIL-21 analog was designed to stabilize the region around helix C through the introduction of a segment grafted from hIL-4. This novel hIL-21 analog was demonstrated to exhibit a 10-fold increase in potency in a cellular assay.

Interleukin (IL) 2-21 is a recently identified type 1 cytokine, which is secreted as a 133-amino-acid protein by activated CD4 + T cells (1). The IL-21 cytokine has been demonstrated to possess potent stimulatory effects on the proliferation, differentiation, and activation of several classes of hematopoietic cells, including B-cells, T-cells, and NK-cells. The biological effects of IL-21 are mediated via activation of the IL-21 receptor complex, which is composed of an IL-21 private receptor chain (IL-21Rα) in complex with the common γ chain (γc), which similarly constitutes an essential component of the signaling receptor complex of the cytokines IL-2, IL-4, IL-7, IL-9, and IL-15. These cytokines thus constitute a subfamily referred to as common γ chain cytokines, with IL-21 being the most recently added member (2).

Within the common γ chain family of cytokines, high resolution structural information has been obtained through x-ray crystallography and NMR spectroscopy for IL-2 and IL-4 (3–8). It is apparent from these studies that IL-2 and IL-4 along with other type 1 cytokines, including IL-6 and granulocyte-macrophage colony-stimulating factor, share a common overall topology in their structures despite a distant homology in sequence (9). The common structural motif of these proteins consists of a central four-helical bundle, arranged in an up-up-down-down topology, connected by loops that are characterized by a high degree of structural freedom, a considerable difference in loop length, and variation in the number and positioning of stabilizing disulfide bridges.

Crystal structures have also been reported for IL-2 and IL-4 in complex with the corresponding private chains and, in the case of IL-2, the common γ chain (10–12). IL-2 is distinct from both IL-4 and IL-21 by having two private receptor chains, IL-2Rα and IL-2Rβ, where IL-2Rβ is homologous to IL-4Rα and IL-21Rα. Only minor structural differences are observed between the free and receptor-bound forms of IL-2 and IL-4, indicating that only slight structural changes occur for these cytokines upon complex formation. These studies accurately identify the residues of the cytokines involved in receptor binding and closely mirror earlier results obtained from mutagenesis studies (13, 14).

To further understand structure-function relationships within the common γ chain family of cytokines and their receptors, we here report studies of hIL-21 by NMR spectroscopy and mutagenesis. Although the structure resolved for the fully folded form of hIL-21 bears a strong resemblance to the structures previously reported for granulocyte-macrophage colony-stimulating factor, IL-2 and IL-4, we have identified some highly distinct features of the hIL-21 molecule. NMR exchange and variable temperature studies of hIL-21 reveal the presence of two distinct yet interchangeable states for a segment extending from Ser 57 to Gly 84. This segment includes and extends beyond helix C in both directions. Chemical shift values and 15N relaxation data show that the Ser 57 –Gly 84 segment is fully folded in one conformer while partially unfolded and missing helix C in the other conformer. In both forms, part of the long CD loop, flanking helix C, is unstructured.

A model of the hIL-21 receptor complex suggests the involvement of the structurally heterogenic helix C of hIL-21 in the interaction with IL-21Rα. Since a segment of hIL-21, which is believed to be critical for receptor binding, exists in alternate conformations, these subpopulations are expected to differ in their inherent binding affinity to the receptor interface. Thus, we undertook the construction of an hIL-21 variant, in which the segment surrounding the unstable helix C region had been...
substituted with the corresponding yet more stable sequence of hIL-4. This analysis led to the identification of a novel hIL-21 variant that possesses a 10-fold increase in potency with respect to hIL-21 receptor activation. The improved potency of this chimeric variant is rationalized to arise as a result of having engineered a higher degree of local stabilization, thus reorganizing hIL-21 for receptor binding, specifically in a domain that is critical for receptor binding.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Met-hIL-21 consists of the sequence of the mature human IL-21 protein as determined by recombinant expression in mammalian cell culture but including an extra methionine residue added recombinantly at the N terminus. The protein is numbered starting with Glu as residue 1. The N-terminal methionine residue that is present when the protein is expressed in Escherichia coli thus becomes residue Met. The cDNA corresponding to the Met-hIL-21 coding sequence was cloned in the pET11c expression vector, expressing the mature hIL-21 in E. coli BL21 DE3 strain, and induced during exponential growth with 1 mM isopropyl-β-D-galactopyranoside for 4 h prior to harvest. Multiple and comparable preparations of the Met-hIL-21 protein were prepared, purified from inclusion bodies, and tested for activity. In the following, Met-hIL-21 expressed in E. coli will be denoted hIL-21.

For NMR analysis, the protein was expressed in E. coli growing in a minimal medium with [15N,15N,13C]glucose and/or [13C6]glucose as the main source for nitrogen and carbon. Inclusion bodies were solubilized in 6 M guanidinium HCl, 100 mM Tris-HCl, 40 mM dithiothreitol at pH 8.0 and refolded by dilution into a refolding buffer containing 0.75 M L-arginine, 40 mM Tris-HCl, 0.005% polyethylene glycol 3350, 1.5 mM dithiothreitol, 4 mM cysteine, 20 mM NaCl, 4 mM MgCl2, 1 mM KCl, 4 mM CaCl2 at pH 7.5 and left overnight at 15 °C with slow stirring. Refolding was stopped by adjusting pH to 5.5 with acetic acid followed by 4-fold dilution into 25 mM sodium acetate, pH 5.5. Misfolded hIL-21 was allowed to precipitate and removed by filtration.

Refolded hIL-21 was captured on a TosoHaas SP550C column and eluted with 1 mM NaCl at pH 8.5 using a step gradient. Fractions with hIL-21 were pooled and diluted 10-fold with 10 mM Tris-HCl, pH 8.5, before being loaded onto a Sepharose SP column. hIL-21 was eluted using a linear gradient from 0.1 to 1 M NaCl in 25 mM Tris-HCl at pH 5.5. Fractions with hIL-21 were pooled and concentrated with a Centriprep centrifugal filter from Millipore (cut-off 10 kDa). The concentrated hIL-21 was allowed to precipitate and removed by filtration.

NMR Experiments—Unless otherwise specified, NMR spectra were acquired at 27 °C on a Bruker Avance 600-MHz spectrometer equipped with a 5-mm TXI cryoprobe. One-dimensional 1H spectra were acquired for hIL-21 samples with concentrations in the range 0.2–2.0 mM.

Sequential backbone assignments were done using established methods (15, 16). Assignment of side chain resonances included the use of HN(CO)HAHB, H(N)(CCC)NH, (H)(CCC)NH, (H)(CH-TOCSY, and (H)CH-TOCSY spectra as well as 15N- and 13C-edited NOEHSQC spectra. The 15N- and 13C-edited NOE-HSQC spectra were acquired on a Varian Inova 800-MHz spectrometer equipped with a 5-mm 1H [15N,13C] TXI cryoprobe, respectively.

Temperature studies were performed by acquiring 15N HSQC spectra at temperatures between 10 and 50 °C. Control spectra at 27 °C were acquired before and after the experiments. 15N HSQC type exchange experiments were acquired with mixing times ranging from 0 to 1000 ms (17). Experiments measuring backbone amide 15N T1 and T2 relaxation times and heteronuclear [1H]-15N NOEs were acquired on a Bruker Avance 600-MHz spectrometer equipped with a 5-mm 1H [15N,13C] TXI cryoprobe (18). T1 and T2 values were determined by least-square fitting of signal intensities to an exponential curve. Heteronuclear [1H]-15N NOEs were calculated as the intensity ratios between 15N HSQC spectra recorded with and without proton saturation.

Cells and Cell Culture—The Baf3/hIL-21Rα cells, which were employed for the activity analysis, were maintained in RPMI 1640 medium, including 10% heat-inactivated fetal bovine serum, 5% penicillin/streptomycin, 1 ng/ml murine IL-3, 200 mg/ml zeocin, and 1 mg/ml neomycin. The cells that have been transfected to stably express hIL-21Rα also harbor an expression reporter cassette in which the luciferase gene is linked to a STAT-inducible promoter element. The Baf3 cell, being a murine pre-B-cell line, expresses endogenously the murine γc, which has been demonstrated to be functionally active when engaged in a complex with the human hIL-21Rα receptor chain.

Construction of IL-21 cDNA Variant—The expression construct encoding the hIL-21 variant, Chim-hIL-21/4, was generated through site-directed mutagenesis of the wild type hIL-21 cDNA. Mutations were introduced by PCR-directed mutagenesis using the overlap extension protocol and Pwo polymerase. The full open reading frame of the mutated hIL-21 cDNA was verified by sequencing.

Activity Assays—The Baf3/hIL-21Rα cells were seeded (50,000 cells/well) in 96-well plates (Packard ViewPlate, catalog number 6005182) in 90 μl of medium and starved in IL-3-free medium for 6 h prior to stimulation. Protein was added to each well in increasing final concentrations, and the cells were stimulated by incubation for 24 h. To each well was added 100 μl of Luciferin reagent (PerkinElmer LuciLite Luminescence Receptor Gene Assay System, catalog number 6016911), and after 30 min in the dark, samples were analyzed in a Packard Topcount microplate scintillation counter. Data were analyzed by GraphPrism. Using this assay, hIL-21 expressed in bacterial and mammalian cells, respectively, has been analyzed in direct comparison. Met-hIL-21 expressed in E. coli and hIL-21 expressed in HEK293 cells have thus yielded potencies of 5.6 × 10^{-10} and

3 C. Herling, personal communication.
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3.1 \times 10^{-10} \text{ M}, respectively (n = 3), and equivalent levels of maximum stimulation.

**Modeling of hIL-21-hIL-2R and Chim-hIL-21/4 Complexes**—Structure alignments were performed with the program LSQMAN (19), and the program Indonesia (20) was used otherwise for sequence alignments and figures. Models were constructed with the program Modeler (21) using the crystal structure of the hIL-4-hIL-4R complex and the NMR structure of hIL-21 as templates. A sequence alignment between the three homologous receptor chains hIL-2R, hIL-4R, and Chim-hIL-21/4 was performed manually using the structural alignment of the binary hIL-2-hIL-2R and hIL-4-hIL-4R complexes as a starting point. Constraints were introduced to guide hydrogen bonding between Asp75 of hIL-21R and Arg76 of hIL-21 and Chim-hIL-21/4.

**RESULTS**

**Resolution of the hIL-21 Structure by NMR**—The \(^{15}\text{N}\) HSQC spectra recorded for hIL-21 display a fairly good dispersion despite a number of signals at random coil chemical shift values. These latter signals are due to the presence of flexible regions in the hIL-21 molecule. Dilution experiments reveal no signs of aggregation in the range of 0.2–2.0 mM hIL-21. Only minor changes were observed in \(^{15}\text{N}\) HSQC spectra acquired at different pH values and salt concentrations (pH between 5.0 and 7.0 and NaCl between 0 and 100 mM).

Backbone resonances were assigned on the basis of a standard set of three-dimensional experiments (15), which were acquired using a uniformly \(^{15}\text{N}/^{13}\text{C}\)-labeled hIL-21 sample. More than 96% of backbone resonances (N, HN, CA, HA, and CO) were assigned. Surprisingly, for residues Ser\(^{57}\)–Gly\(^{84}\), two distinct signals were observed for each residue, demonstrating the presence of two different species of hIL-21. Side chain resonance assignments are nearly complete (excluding the minor form at 27 °C of Ser\(^{57}\)–Gly\(^{85}\)), with only 23 nonlabile protons missing.

A plot of the difference between the observed chemical shifts and the tabulated values for random coil chemical shifts for backbone CA atoms (22) against sequence number is shown in Fig. 1. Here four regions displaying positive secondary chemical shift deviations reveal the presence and the position of the four \(\alpha\)-helices (A–D) observed within the major form of hIL-21. Helices A (Met\(^{37}\)–Asp\(^{26}\)) and D (Pro\(^{104}\)–Ser\(^{124}\)) are significantly longer than the helices B (Trp\(^{44}\)–Lys\(^{52}\)) and C (Asn\(^{63}\)–Lys\(^{73}\)). In the minor form of hIL-21 at 27 °C, the segment Ser\(^{57}\)–Gly\(^{84}\) shows no sign of helical structure (Fig. 1), suggesting that an extended, unordered conformation of this segment, which includes helix C sequence.

A total of 1235, 2994, and 449 peaks from \(^{15}\text{N}\)-separated, \(^{13}\text{C}\)-separated and two-dimensional NOESY spectra, respectively, were included in structure calculations. Peaks from the unfolded form of helix C (see below) were not included in these calculations. Together with chemical shifts for the assigned resonances, the NOEs were analyzed with Cyana using the candid protocol for automatic NOE assignment and structure calculation (23). Initial structure calculations were used to calibrate upper distance limits. Subsequently, all peak intensities were downscaled by a factor of 2 to correct for effects on peak intensities due to the presence of the unfolded form.

Additional sources of structural information were included in the calculations. Thus, two disulfide bonds were enforced between Cys\(^{42}\) and Cys\(^{49}\) and between Cys\(^{49}\) and Cys\(^{96}\). This disulfide pattern has been established for the hIL-21 molecule through an analysis that combined protease cleavage, Edman degradation, and mass spectrometry. Test calculations without disulfide bond constraints supported this pattern (not shown). Chemical shift values for HA, CA, CB, N, and CO atoms were analyzed to predict \(\phi\) and \(\psi\) backbone angles using the computer program Talos (24). Talos gave good predictions for 78 residues, and 156 angle \(\phi/\psi\) angle constraints were included in the calculations with an uncertainty of \(\pm 30^\circ\). From the HNHA spectrum, 72 J(HA-HN) scalar coupling constants were extracted and included in the structure calculations. Hydrogen bond constraints were added for 20 backbone amide protons, which exchange slowly in deuterium exchange experiments. Hydrogen bond patterns were established from structures calculated without hydrogen bond constraints.

An ensemble of 20 structures was calculated with Cyana (Table 1). No distance violations greater than 0.5 Å were observed. In only four cases was a dihedral constraint violated by more than 5°. Segments of the protein at the N terminus (Met\(^{37}\)–His\(^{10}\)) and at the C terminus (Ser\(^{124}\)–Ser\(^{133}\)) are disordered, as is part of the loop connecting helices C and D (Pro\(^{79}\)–Thr\(^{93}\)). Except for Met\(^{37}\)–His\(^{10}\)}, and at the C terminus (Ser\(^{124}\)–Ser\(^{133}\)) are disordered, as is part of the loop connecting helices C and D (Pro\(^{79}\)–Thr\(^{93}\)).

\(4\) I. Fabrin and S. Bayne, personal communication.
for these regions, the ensemble is well ordered, with a root mean square deviation of 0.64 Å for backbone heavy atoms (Fig. 2).

hIL-21 Is a Four-helical Bundle with an Up-Up-Down-Down Topology—hIL-21 forms a four-helical bundle arranged in an up-up-down-down topology, which is shared by all members of the family of short-chain cytokines (Fig. 3). As a consequence of this topology, two long overhand loops connect helices A and B and helices C and D and wrap around the helix bundle on the face formed by helices B and D. Part of the two connecting loops forms a small β-sheet, a structural element that is generally conserved within the short-chain cytokines. A shorter loop connects the helices B and C, which themselves are remarkably short in comparison with other family members, being composed of 9 and 11 amino acids, respectively. A novel feature of hIL-21, which is not shared by other short-chain cytokines, is the presence of a β-sheet-like contact between the connecting loops AB and BC, where a hydrogen bond is formed between the carbonyl oxygen of Lys56 and the amide group of Val28. This β-sheet-like contact is possible due to the short B helix, which is followed by a segment (Ala53–Asn59) presented in an extended conformation and packing against the sequence, Leu27–Phe31, from the first part of the AB connecting loop. The long overhanging CD loop follows the short helix C. The N-terminal part of this loop, residues Lys75–Lys77, adopts an extended conformation, which is followed by 16 unstructured residues (Pro78–Cys93).

Two Distinct Conformers of hIL-21 Exist in Equilibrium—During backbone assignment, two forms of hIL-21 were observed for residues within the segment Ser57–Gly84. The major form at 27 °C comprised helix C and the flanking loop regions, whereas the minor form showed no sign of helix formation. Presence of multiple signals for a resonance is due to either distinct chemical entities in the NMR samples or several conformers exchanging slowly on an NMR time scale. To further investigate the two sets of signals identified, NMR exchange spectra were recorded (17). In these 15N HSQC type spectra, a mixing period is inserted between the two evolution times. If a conformational exchange takes place between two states of the protein during the mixing period, four peaks will appear for each exchanging amide group. Two of these peaks represent the normal 1H,15N correlation peaks for the two conformers. The two additional peaks are due to a conformational exchange occurring during the mixing period. Exchange peaks were observed for residues Ser57–Gly84, thus proving the existence of two distinct states of hIL-21, which exchange on a millisecond to second time scale (Fig. 4A).
A temperature dependence of the relative peak intensities for signals attributable to the major and minor form of the Ser57 to Gly84 segment was observed in 15N HSQC spectra (Fig. 4B). Below 35 °C, the form in which helix C is present is the most abundant, whereas at higher temperatures the form lacking helix C predominates. Spectra acquired at 27 °C before and after the temperature experiments were very similar, thus demonstrating that the conformational changes taking place are reversible.

The degree of internal motion in hIL-21 was probed on a microsecond to nanosecond time scale via backbone amide15N T2 relaxation times and heteronuclear {1H}-15N NOEs (18) (Fig. 5A). For residues present within the four helices, the relaxation properties are fairly uniform, indicating a stable structure for the helix bundle of hIL-21. In contrast, the N-terminal residues, Met0–His5, as well as the C-terminal Ser125–Ser133 display increased 15N T2 values and decreased {1H}-15N NOE values (15N T1 values also decrease, but the effect is less pronounced), showing that the termini of the protein exhibit increased mobility. The three loops connecting the helices show a different dynamic behavior. The loop AB does not show increased dynamics relative to the helix bundle, thus indicating that this loop packs stably with the helix bundle. This is in agreement with the hIL-21 structure, in which part of the loop was observed to form β-sheets with both the BC and CD loops.

The rest of the loop packs between the helix bundle and the CD loop. The short BC loop shows increased dynamics for residues Gly61 and Asn62, both located at the turn between helix B and C and exposed to water. Residues in the first part of loop CD, extending from Pro79 to Pro94, form the most mobile segment of hIL-21, when excluding the C- and N-terminal residues. This is in accordance with the highly disordered structure of the segment, which was indicated in the NMR structure by the lack of long range NOEs. The last part of the CD loop is, however, ordered like the helix bundle and also partakes in the β-sheet described for the AB loop. Moreover, the loop is connected to helix B via two disulfide bonds (Cys42–Cys93 and Cys49–Cys96). The pattern of mobility in the unfolded form of Ser57 to Gly84 mirrors that of the folded form just at a higher level. T2 values are mapped onto the NMR structure in Fig. 5B for the folded form and Fig. 5C for the unfolded form (the two plots are identical outside the Ser57–Gly84 region). It is evident that the partial unfolding involves the segment around and including helix C. The segment is flanked on the C-terminal side by the highly flexible part of the CD loop, which perhaps destabilizes helix C.

A picture of a structurally unique region emerges for the segment of hIL-21 surrounding helix C. Together with the flanking loop residues (Ser57–Gly84), helix C thus exists in two distinct conformations present in equilibrium: a folded form, which predominates below 35 °C, and an unfolded and highly dynamic form, lacking helix C, which is dominant above 35 °C. This region overlaps with 16 unstructured residues (Pro78–Cys83) in the CD loop, which are devoid of secondary structural elements in both forms.

Stabilization of Helix C and the CD Loop upon Receptor Binding—The IL-21 receptor complex is composed of IL-21Rα and γc, the latter being shared by the cytokines IL-2, IL-4, IL-7, IL-9, and IL-15. Recently, crystal structures of a binary hIL-4-hIL-4Rα complex (11) and a quaternary hIL-2/hIL-2Rα/hIL-2β/γc complex (10) have been resolved. In these complexes, the hIL-2β and the hIL-4Rα chains are homologous to IL-21Rα. In both complexes, the hIL-4/hIL-4Rα and hIL-2/hIL-2β receptor interfaces are assembled around residues positioned mainly within helices A and C, whereas the hIL-2-γc receptor interface is assembled around residues on helices A and D.

The hIL-4/hIL-4Rα interface is formed around the two key positions Glu61 (helix A) and Arg88 (helix C) of hIL-4. Residue Glu61 docks into a pocket formed by Tyr13, Tyr127, and Tyr183 of hIL-21Rα. In both complexes, the hIL-4/hIL-4Rα and hIL-2/hIL-2β receptor interfaces are assembled around residues positioned mainly within helices A and C, whereas the hIL-2-γc receptor interface is assembled around residues on helices A and D.
Asp\textsuperscript{20} forms hydrogen bonds with His\textsuperscript{133} and Tyr\textsuperscript{134} in hIL-2R\textsubscript{H9252}, and Asn\textsuperscript{88} interacts with the Arg\textsuperscript{42} and Gln\textsuperscript{70} backbone carbonyls. However, superposition of the two complexes reveals that the two sets of interaction points are shifted roughly one turn toward the N terminus in the IL-4 complex compared with the IL-2 complex (Fig. 6, B and C).

To explore differences in receptor interfaces between IL-2, IL-4, and IL-21 and their cognate private receptors, a model of the hIL-21\textsubscript{H18528}hIL-21R\textsubscript{H9251} complex was constructed based on the crystal structures of the hIL-2 and hIL-4 receptor complexes. Sequence-based alignments of members of the IL-2 family are of poor quality due to the low sequence identity, which averages 19\% (10) between the individual members. The present structure of hIL-21 and those reported for hIL-2 and hIL-4 have, however, enabled a structure-based sequence alignment of these family members (Figs. 6, A and B). For hIL-2 and hIL-4, the binary hIL-2\textsubscript{H18528}hIL-2R\textsubscript{H9252} and hIL-4\textsubscript{H18528}hIL-4R\textsubscript{H9251} complexes were used for the alignment. Despite low sequence identities, hIL-4 superimposes well with both hIL-2 and hIL-4. The sequence alignment between the three homologous receptor chains hIL-2R\textsubscript{H9252}, hIL-4R\textsubscript{H9251}, and hIL-21R\textsubscript{H9251} is shown in Fig. 6C.

In the model of the hIL-21-hIL-21R\textsubscript{H9251} complex, interactions corresponding to Asp\textsuperscript{20} (helix A) and Asn\textsuperscript{88} (helix C) of hIL-2 are lost, since critical residues are conserved neither between hIL-2 and hIL-21 nor between hIL-2R\textsubscript{H9252} and hIL-21R\textsubscript{H9251} (Fig. 6, B and C). In contrast, interactions that correspond to those involving Glu\textsuperscript{9} of hIL-4 are preserved in the hIL-21-hIL-21R\textsubscript{H9251} complex (Fig. 6, B and C). Thus, residue Glu\textsuperscript{9} in hIL-4 corresponds to Gln\textsuperscript{12} in hIL-2, and the cluster of the three interacting tyrosines in hIL-4R\textsubscript{H9251} (Tyr\textsuperscript{13}, Tyr\textsuperscript{127}, and Tyr\textsuperscript{183}) are conserved in hIL-21R\textsubscript{H9251} (Tyr\textsuperscript{13}, Tyr\textsuperscript{131}, and Tyr\textsuperscript{193}). In the hIL-21 complex, Gln\textsuperscript{12} forms two hydrogen bonds to Tyr\textsuperscript{13} and Tyr\textsuperscript{193} in hIL-21R\textsubscript{H9251} in analogy to the interactions involving Glu\textsuperscript{9} of hIL-4. According to the structural alignment, the other important interaction, Arg\textsuperscript{88} in hIL-4 with Asp\textsuperscript{72} in hIL-4R\textsubscript{H9251}, is similarly conserved in the hIL-21 ligand-receptor complex, the corresponding residues being Arg\textsuperscript{76} (hIL-21) and Asp\textsuperscript{75} (hIL-21R\textsubscript{H9251}).

Residue Arg\textsuperscript{88} is present within the helix C segment of hIL-4, whereas Arg\textsuperscript{76} of hIL-21 is positioned beyond, on the C-terminal side of helix C within the extended CD loop as a consequence of helix C being significantly shorter in hIL-21 compared with hIL-4. Although Arg\textsuperscript{76} is exposed to the solvent in the hIL-21 structure, the conformation in which it is presented is still less optimal for receptor interaction compared with the positioning of Arg\textsuperscript{88} in hIL-4. In addition, in hIL-21, the extended region Lys\textsuperscript{75}–Lys\textsuperscript{77} is part of the region that partially unfolds and is followed by the highly flexible part of the CD loop. Upon receptor binding, Lys\textsuperscript{75}–Lys\textsuperscript{77} probably changes into a helical conformation, with the CD loop adopting a structurally better defined conformation and the equilibrium being shifted toward the folded form, thus positioning Arg\textsuperscript{76} in...
a considerably more favorable position to interact with Asp\textsuperscript{75} in hIL-21R. 

**Design of a Novel and Highly Potent Analog of hIL-21**

A major structural rearrangement of hIL-21 taking place upon receptor binding invariably will have adverse effects on the binding affinity toward the receptor, since part of the ligand-receptor binding free energy will be spent on stabilizing the structure of hIL-21 in the receptor-associated state. The loss of binding free energy due to a structural rearrangement might be circumvented through preorganization of unbound hIL-21 into a conformation resembling the receptor-bound state, thus leading to a higher binding affinity.

We have designed a variant of hIL-21, which we hypothesize will be preorganized in a receptor-bound state. In this variant, part of the structurally unstable region of hIL-21 around helix C and the CD loop has been replaced with the homologous, but structurally more stable, region of IL-4. In total, a stretch of 16 amino acids (Lys\textsuperscript{77}–Thr\textsuperscript{92}) located within the CD loop of hIL-21 was exchanged for the corresponding, yet shorter stretch of 10 amino acids originating from the hIL-4 sequence. This stretch of hIL-4-derived sequence is flanked on the N-terminal side by a conserved region in the two proteins (Lys\textsuperscript{72}–Arg\textsuperscript{88} in hIL-21 and Lys\textsuperscript{84}–Arg\textsuperscript{88} in hIL-4). On the C-terminal side, the stretch is flanked by a conserved disulfide linkage. This hIL-21/hIL-4 construct has been denoted Chim-hIL-21/4 (Fig. 8A).

To evaluate the design, a model of the complex formed between Chim-hIL-21/4 and the hIL-21R\textsubscript{1025} receptor chain was obtained under the assumption that the hIL-4 sequence, which was introduced into the hIL-21 backbone in Chim-hIL-21/4, would adopt the conformation present in the context of the native hIL-4 structure. Under this assumption, helix C will be extended by 8 residues in Chim-hIL-21/4 compared with the structure resolved for the wild-type hIL-21 protein. Position Arg\textsuperscript{88} in Chim-hIL-21/4 (corresponding to Arg\textsuperscript{88} in hIL-4 and Arg\textsuperscript{88} in hIL-21) is now an integral part of the helix C structure and presented more favorably to interact with Asp\textsuperscript{75} of hIL-21R (Fig. 8B).
A $^{15}$N HSQC spectrum was acquired on uniformly $^{15}$N/$^{13}$C-labeled Chim-hIL21/4 (Fig. 8C). Using a HNCO spectrum, 121 peaks in the HSQC spectrum could be assigned to backbone amide groups. This is in perfect agreement with Chim-hIL21/4 consisting of 122 residues excluding proline residues. A few very weak additional signals were also observed; however, these peaks did not display any exchange cross-peaks in NMR exchange spectra (data not shown). Thus, no conformational exchange was observed for Chim-hIL21/4, which seems only to be present in one form.

**DISCUSSION**

The hIL-21 structure is characterized by the typical cytokine topology composed of a central and rather rigid core, the four-helical bundle, and the connecting loop segments, which exhibit a significant degree of flexibility. The short $\beta$-sheet conserved throughout the family of short-chain cytokines and formed between the AB and CD loops is present in hIL-21. Helices B and C are quite short compared with hIL-2 and hIL-4, and deuterium exchange data indicate that helices A and D together with the conserved $\beta$-sheet constitute the most stable part of the structure. Due to the short helix B, the first part of the AB loop is able to form a $\beta$-sheet-like contact with the extended part of the BC loop. This novel feature of hIL-21 is possible neither in hIL-2 nor in hIL-4 due to their longer B helices.

A common feature for hIL-2, hIL-4, and hIL-21 is the disordered CD loop. NMR structures have previously been reported for IL-2 (4) and IL-4 (5, 6, 25). Compared with the four-helix bundle, the CD loop, excluding the $\beta$-sheet, is poorly defined in both structures. Backbone amide $^{15}$N relaxation measurements on hIL-4 showed that poorly defined loop regions are due to the internal motion (26). Similarly, part of the CD loop was not well defined in the crystal structure of hIL-2 (3). Thus, the CD loop seems to be inherently flexible within the family of common $\gamma$ chain cytokines.

In the hIL-21 structure, the structurally disordered region extends beyond the CD loop and includes helix C. Furthermore, the segment surrounding and including helix C exists inherently in two alternate and interchangeable conformations, a folded structure form and a flexible unfolded form. Such structural inhomogeneity has been observed neither for hIL-2 nor hIL-4 nor, to the extent of our knowledge, for any other of the several cytokine proteins that have thus far been structurally characterized. Other members of the family of short-chain four-helical cytokines display flexibility (e.g. both erythropoietin (27) and IL-13 (28) have a flexible CD loop). Part of the flexible CD loop in erythropoietin exists in two distinct forms, probably due to proline $cis$/$trans$ isomers (27). However, unlike hIL-21, helix C in erythropoietin does not display conformational heterogeneity.

Crystal structures of the quaternary hIL-2-hIL-2R$\alpha$-hIL-2R$\beta$$\gamma$, and binary hIL-4-hIL-4R$\alpha$ complexes (10, 11) have provided the basis for our current understanding of ligand-recep-
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Both in free hIL-4 and in the hIL-4-hIL-4Rα receptor complex, the region surrounding Arg$^{86}$ forms an α-helical structure, whereas the corresponding region in hIL-21, according to the NMR structure, adopts an extended structure. As a consequence, Arg$^{76}$ in hIL-21 is not optimally positioned to engage in the ion pair contact to hIL-21Rα Asp$^{73}$. It would thus appear very likely that upon receptor binding, the extended stretch following helix C in hIL-21 will rearrange into a helical conformation. In this way, structural stabilization would be attained upon complex formation.

In analogy, structural stabilization upon receptor binding is implied for hIL-4 from x-ray crystallographic studies. For helix C and the CD loop in IL-4, temperature factors are higher in the two original crystal structures of free hIL-4 (7, 8) compared with the hIL-4-hIL-4Rα complex (11) (data not shown). IL-4 receptor complex formation thus stabilizes the region corresponding to the structurally unstable region surrounding helix C in hIL-21. Likewise, we would expect stabilization of the region around the CD loop to take place in hIL-21 upon receptor binding. No structure of the binary hIL-2-hIL-2β complex has been determined, and the effect on the CD loop of hIL-2 as a consequence of hIL-2β binding is therefore not known. However, binding to hIL-2Rα has been shown to stabilize the hIL-2 structure and prime the formation of the hIL-2Rβ binding site (e.g. the affinity of hIL-2β is 2-fold higher toward the binary hIL-2-hIL-2Rα complex compared with hIL-2 alone (29)). Resolution of the hIL-2-hIL-2Rα complex has recently demonstrated that the CD loop of hIL-2, which is disordered in the free, unbound form of the ligand, becomes ordered when present in the receptor-bound form (12).

An ordering of the CD loop upon receptor binding requires expenditure of free energy and must be driven by binding free energy. A high degree of interface complementarity is required, which may only be supplied by the cognate receptor. The flexible CD loop within the family of common γ chain cytokines may therefore confer selectivity among the individual family members at the expense of binding affinity. At physiologically relevant temperatures, around 37 °C, hIL-21 exists in a close to 1:1 equilibrium between a form in which the helix C segment is unordered and a more folded form. We propose that the latter form more closely resembles a receptor-bound state of the protein, which possesses an enhanced affinity with respect to receptor interaction. In this way, nature may utilize molecular disorder or partial unfolding to control the thermodynamics of receptor activation and to provide selectivity.

In the hybrid protein, Chim-hIL-21/4, a segment of the hIL-21 sequence has been exchanged for the corresponding segment of hIL-4, specifically the C-terminal part of helix C and the first part of the CD loop. The rationale behind our design of this chimeric exchange has been that the helix C of the hIL-21/hIL-4 hybrid protein would be extended compared with native hIL-21, leading to a shortening of the CD loop and reduced flexibility. Indeed, no conformational exchange was observed for Chim-hIL-21/4, indicating that this protein is stabilized in one conformation. Prolongation of helix C would furthermore position Arg$^{76}$ of the Chim-hIL-21/4 protein in a structural context resembling Arg$^{86}$ of hIL-4 and in this way facilitate interaction with the conserved Asp$^{73}$ in the hIL-21Rα. Accord-

**FIGURE 7.** Ribbon plot of hIL-4-hIL-4Rα complex (A), hIL-21-hIL-21Rα (B), Chim-hIL-21/4-hIL21Rα (C). Side chain heavy atoms are shown for the conserved ion pair Arg$^{86}$–Asp$^{73}$ in the hIL-4-hIL-4Rα complex and Arg$^{76}$–Asp$^{73}$ in the hIL-21-hIL21Rα and Chim-hIL-21/4-hIL21Rα complexes.

A model of the hIL-21-hIL-21Rα complex has revealed a close resemblance between the hIL-21-hIL-21Rα and the hIL-4-hIL-4Rα interfaces. Important contacts in the hIL-4-hIL-4Rα interface are thus conserved in the hIL-21-hIL-21Rα complex (e.g. Arg$^{86}$ of hIL-4 (Arg$^{76}$ of hIL-21), which forms an ion pair with Asp$^{73}$ of IL-4 (Asp$^{73}$ of hIL-21)). It thus seems very likely that key interactions are conserved between these two receptor complexes.
ing to this view, Chim-hIL-21/4 would represent a preorganized and stabilized form of the protein conformation similar to that present in the receptor complex, and in this way lead to a free energy advantage upon complex formation relative to the native hIL-21. Although major receptor-cytokine contacts seem to be conserved between hIL-4 and hIL-21, we cannot rule out the possibility that contacts specific for hIL-21 are lost upon introduction of the hIL-4 segment. However, the 10-fold increase in potency displayed by the Chim-hIL-21/4 hybrid reveals that potential hIL-21-specific contacts are of minor importance at most.

A previous study has in a similar fashion demonstrated that exchanging segments of cytokines that despite a very limited sequence homology are believed to share a common overall three-dimensional structure can lead to functionally active hybrid proteins (30). Here we demonstrate that the exchange of a highly flexible segment of the hIL-21 protein with a segment of hIL-4 that represents a significantly more ordered structure leads to a hybrid protein that is not only functionally active with respect to stimulating the hIL-21 receptor signaling pathway but even exhibits a 10-fold enhanced potency.

The literature includes numerous examples of proteins that lack intrinsic structure either for the entire polypeptide chain or a portion thereof. In many such cases, the unstructured segments become increasingly structured upon binding to target molecules (31). This induction of structure upon complex formation has been linked both to the ability to recognize several targets (32) as well as to the contribution of specificity toward a single target (33). The hIL-21 structure represents an intermediate state between a folded protein and a protein lacking intrinsic structure and may reflect a fine tuning of protein-protein interactions in the IL-21 receptor complex.

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