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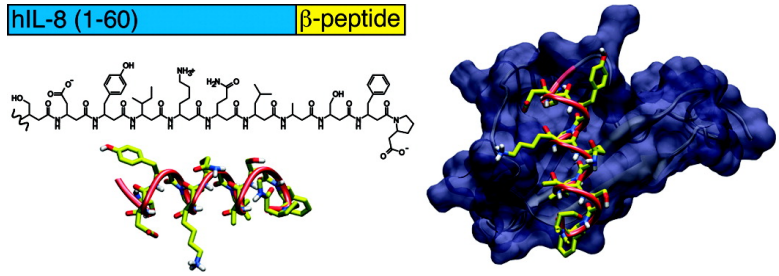

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Artificial Chemokines: Combining Chemistry and Molecular Biology for the Elucidation of Interleukin-8 Functionality

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Abstract: How can we understand the contribution of individual parts or segments to complex structures? A typical strategy to answer this question is simulation of a segmental replacement followed by realization and investigation of the resulting effect in structure−activity studies. For proteins, this problem is commonly addressed by site-directed mutagenesis. A more general approach represents the exchange of whole secondary structure elements by rationally designed segments. For a demonstration of this possibility we identified the α-helix at the C-terminus of human interleukin-8 (hIL-8). Since this chemokine possesses four conserved cysteine residues, it can easily be altered by ligation strategies. A set of different segments, which are able to form amphiphilic helices, was synthesized to mimic the C-terminal α-helix. Beside sequences of α-amino acids, oligomers of non-natural β^3-amino acids with the side chains of canonical amino acids were introduced. Such β-peptides form helices, which differ from the α-helix in handedness and dipole orientation. Variants of the semisynthetic hIL-8 proteins demonstrated clearly that the exact side chain orientation is of more importance than helix handedness and dipole orientation. The activity of a chimeric protein with a β-peptide helix that mimics the side chain orientation of the native α-helix most perfectly is comparable to that of the native hIL-8. Concepts like this could be a first step toward the synthesis of proteins consisting of large artificial secondary structure elements.

1. Introduction

Proteins are complex three-dimensional structures in which individual segments might influence the overall topology, even if they are far away from the active center. With current methods it is difficult to address the impact of whole secondary structure elements on the overall function of a specific protein, because structure−activity studies are mainly limited to the replacement of single amino acid residues by site-directed mutagenesis. In rare cases, noncanonical amino acids have been introduced either by ribosomal displace techniques, mischarged RNA, or native chemical ligation, but again they are confined to the exchange of single residues or dipeptide moieties.[1] The replacement of whole secondary structure elements by rationally designed segments of non-natural amino acids represents a promising approach.

In order to demonstrate the influence of a complete secondary structure element on the overall protein function, we selected human interleukin 8 (hIL-8). hIL-8 is a pro-inflammatory chemokine, which acts predominantly at neutrophile granulocytes. It is expressed in several active isoforms with 69 to 79 amino acids. All possess an N-terminal Glu-Leu-Arg (ELR) motif, which is essential for receptor binding and activity. The structure of hIL-8 was determined by X-ray crystallography and NMR methods.[2,3] It is characterized by a central β-sheet motif, stabilized by an amphiphilic C-terminal α-helix via hydrophobic interactions. The role of this helix with respect to the biological function of hIL-8 is discussed controversially.[4−6] A further characteristic element consists of four conserved cysteine residues that form two disulfide bridges to stabilize the structure.[7] Since the first two cysteines are separated by one glutamine residue, hIL-8 belongs to the Cys-Xaa-Cys (CXC) chemokine family.[8] The activity is mediated by two seven-helical-transmembrane receptors CXCR1 and CXCR2, which both couple to an inhibitory G-protein.

To understand the role of the C-terminal α-helix of hIL-8, we replaced this secondary structure element by different artificial segments employing the expressed protein ligation

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strategy.[9,10] For this purpose, special α-peptide sequences and various optimized β-peptide oligomers were selected considering the aspects of helix dipole orientation, handedness, and amphiphilicity for stabilization of the central β-sheet and, thus, the overall tertiary structure of hIL-8. The applied strategy for the construction of the hIL-8 analogues was expressed protein ligation with Cys 55 of hIL-8 (1−77) as ligation site.

Oligomers of β^3-amino acids (β-peptides) with proteinogenic side chains form stable helices with as few as six β-amino acid residues[11−14] in contrast to α-peptides, for which helical structures evolve only in longer peptide chains. Accordingly, segments consisting of β^3-amino acids would be excellent candidates to investigate the function of a specific helical segment within a protein. Studies on the interaction with hyaluronic acid and CXCR1 activation were performed to examine the biological function of the corresponding semisynthetic hIL-8 analogues.

2. Experimental Section

2.1. Synthesis of hIL-8 and Analogues. All hIL-8-analogues were synthesized by expressed protein ligation as described earlier.[9,10] hIL-8 (1−54)-MESNA-thioester was made by using the IMPACT-CN-system. hIL-8 (55−60), hIL-8 (61−77), CAP-18 (105−121), and hIL-8 (55−60)-CAP-18 (105−121) were synthesized by automated solid-phase peptide synthesis using Fmoc/^{t}Bu-strategy.[15] The chimeric hIL-8 (55−60)-β-peptides were coupled manually onto Wang resin (loading 0.64 mmol/g) using the Fmoc/^{t}Bu-protected monomers (α-amino acids purchased from Novabiochem-Merck, Schwalbach, Germany; β-amino acids from Fluka, Buchs, Switzerland) and 1-hydroxybenzotriazole and diisopropylcarbodiimide for activation.

The peptide (55−60) was ligated to the hIL-8 (1−54) thioester in 5:1 ratio, and the chimeric (55−60)-β-peptides and the (55−60)-CAP18 (105−121) peptide were ligated in an 1:1 ratio in 50 mM TRIS-buffer pH 8.5 containing 0.2 M MESNA, 0.5 M NaCl, and 10 mM TCEP for 48 h. After ligation, a refolding procedure was performed as described earlier.[16] The ligation products were purified by HPLC to homogeneity >95%. Identity was proven by FT-ICR mass spectrometry. Spectra and chromatograms are provided in the Supporting Information (Figures S3−S5).

2.2. Molecular Modeling. Molecular modeling studies were done employing the Molecular Operating Environment suite MOE2005 (Chemical Computing Group, Inc., Montreal, Canada). The 3_{14}-β-peptide helices were built with the backbone dihedral angles given in refs 13 and 17. On the basis of the NMR structure of hIL-8 (residues 7−77, PDB entry 1IL8),[2] the hIL-8 models were constructed replacing the C-terminal part by the synthesized peptides. After construction of the models, the systems were subjected to an energy minimization applying the CHARMM22 force field incorporated in MOE2005. Subsequently, molecular dynamic (MD) simulations over 1 ns at 300 K were performed with implicit solvent consideration ($\varepsilon = 80$). The constructed models kept their structure during the entire MD run. The maximal root-mean-square deviations of the peptide backbones were below 1.5 Å in all cases.

2.3. Cloning of the Receptor CXCR1. The cDNA of eGFP was amplified by PCR from a pEGFP-N1 vector using a forward primer 5′-GGCGGCGAATTCGCCACCATGGTGAGCAAGGG-CGAG-3′ containing an EcoR1 cleavage site and a reverse primer 5′-GCCGCCGCTAGCTTACTTGTACAGCTCGTCCA-3′ containing a Nhe1 cleavage site for introduction of the EGFP-cDNA to a pVITRO2-vector (Invivogen, San Diego, CA). After Nhe1/EcoR1-digestion and purification, the cDNA was introduced into the second multiple cloning site of the digested vector. For introduction of the receptor to the first multiple cloning site of pVITRO2, its cDNA was amplified by PCR using a CXCR1-forward primer 5′-AGAGAGACCGGTGCCACCATGTCAAATATTACAGATCCAC-3′ containing an Age1 cleavage site and a CXCR1-reverse primer 5′-TCTCTCGTCGACTCAGAGGTTGGAAGAGACATTGA-3′ containing a Sal1 cleavage site. After Age1/Sal1 digestion and purification, the receptor-cDNA was introduced into the first multiple cloning site of the digested pVITRO2-vector.

2.4. Peptide Binding to Hyaluronic Acid. Samples of 10 μM hIL-8 (1−77), hIL-8 (1−60), hIL-8 (1−54), hIL-8 (1−60)-β-peptide 1, hIL-8 (1−60)-β-peptide 2, and hIL-8 (1−60)-CAP18 (105−121) were prepared containing 0, 0.5, 1, and 2 mg per mL of hyaluronic acid (Fluka, Buchs, Switzerland) in water. After 30 min incubation at room temperature, 20% glycerol was added and 20 μL samples were separated by gel electrophoresis. One percent agarose gels (pH 5, 8 cm × 10 cm) were prepared on glass plates as described in ref 18. After the samples were loaded to the wells, gel electrophoresis was performed at room temperature for 40 min at 50 V in an electrophoresis buffer containing 0.1 M sodium acetate, 50 mM MOPS, 0.5% CHAPS, pH 7. Afterwards, agarose-gel electrophoresis gels were stained in 20% ethanol, 10% acetate in water with 10 μM RuBPS, which was synthesized according to the method of Rabilloud.[19] Gels were analyzed by using a Molecular Imager FX (Biorad, Hercules, CA) with an excitation wavelength of 532 nm.

2.5. Cell Culture and Inositol Phosphate Accumulation Assays. COS-7 (African green monkey kidney) cells were grown in DMEM medium (PAA Laboratories, Pasching, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS) at 37 °C and 5% CO_2 in 12-well plates (1.5×10^5 cells/well) and transiently transfected with 0.4 μg vector DNA encoding the human CXCR1-receptor and with 0.1 μg of plasmid DNA coding for the chimeric G-protein (kindly provided by E. Kostensis) using 1.5 μL of metafectene (Biontex, Munich, Germany) per well. One day after transfection, cells were incubated with 2 μCi/mL of myo-^{3}H-inositol (25.0 Ci/mmol; Perkin-Elmer Life Sciences, Wellesley, MA) and washed after 16 h with 1 mL culture media containing 10 mM LiCl. Cells were washed and stimulated in media without FCS containing 10 mM LiCl in the absence or with increasing concentrations of agonist for 1 h at 37 °C.[20] The reactions were stopped by aspiration of medium and cell lysis with 300 μL of 0.1 M NaOH. After the addition of 100 μL of 0.2 M formic acid and sample dilution, intracellular IP levels were determined by anion-exchange chromatography as described in refs 21 and 22. Additionally, concentration−response curves were performed in the presence of 0.25, 0.5, and 1 mg/mL hyaluronic acid in DMEM

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Table 1. Sequences and Characteristics of the Synthesized Helix-Forming Peptides

peptide[a]	sequence	length (Å)	handedness	faces	CD minima (nm)
hIL-8 (61−77)	N**W**VQRVVEK**F**LKRAENS	23	right	three	207, 222
CAP18 (105−121)	GGLRKRLRK**F**RNKIKEK	23	right	two	207, 222
β-peptide 1	SDYIKQLAS**F**P	18	left	two	212
β-peptide 2	**W**RQIKE**F**RAVKEAN	23.3	left	three	215

[a] β-peptides consist of β^3-homoamino acids with side chains of canonical amino acids and are underlined. The homologous Phe-residue and the Trp in hIL-8 (61−77) and in β-peptide are marked in bold.

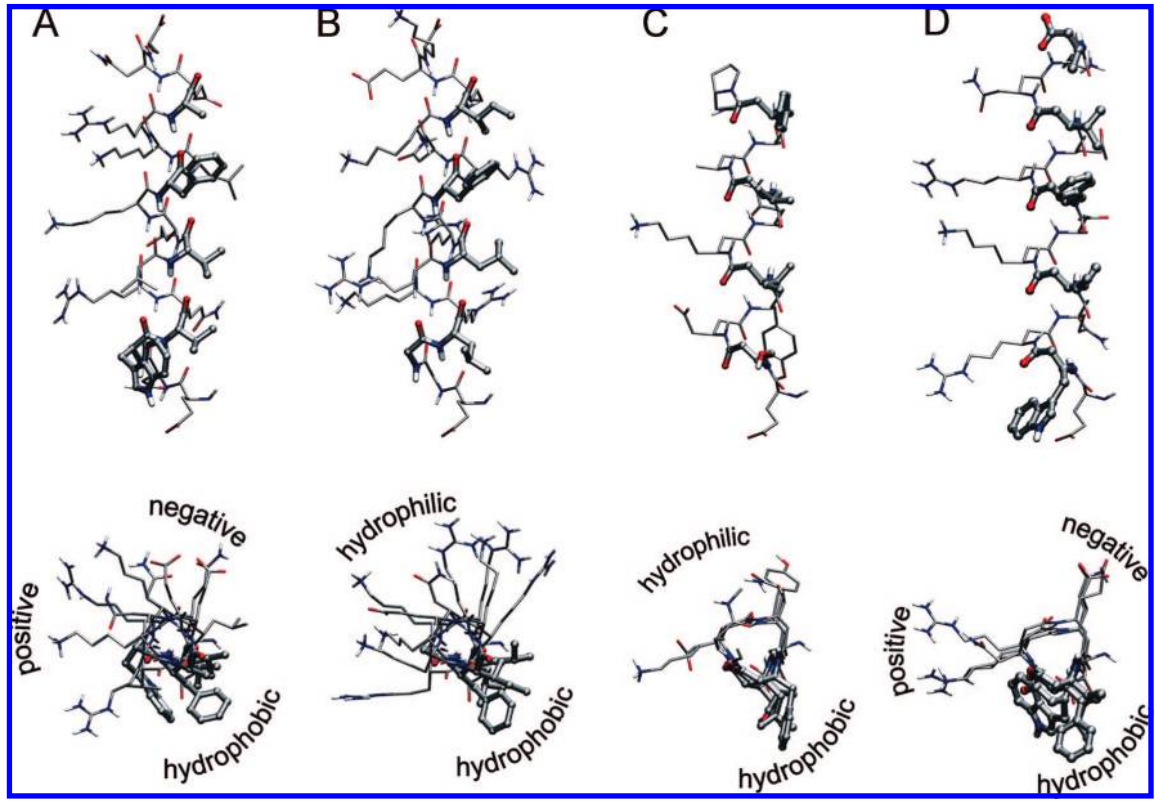

Figure 1. Comparison of (A) the C-terminal α-helix of residues 61−77 of hIL-8 (PDB entry 1IL8), (B) the α-helix of the residues 105−121 of CAP18 (PDB entry 1LYP), (C) the proposed structure of a 3_{14}-helix of β-peptide 1, and (D) β-peptide 2.

containing 10 mM LiCl for 1 h at 37 °C. All assays were performed at least three times as biological duplicates and analyzed by Graphpad Prism (GraphPad Software, Inc., La Jolla, CA).

3. Results

3.1. Sequence Selection and Optimization by Molecular Modeling. On the basis of the NMR structure of human interleukin-8,[2] the C-terminal part (residues 61−77) was replaced by the synthetic peptides given in Table 1. At first, we examined whether the native amphiphilic α-helix could simply be substituted by any other amphiphilic α-helix. Thus, we selected the residues 105−121 of the CAP18 segment (cationic antimicrobial peptide),[23] which are part of an amphiphilic α-helix.

If amphiphilicity proves to be of importance for biological function, it could even be possible to mimic the α-helix by amphiphilic helices of nonproteinogenic amino acid sequences (foldamers).[24] The most examined class of foldamers are β-peptides consisting of β^3-amino acids. A preferred secondary structure type in these β-peptides is a 3_{14}-helix.[13,17,25] Therefore, a previously prepared[26] sequence of β-peptide 1 (Table 1) could be suited for our purposes, since it forms such an amphiphilic 3_{14}-helix. Accordingly, β-peptide 1 was selected, because it has been reported to form a stable helical secondary structure. However, the comparison with the hIL-8-helix of 23 Å length showed that the β-peptide 1 helix is shorter (18 Å). Thus, the sequence of β-peptide 2 was designed, in which the side chains fit best to the hIL-8 sequence and the length of 23.3 Å corresponds closely to that of the native helix after elongation with another three β^3-amino acid residues (Figure 1D). Key anchor residues were maintained, and the three faces of the hIL-8 helix were mimicked. The introduction of β-peptide helices includes further aspects of binding and activity, since a 3_{14}-helix composed of β-amino acids with this configuration is left-handed and has an opposite orientation of the helix dipole in comparison to the native α-helix.[11]

Molecular modeling has to consider that expressed protein ligation was chosen to introduce the artificial peptides. Therefore, replacement of the C-terminal residues 61−77 by the

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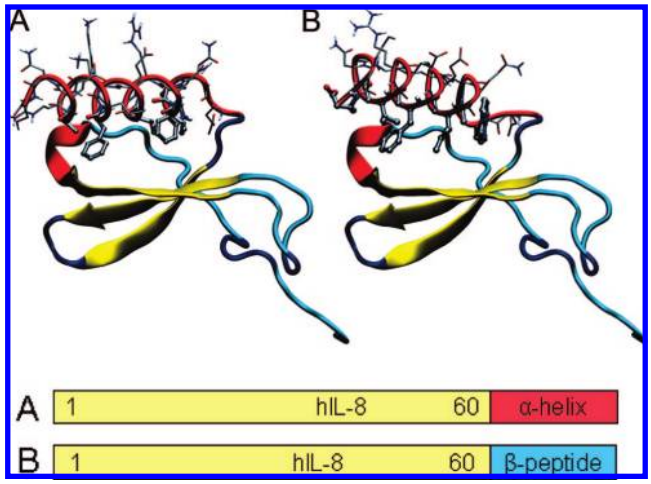

Figure 2. Comparison of (A) the NMR structure of hIL-8 with (B) the modeled hIL-8 (1−60)-β-peptide 2 and schematic drawing of the chimer construction. For clarity, only the C-termini are shown in detail. The hydrophobic side chains at the interface are represented as ball-and-sticks.

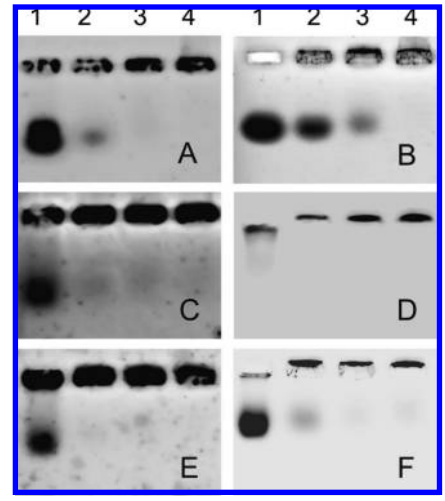

Figure 3. Binding of (A) hIL-8, (B) hIL-8 (1−60)-CAP18 (105−121), (C) hIL-8 (1−60), (D) hIL-8 (1−54), (E) hIL-8 (1−60)-β-peptide 1, and (F) hIL-8 (1−60)-β-peptide 2 to hyaluronic acid (1 = 0 mg/mL; 2 = 0.5 mg/mL; 3 = 1 mg/mL; 4 = 2 mg/mL).

model peptides required the maintenance of the native amino acids 55−60. Consequently, the sequence of the artificial peptides begins always with these amino acids. As suggested by the modeling studies, an immediate connection of β-peptide 1 with the hIL-8 sequence 1−54 via Cys 55 does not result in a stably folded protein although ligation was performed successfully (data not shown).

The top views in Figure 1A and 1D show a rather perfect agreement between the positively charged, negatively charged, and hydrophobic faces of the native α- and the β-peptide 2 helices. The correspondence is not so perfect for the helix of β-peptide 1 and the α-helical CAP18 segment (105−121). Nevertheless, the top views in Figure 1B and 1C indicate the amphiphilic nature also for these two helices. All helices possess a Phe-side chain at nearly the same position, which could act as a hydrophobic anchor for the β-sheet of hIL-8. Figure 2 illustrates the rather perfect fit of the β-peptide 2 and the native hIL-8 structure. Although the handedness of the incorporated β-peptide helices is opposite to that of the native α-helix, the orientation of the side chains is in close correspondence.

3.2. Chimeric Proteins by Ligation Strategies. The C-terminal peptides hIL-8 (55−60)-CAP18 (105−121), hIL-8 (61−77), and CAP18 (105−121) were prepared by automated solid phase peptide synthesis on Wang resin using the Fmoc-strategy. TFA-cleavage resulted in more than 95% pure peptides. The β-peptides were coupled by manual synthesis on Wang resin followed by coupling of the α-amino acids of hIL-8 (55−60). Each coupling step was performed twice. β-Peptide 1 was obtained in a purity of 70%; β-peptide 2 was only 30% pure. Impurities were removed by preparative HPLC.

The hIL-8 (1−54) thioester was constructed employing the pTXB1 vector as described in ref 9. Mercaptoethane-sulfonic acid (MESNA) was used to obtain a more stable thioester compared to DTT. Reaction with hIL-8 (55−60) as control peptide led to hIL-8 (1−60) in a yield of 90%. Ligation with hIL-8 (55−60)-β-peptide 1 yielded 70%, with hIL-8 (55−60)-β-peptide 2 35%, and with hIL-8 (55−60)-CAP18 (105−121) 52% of product. After applying a refolding step with cysteine/cystine buffer, disulfide bridge formation could be observed by FT-ICR-mass spectrometry. Educts were removed by HPLC. Basic hydrolysis of hIL-8 (1−54)-thioester led to C-terminally free hIL-8 (1−54). hIL-8 (1−77) as a reference was obtained by using the IMPACT system.

3.3. CD Spectroscopy Revealed Structural Elements. The CD spectrum of hIL-8 is dominated by a Cotton effect typical for an α-helix. The removal of the helix-forming part leading to hIL-8 (1−60) resulted in a random coil signal, which was even stronger in the case of hIL-8 (1−54), in which only one disulfide-bridge is formed. After ligation of the hIL-8 (55−60) CAP18 (105−121) segment, the typical α-helical signal appeared again. None of the peptides itself, neither hIL-8 (61−77), nor CAP18 (105−121), nor any of the β-peptides, formed a helix in phosphate buffer. After addition of TFE, helix formation was induced in all peptides. hIL-8 (61−77) and CAP18 (105−121) exhibited the typical negative Cotton effects at 207 and 222 nm for α-helices, whereas the β-peptides showed a pronounced minimum at 212−215 nm corresponding to a 3_{14}-helix.[11] This supports the assumption that the β-peptide fragments could behave like the α-helix-forming peptides when replacing the helix of hIL-8.

The CD spectrum of hIL-8 (1−60)-β-peptide 1 had the same shape as that of hIL-8 (1−60), indicating that β-peptide 1 does not influence structure formation of hIL-8. The introduction of β-peptide 2, in which the side chains were chosen as in the hIL-8 helix, resulted in a dramatic loss of the random coil signal (see Figure S1, Supporting Information).

3.4. Binding of hIL-8 Analogues to Hyaluronic Acid. Interaction with the extracellular matrix plays an important role for chemokines. A too strong binding to hyaluronic acid (HA) prevents the release of the chemokine from the extracellular matrix. Therefore, binding of hIL-8 analogues to HA was investigated with increasing amounts of HA and monitored by agarose gel electrophoresis (Figure 3).

HA and the bound peptides remained in the gel pocket, and free ligand was moving to the cathode because of the positive charge at pH 5. It could be observed that the C-terminally truncated peptides hIL-8 (1−54) and hIL-8 (1−60) showed the strongest interaction, because moveable chemokine was not detected at a HA-concentration of 0.5 mg/mL. Interestingly, the hIL-8 (1−60)-β-peptide 1 behaved in the same way (Figure 3C−E). Surprisingly, the stronger positively charged hIL-8 (1−60)-CAP18 (105−121) showed a lower affinity to HA. Only

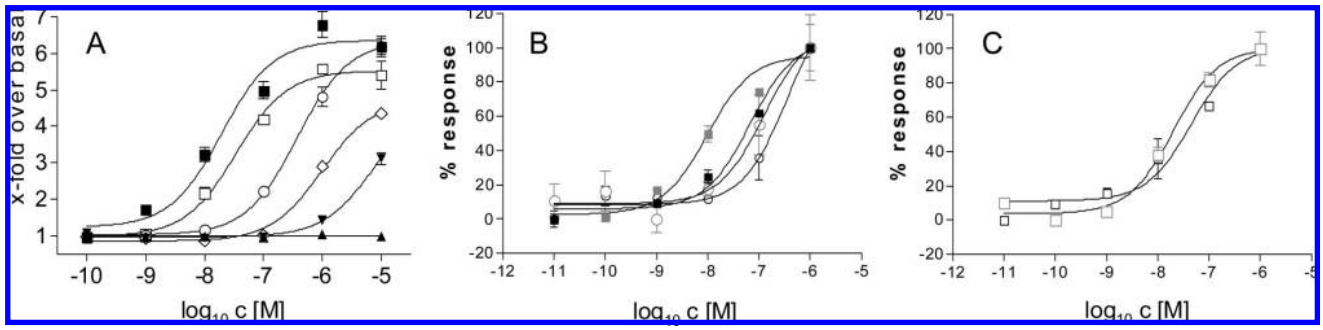

Figure 4. IP_3 formation after stimulation of free CXCR1 with hIL-8 analogues. IP_3 formation was observed with $G\alpha_{\Delta 6qi4myr}$ after stimulation with ligands. (A) hIL-8 (■), hIL-8 (1−60) (▼), hIL-8 (1−54) (▲), hIL-8 (1−60)-CAP18 (105−121) (□), hIL-8 (1−60)-β-peptide 1 (◇), and hIL-8 (1−60)-β-peptide 2 (○) (concentration in M); (B) hIL-8 (■) and hIL-8 (1−60)-β-peptide 2 (○) in the presence of 0.5 mg/mL (gray) and 1 mg/mL (black) hyaluronic acid; (C) hIL-8 (1−60)-CAP18 (105−121) (□) in the presence of 0.5 mg/mL (gray) and 1 mg/mL (black) hyaluronic acid. Signal is less influenced by hyaluronic acid compared to hIL-8 and hIL-8 (1−60)-β-peptide 2 in part B. For hIL-8 (1−60)-β-peptide 1 signal transduction was too much reduced in the presence of hyaluronic acid.

at 2 mg HA/mL was no movable ligand observed (Figure 3B). hIL-8 and hIL-8 (1−60)-β-peptide 2 behaved identically. In both cases 1 mg HA/mL was required for full HA-binding.

3.5. Confirmed Activity at CXCR1 by IP_3 Accumulation Assays. Replacement of C-terminal amino acids of $G\alpha_q$ with the corresponding $G\alpha_i$ residues confers the ability to stimulate the PLC-β pathway onto G_i-coupled receptors.[22,27,28] Compared to the measurement of adenylate cyclase inhibition, this gain-of-signal assay is much more sensitive. To apply this assay to hIL-8 and the analogues, the cDNA for the receptor CXCR1 was cloned into the first multiple cloning site (MCS) of a pVITRO2-vector, and coexpression with the chimeric $G\alpha_{\Delta 6qi4myr}$ was performed. The DNA which encodes the enhanced green fluorescent protein (eGFP) was cloned into the second MCS. These constructs allow the expression of the CXCR1 receptor in eukaryotic cells and the transfection control by the fluorescence of eGFP.

The dose−response curve of hIL-8 (1−60)-β-peptide 1 showed an EC_{50} value of 771.6 ± 215.4 nM. As the formation of IP_3 was only 4-fold over the basic value, neither activity nor efficacy was in the range of those for hIL-8. The only analogue that led to full activation of the receptor CXCR1 was the hIL-8 analogue with the tailored β-peptide 2 although the activity was slightly reduced (EC_{50} = 331.3 ± 107 nM). This shows that restoration of the activity is possible by introduction of a β-peptide segment (Figure 4A).

In order to link affinity to hyaluronic acid and receptor activity, we performed IP_3 assays in the presence of 0.25, 0.5, and 1 mg/mL hyaluronic acid. Whereas hIL-8 (1−60)-CAP18 (105−121) showed no change in the activity (EC_{50} = 40.2 ± 5.2 nM, Figure 4C), a mild shift was found for hIL-8 and hIL-8 (1−60)-β-peptide 2 (EC_{50} 2−3 fold increased with 1 mg/mL hyaluronic acid, Figure 4B). The strongest effect was found for hIL-8 (1−60)-β-peptide 1, which led to a 20-fold increase in activity and a 50% reduction of efficacy at 0.5 mg/mL hyaluronic acid. This is in nice agreement with the gel shift data and confirms the dual role of hIL-8 in the body.

4. Discussion

The aim of the project was to identify the role of individual secondary structure elements on the overall function of hIL-8. In hIL-8, the C-terminal α-helix stabilizes the structure by covering the surface formed by an antiparallel β-sheet motif.[2,3] The ELR-motif, which is essential for receptor recognition and activity,[29,30] is located at the opposite site of the protein. This led to the assumption that the C-terminal helix does not contribute to the activity of the molecule, which is determined by a structural scaffold provided by both disulfide bridges and the 35−40-turn including the ELR-motif.[7] In high concentrations, however, it could be shown that N- and the C-terminal segments of hIL-8 bind to the chicken CXCR1 receptor.[31] Furthermore, removal of the helix led to inactive proteins.[32] Thus, the role of the helix remained unclear.

Since chemokines belong to a group of small proteins with 70−120 amino acids they are not easy to obtain by peptide synthesis on a solid support. The occurrence of four cysteines allows the application of native chemical ligation strategies. Thus, human interleukin-8 has been synthesized by this approach.[33] This method can not only be used to produce native hIL-8 but also allows incorporation of non-natural amino acids into the protein as shown by expressed protein ligation of hIL-8 analoga, in which a carboxyfluoresceine-label was coupled at the Lys 69-side chain[9] or photoactivatable amino acids were introduced.[16] Employing this strategy, a whole structural segment was replaced by several peptide mimetics in this study. In particular, it could be demonstrated that oligomers of β-amino acids (β-peptides) are able to mimic longer secondary structure elements in proteins under preservation of biological activity.

To elucidate which type of secondary structure elements exists in the synthesized hIL-8 analogues, CD-spectroscopy was performed. In the native hIL-8 (1−77), an α-helix predominates as could be seen by the typical Cotton effect. For the N-terminal fragment (1−54) no defined structure was recorded, which emphasized the role of the disulfide bridge between Cys 13 and Cys 55 in the formation of a structural scaffold. This has been proven by the negative Cotton effect at 200 nm measured for hIL-8 (1−60), in which this disulfide bridge is formed.

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Table 2. Analytical and Pharmacological Data of hIL-8 Analogues

protein	HPLC, R_t (min)	mass[a] (Da) calcd	mass[a] (Da) expt	CXCR1, EC_{50} (nM)	E_{max} (*x*-fold over basal)
hIL-8 (1−77)	18.3[b]	8918.5	8917.8	25.9 ± 6.4	6.5
hIL-8 (1−60)	22.8[c]	6833.1	6832.7	>5000	3.0
hIL-8 (1−54)	14.3[b]	6149.3	6149.4	nd[d]	nd[d]
hIL-8 (1−54)-β-peptide 1	19.4[b]	7569.1	7567.0	nd[d]	nd[d]
hIL-8 (1−60)-β-peptide 1	17.7[b]	8237.6	8237.6	771.6 ± 215.4	4.0
hIL-8 (1−60)-β-peptide 2	18.3[c]	8785.9	8785.8	331.3 ± 107	6.5
hIL-8 (1−60)-CAP18 (105−121)	18.0[c]	8942.7	8942.3	34.7 ± 11.6	5.1

[a] Calculated masses correspond to oxidized cysteine residues and are average masses. [b] Gradient system 20% to 70% acetonitrile with 0.1 trifluoroacetic acid within 30 min. [c] Gradient system 10% to 60% acetonitrile with 0.1 trifluoroacetic acid within 30 min. [d] Not detectable.

CD spectroscopy shows that the peptides hIL-8 (61−77) and CAP18 (105−121) do not form helical structures in buffer but do form them after addition of 30% TFE. A similar effect was observed for both β-peptides. Addition of 30% TFE elicits the typical Cotton effect of a 3_{14}-helix. This is in agreement with structural data on β-peptides indicating helix formation in sequences with only six amino acids.[11−14] The helix formation is less pronounced in aqueous solution,[34,35] but when such peptide mimetics come in contact to TFE, methanol, or hydrophobic surfaces, the helices are formed.[36] Obviously, the hydrophobic surface of the β-sheet scaffold of hIL-8 (1−60) represents such a helix-promoting environment. The CD-spectrum of the chimeric hIL-8 (1−60)-β-peptide 1 did not show a defined structure, indicating that it does not exactly fit onto the surface of the sheet-forming parts in hIL-8. In contrast, the introduction of the rationally designed β-peptide 2 led to an induction of a defined structure indicated by the decrease of the random coil signal. Interestingly, however, when hIL-8 (1−54) is directly linked to the β-peptide segment, no stable structure and no activity is found. This further indicates that a defined interaction has to take place. Of course, the exact interaction of helix and β-sheet should be determined by additional variants, e.g., those in which the possible hydrophobic interaction points are replaced by more hydrophilic ones.

Recently, it has been found for a GROα/IL-8 chimeric protein that the helical segments are not easily exchangeable.[37] Both proteins show a high sequence similarity and bind to the same receptor CXCR2.[38] NMR studies on a mixed chemokine suggested, however, that the helix does not contact the β-sheets but points to the surrounding media.[37] Nevertheless, in the case of the chimeric hIL-8 (1−60)-β-peptide 2 our CD-data provide evidence for interaction of the β-peptide helix with the β-sheet scaffold of hIL-8.

Glycosaminoglycan binding plays an important role for the in vivo activity of certain chemokines.[39] The addition of heparin sulfate to hIL-8 enhances the chemotaxis of neutrophiles.[4] All hIL-8 analogues were tested for binding to the extracellular matrix component hyaluronic acid. In contrast to ref 40, which suggests the C-terminal helix as the strongest determinant for glycosaminoglycan binding, we found the opposite. Analogues without or with not well-structured C-terminal segments bind best to hyaluronic acid, whereas hIL-8 (1−60)-CAP18 (105−121) showed the weakest binding despite its strong α-helicity. These results could be nicely confirmed in activity assays that were performed in the presence of hyaluronic acid. As hIL-8 and hIL-8 (1−60)-β-peptide 2 behaved nearly identically in binding and receptor activation was only 2−3 fold reduced in the presence of hyaluronic acid, it can be concluded that the three faces of the C-terminal helix provide optimal structural conditions for hIL-8 binding to and dissociation from the extracellular matrix, which is crucial for the best cell interaction. Thus, the helix might play an important role on the release of the chemokine from the extracellular matrix to prevent a too strong binding.

To investigate the activity of the semisynthetic hIL-8 analogues at CXCR1, a signal switch from the cAMP pathway to the IP_3 pathway was applied by cotransfection of the receptor construct with a chimeric G_q-protein.[27] The switch to the phospholipase C-pathway provides a much better signal-to-noise ratio than the cAMP assay for Gi-coupled receptors.[20] Activation with an EC_{50} of 25.9 nM could be observed after cotransfection with $G\alpha_{\Delta 6qi4myr}$, which is in agreement with ref 41 where the 10-fold more reactive hIL-8 (1−72)[42] shows an EC_{50} value of 2.5 nM. The assay provides two results (Table 2). EC_{50} values represent the activity of the analogues that frequently correspond to the binding of the ligands. On the other side, E_{max} values are provided, which reflect the efficacy and accordingly how the agonists can stabilize the active receptor conformation. Even at 10 μM concentration hIL-8 (1−54) did not affect the IP_3 level, which indicates that a correctly folded β-sheet scaffold is required for receptor activation.[7] The variant hIL-8 (1−60), which possesses both disulfide bridges, showed only low activation of the receptor as well. The EC_{50} value is more than 200-fold higher compared to hIL-8, suggesting that the C-terminal helix plays an important role in tertiary structure formation, receptor binding, and activation. Molecular modeling of IL-8 in the CXCR2-bound state showed indeed that the C-terminal helix of IL-8 might have contact to the extracellular domains 3 and 4 of the receptors.[43]

Replacement of the C-terminal α-helix with the amphiphilic CAP18-segment led to a slightly decreased activity, and the full level of efficacy was not reached. The introduction of the

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β-peptide segment 1 led to only partial restoration of the activity and a loss of efficacy compared to hIL-8. The tailored analogue hIL-8 (1−60)-β-peptide 2, in which the β-peptide helix contains the same side chains as the native helix resulted in full activation of CXCR1 in comparison to hIL-8. The about 10-fold increased EC_{50} value and, thus, the lower activity to the receptor could be caused by the reversed dipole direction and the opposite handedness of the 3_{14}-helix. However other explanations might also be possible, e.g., backbone interactions or slight differences in the orientation of the side chains. Additional structure–activity studies will be required for detailed analysis. Comparing the various amphiphilic helices, close correspondence of the side chains and their orientations to the native protein seems to be more important than dipole orientation and handedness of the helices. With respect to the amphiphilic helix of hIL-8 we found that this element in hIL-8 obviously has two functions. On one side, the helix is important for high affinity to the receptor. This can be transmitted by any amphiphilic α-helix. Either the dipole moment or any backbone interactions might contribute. However, to fully stabilize the active receptor conformation, neither the backbone, nor the dipole moment, nor the handedness plays a role, but obviously three faces are required. This could be best realized by the β-peptide-2.

5. Conclusions

Our results show that larger secondary structure elements of peptides and proteins can be completely replaced by designed non-natural peptide sequences via chemical ligation approaches. The maintenance of the nearly full activity after substitution of the C-terminal α-helix of hIL-8 by a designed sequence of 14 β^3-amino acids with side chains corresponding to the native structure shows that foldamers like β-peptides are able to mimic important features of native proteins. Our concept might be extended by using more constrained β-amino acids or other foldamers. Thus, introduction of conformationally restricted amino acids like cyclic amino acids[12,44] or salt bridges[26] into the β-peptide might support the selective formation of special folding patterns. This could be a promising step toward the synthesis of proteins consisting of large artificial secondary structure elements.[45,46]

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Supporting Information Available: Supporting Information includes a detailed description on cloning of hIL-8 cDNA, purification of hIL-8 (1−77), and CD, HPLC, and FT-ICR mass spectra of the various hIL8-analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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