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Epitope Identification and Affinity Determination of an Inhibiting Human Antibody to Interleukin IL8 (CXCL8) by SPR- Biosensor−Mass Spectrometry Combination

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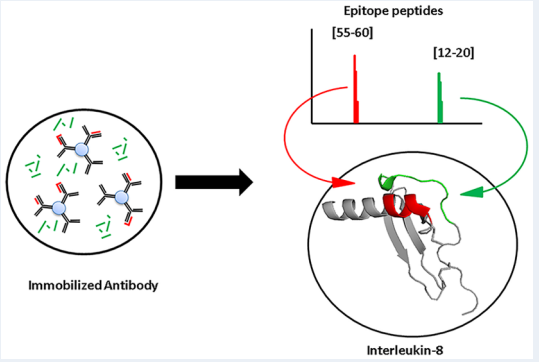
Supporting Information

ABSTRACT: The polypeptide chemokine Interleukin-8 (IL8) plays a crucial role in inflammatory processes in humans. IL8 is involved in chronic inflammatory lung diseases, rheumatoid arthritis, and cancer. Previous studies have shown that the interaction of IL8 with its natural receptors CXCR1 and CXCR2 is critical in these diseases. Antibodies have been used to study the receptor interaction of IL8; however, the binding epitopes were hitherto unknown. Identification of the antibody epitope(s) could lead to a molecular understanding of the inhibiting mechanism and development of improved inhibitors. Here, we report the epitope identification and the affinity characterization of IL8 to a monoclonal anti-human IL8 antibody inhibiting the receptor binding by a combination of surface plasmon resonance (SPR) biosensor analysis and MALDI-mass spectrometry. SPR determination of IL8 with the immobilized antibody revealed high affinity (K_D, 82.2 nM). Epitope identification of IL-8 was obtained by proteolytic epitope-extraction mass spectrometry of the peptide fragments upon high pressure trypsin digestion, using an affinity microcolumn with immobilized anti-IL-8 antibody. MALDI-MS of the affinity-bound peptide elution fraction revealed an assembled (discontinuous) epitope comprising two specific peptides, IL8 [12−20] and IL8 [55−60]. Identical epitope peptides were identified by direct MALDI-MS of the eluted epitope fraction from the immobilized anti-IL8 antibody on the SPR chip. SPR determination of the synthetic epitope peptides provided high affinities confirming their binding specificity. The previously reported finding that the anti-Il8 antibody is inhibiting the IL8−CXCR1 interaction is well consistent with the overlapping region of epitope interactions identified in the present study.



KEYWORDS: *inflammation diseases, chemokine inhibitors, interleukin-8, antibody epitope, surface plasmon resonance, affinity-mass spectrometry, proteolytic epitope extraction, discontinuous epitope*

INTRODUCTION

The 72 amino acid chemokine interleukin-8 (IL8 or CXCL8) is a biomarker for inflammation.[1] IL8 functions as an inflammation mediator and is involved in several chronic inflammatory diseases such as rheumatoid arthritis.[2] In a bacterial infection IL8 causes recruitment of leukocytes, particularly neutrophil granulocytes, from the site of inflammation to combat the pathogens. Dysfunction of the chemotactic recruitment of leukocytes leads to chronic diseases such as rheumatoid arthritis,[2] bronchiolitis obliterans,[3] COPD,[4] and psoriasis.[5] Furthermore, IL8 is an angiogenesis factor; i.e., it causes new formation of blood vessels. In several types of cancer the release of IL-8 causes blood vessels to grow toward the tumor and supplies it with nutrients.[6,7] The structure of IL8 has been elucidated,[8,9] and its interaction with the cognate receptors CXCR1 and CXCR2 has been intensively studied[10−15] (Figure 1; Supplementary Table 1). The binding mechanism of IL8 to CXCR1 has been proposed to consist of (i) binding of the N-terminus to the IL8 N- terminal loop; (ii) activation of the receptor by specific interaction of the IL8 N-terminus with extracellular domains (ECD) 3 and 4 of CXCR1.[13]

A murine monoclonal anti-human IL8 antibody from hybridoma cell line 6217.11 (IgG1; Sigma-Aldrich I2519) has been shown to inhibit the IL8−CXCR1 interaction. However, the binding epitope of the interaction has been hitherto unknown.[16−20] For the identification of antibody epitopes, efficient tools have been developed by combination of

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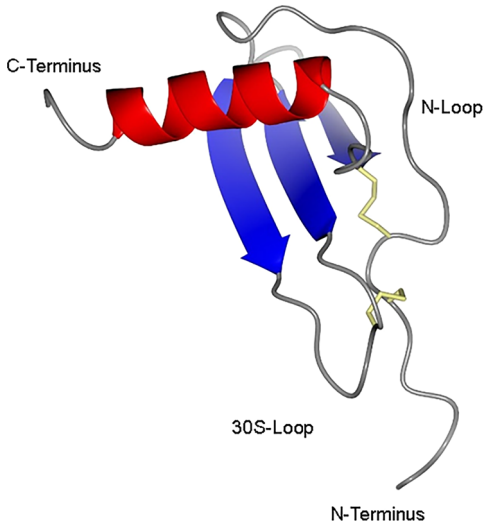

Figure 1. Crystal structure of IL8 (single monomer from the dimer structure; PDB 1IL8). Disulfide bonds are shown in yellow, N-terminus, C-terminus, α-helix (red; binding site to GAGs), β-sheets (blue; dimerization site of β_3 and the α-helix), and loops (gray, binding sites for CXCR1).

selective proteolytic excision and extraction with ESI and MALDI mass spectrometry and successfully employed to both linear and assembled (discontinuous) epitope identifications in protein–antibody complexes, protein–peptide interactions, and carbohydrate–protein complexes[21−27] (Figure 2). The combination of proteolytic epitope excision and extraction–mass spectrometry and surface plasmon resonance (SPR) biosensor analysis was recently developed as a highly efficient tool for the identification of epitopes and determination of binding affinities (PROTEX-SPR-MS).[24,26,27,30] Here, we report the identification of the interaction epitopes on IL8 to a monoclonal antibody that binds with high affinity and specificity. A monoclonal anti-IL8 antibody was immobilized on an affinity microcolumn and incubated with a proteolytic digestion mixture of IL8. After washing off supernatant and nonbinding peptides, the eluted epitope fraction was analyzed by MALDI-MS, and peptide sequences were determined by tandem-MS analysis. The MS analysis revealed a discontinuous (assembled) epitope comprising two specific peptide sequences, in agreement with structural and binding data of IL8.[18,20]

EXPERIMENTAL SECTION

Protein Reduction and Alkylation. A solution of 20 μg (0.5 mg/mL) IL8 in 40 mM NaH_2PO_4 containing 35 mM NaCl, pH 7.4, was diluted with 40 μL of ammonium hydrogen carbonate (100 mM in Milli-Q-water). A 10 mM solution of dithiotreitol (DTT; 3 μL in Milli-Q-water) was added, and the mixture was incubated for 15 min at 95 °C. After the mixture was cooled to 20 °C, 6 μL of 100 mM iodoacetamide (IAA) in Milli-Q-water was added and the mixture incubated for 1 h in the dark. Afer further addition of 5 μL of DTT solution (100 mM in Milli-Q-water) the reaction mixture was incubated for 30 min in the dark to provide reduced and alkylated IL8.

Protein Digestion at Standard Conditions. A 1 μL solution (0.5 mg/mL) of trypsin (Promega, Mannheim, Germany; sequencing grade) was added to an aliquot of 20 μg of reduced and alkylated IL8. The reaction mixture was then incubated for 18 h at 37 °C and characterized by MALDI MS.

High-Pressure Protein Digestion. A 1 μL solution of (0.5 mg/mL) of trypsin (Promega, sequencing grade) was added to an aliquot of 20 μg of reduced and alkylated IL8 as described above. High-pressure digestion was performed with a Barocycler 2320GTX (Pressure Biosciences Inc., Boston, MA). The sample in the high-pressure Barocycler cell undergoes pressure cycles of 50 s 20 kpsi pressure and 10 s pressure release to atmospheric pressure. For complete digestion, 90–120 cycles were executed at 50 °C. Subsequently, the digestion mixture was characterized by MALDI-MS.

Both proteolytic digestions at high pressure and at ambient conditions were monitored for completion by 1D SDS gel electrophoresis, and digestion yields >95% were estimated.[30]

Affinity Column Preparation. A suspension of 15 mg of CNBr-activated sepharose 4B (GE Healthcare, Uppsala, Sweden) was prepared in a microcolumn (MoBiTec

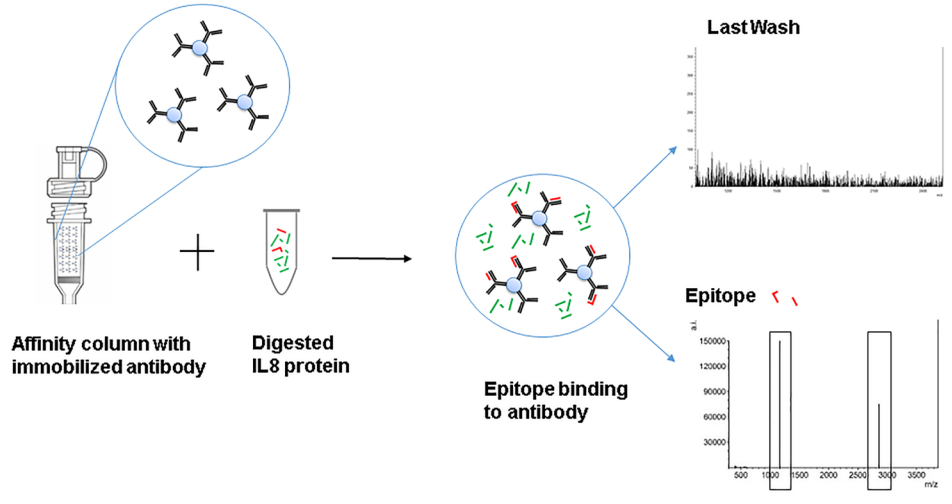

Figure 2. Epitope extraction method for epitope identification by proteolytic extraction–affinity mass spectrometry: (1) incubation of the sepharose-affinity column with the IL8 digestion mixture; (2) nonbinding supernatant material is washed away; (3) elution of affinity-bound peptide(s); (4) MS analysis of epitope peptide(s).

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GmbH, Goettingen, Germany) in 0.5 mL of 0.1 M hydrochloric acid for 30 min in a shaker. After the sepharose was washed off with 3 mL of coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3), a solution of 50 μg of murine monoclonal anti-human IL8 antibody (Sigma-Aldrich, Taufkirchen, Germany, No. I2519) in 0.5 mL of coupling buffer was added and incubated for 3 h in a shaker. Washing with 3 mL of blocking buffer (0.1 M ethanolamine, 0.5 M NaCl, pH 8.3) and incubation with 0.5 mL of blocking buffer was carried out for 3 h, followed by washing with 5 mL of washing buffer (0.2 M Na acetate, 0.5 M NaCl, pH 4.0) and 5 mL of ammonium bicarbonate buffer (10 mM, pH 7.5). The affinity column was then stored in ammonium bicarbonate buffer at 4 °C. A control column was prepared in the same manner without the antibody incubation step.

Proteolytic Epitope Extraction Mass Spectrometry. A solution of 20 μg of the digestion mixture of reduced and alkylated IL8 was incubated for 12 h at 20 °C on the affinity column. Subsequently, the column was washed with 15 mL of 10 mM ammonium bicarbonate buffer. After the proteolytic mixture was incubated for 12 h, the column was washed three times with ammonium bicarbonate buffer until no more signal was observed in the MALDI mass spectrum. The epitope peptide fraction was then eluted with 200 μL of 0.01% TFA, pH 2.5. The eluate sample was analyzed by MALDI-MS for epitope identification. All solutions were concentrated to 20 μL in an Eppendorf concentrator (Sigma-Aldrich) prior to MS analysis (supernatant, last wash fraction, and elution fraction). Epitope identification using chymotrypsin as protease was performed with the same procedure. The identical procedure was carried out with the control column for the identification of unspecific binders to the column material.

Proteolytic Epitope Extraction-MS from an SPR Chip. SPR chips were prepared as described in the section Affinity Determination by modification with a 16-mercaptohexdecanoic acid self-assembled monolayer and immobilization of 12 μg anti-IL8 antibody. The PBS-T running buffer was exchanged by Milli-Q-H_2O, and the flow rate for sample injection/washing set to 5 μL/min and then finally raised to 50 μL/min for regeneration of the chip surface with 0.1% aqueous TFA. A 2.5 μg digestion mixture of reduced and alkylated IL8 was diluted with Milli-Q-H_2O to a final volume of 250 μL for the SPR injection. A 250 μL portion of digested IL8 was then injected at 5 μL/min and eluted for 50 min over the immobilized anti-IL8 antibody. Fractions were collected, with each fraction being collected over a period of 20 min (overall 100 min). Subsequently washing was performed for 120 min at 5 μL/min with running buffer, the flow rate was then set to 50 μL/min, and an injection of 0.1% TFA in Milli-Q-water was carried out. The volume of each fraction was then reduced to 10−30 μL prior to MALDI-MS analysis.

MALDI-TOF Mass Spectrometry. MALDI-TOF-MS was performed with a Bruker Autoflex-III Smartbeam (Bruker Daltonics, Bremen, Germany) in the positive mode. For protein analysis, a 50 mg/mL SDHB matrix solution (Super DHB; Bruker Daltonics) in 50:50 (v/v) acetonitrile/0.1% TFA in Milli-Q-H_2O was prepared and used, while peptides were analyzed with a 20 mg/mL 2,5-DHB matrix solution (Bruker Daltonics) in 50:50 (v/v) acetonitrile/0.1% TFA matrix was prepared in Milli-Q-H_2O. A 0.5 μL portion of sample was spotted on a stainless steel MALDI target and mixed on the target with 0.5 μL of matrix.

Synthesis and Purification of Epitope Peptides. *N*,*N*-Dimethylformamide (DMF), *N*-methyl-2pyrrolidone (NMP), dichloromethane (DCM), (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), methyl *tert*-butyl ether (MTBE), diisopropylethylamin (DIPEA), and trifluoracetic acid (TFA) were purchased from Applied Biosystems (Darmstadt, Germany), piperidine was obtained from Acros Organics (Fisher Scientific) and triisopropylsilane (TIS) from Alfa Aesar (Landau, Germany). Fmoc-protected derivatives were purchased from Novabiochem (Darmstadt, Germany).

Fmoc solid-phase peptide synthesis (Fmoc-SPPS) was carried out on an ABI 433 A peptide synthesizer (Applied Biosystems; Darmstadt Germany). The epitope peptides ENWVQR and TYSKPFHPK were synthesized on preloaded PS-PHB-Arg(PMC)-Fmoc resin (capacity 0.59 mmol/g) and on a PS-PHB-Lys(Boc)-Fmoc resin (capacity 0.55 mmol/g) from Rapp Polymere GmbH (Tuebingen, Germany). An amount of resin corresponding to 0.1 mmol was used for each peptide, and Fmoc-protected amino acids were employed in all steps. Removal of side-chain protecting groups and cleavage of the peptides from the resins were achieved by incubating the resin in 5 mL of 95 % TFA, 2.5% TIS, and 2.5% Milli-Q-water for 3 h at room temperature. The peptide was then precipitated by pouring the reaction mixture into 20 mL of MTBE followed by centrifugation and decanting. The peptide was then washed twice with MTBE, centrifuged, and decanted. Peptides were purified by HPLC using a 2795 Alliance HT instrument (Waters, Milford, MA) and a C18 column (250 mm × 4.6 mm, Interchrom KR5C18-25QK).

Affinity Determination. SPR measurements were performed with an Ametek-Reichert 2Ch7500 SPR instrument (Ametek-Reichert, Buffalo, NY) using glass slides of 0.9 mm thickness and an area of 1 cm^2 with a 50 nm gold layer bearing a self-assembled monolayer (SAM) functionalized with carboxymethyl dextran hydrogel or a 16-mercaptohexadecanoic acid self-assembled monolayer (both surfaces were functionalized in the same manner to the antibody). The flow rate for all experiments was set to 25 μL/min. For surface activation, a mixture of 125 μL (40 mg/mL) of EDC and 125 μL (10 mg/mL) of NHS in Milli-Q-H_2O was injected over the gold chip. After immobilization of 250 μL (0.06 μg/μL, 10 mM sodium acetate buffer pH 5.2) anti-IL8 antibody, the chip was blocked with 1 mL (1 M, pH 8.5) of ethanolamine in Milli-Q-H_2O. The antibody was immobilized in one of the two channels of the dual-channel detector chip to account for unspecific interactions that were subtracted in the data analysis. For affinity determinations, the chip was washed for 15 min with PBS-T buffer (PBS, 0.05% Tween, pH 7.5), and subsequently dilution of a series of IL8 (concentration range between 47 nM and 1500 nM) in the same PBS-T buffer was measured. Sample injection for 4 min (association) was followed by PBS-T buffer injection for 4 min (dissociation), and sensograms were recorded. For complete regeneration, the chip was washed for 4 min with 0.1% formic acid in Milli-Q water and then washed for 8 min with PBS-T buffer. K_D values were determined using the TraceDrawer 1.7.1 software (Ridgeview Instruments AB, Vange, Sweden).

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All SPR determinations were carried out in triplicate with errors estimated at ca. 15%. Affinities of synthetic epitope peptides were determined in the same manner using dilution series of peptides within concentration ranges of 600 nM to 3.5 μM.

RESULTS AND DISCUSSION

Affinity Characterization of the Anti-IL8 Antibody. To characterize the affinity of the murine anti-human IL8 antibody to IL8, the dissociation constant K_D was determined by SPR analysis on carboxymethyl- functionalized SAM gold chips by immobilization of the antibody on the chip surface using standard EDC/NHS chemistry as described in the Experimental Section. For the K_D determination, a dilution series of IL8 was injected and sensograms were recorded and evaluated.

The SPR analyses provided a binding constant K_D of 82.2 nM for the antibody interaction of intact native IL8 (Table 1; Supplementary Figure S1); in contrast, reduced unfolded IL8 had a >100-fold lower affinity (low μM K_D). It was noted that at higher concentrations of IL8 the deviation of the recorded signal from a one-to-one binding curve was unusually high. This observation could be explained by the known dimerization of IL8 at higher concentrations.[28,29]

Table 1. SPR Determination of Affinity Binding Constants of the IL8−Anti-IL8 Antibody Complex[a]

	K_D (nM)	k_a (1/M·s))	k_d (1/s)
value	82.2	3.48×10^3	2.86×10^{-4}
error	7.54	0.05×10^3	0.22×10^{-4}

[a]The anti-IL8 antibody was immobilized on a sepharose functionalized gold chip with the running buffer PBST (see Supplementary Figure 2).

Structural Characterization of Interleukin-8 by Mass Spectrometric Peptide Mapping. Prior to proteolytic digestion, the disulfide bridges of IL8 were reduced and the cysteine residues alkylated in order to provide a suitably unfolded protein and facilitate enzymatic digestion, as described in the Experimental Section. The alkylated IL8 was characterized by MALDI-TOF MS (Supplementary Figure S2) and by mass spectrometric peptide mapping of proteolytic fragment mixtures. Tryptic digestions of IL8 were performed under standard conditions (18 h, atmospheric pressure) and with a high-pressure proteolyzer at approximately 30 kpsi (120 min); the corresponding mass spectra of the digest mixtures are compared in Figure 3. Both digestion procedures provided identical peptides; however, the high-pressure digestion proceeded with significantly higher rate, while both digestion procedures provided >90% sequence coverage. Notably, trypsin fully maintained its cleavage specificity under high pressure conditions, indicating that the pressure-induced unfolding of IL8 appears to be responsible for the considerably shorter digestion times.[31]

Identification of Antibody Epitope Peptides of IL-8. Epitope extraction with the antibody immobilized on a sepharose microaffinity column (Figure 2) followed by removal of supernatant peptides by washing with aqueous solvent at binding conditions (pH 7) did not show unbound peptides in the last washing fraction. Subsequent elution was performed with slightly acidified solvent containing 0.1% TFA. MALDI-MS analyses of the elution fractions from epitope extraction of the IL8 tryptic digestion mixture from

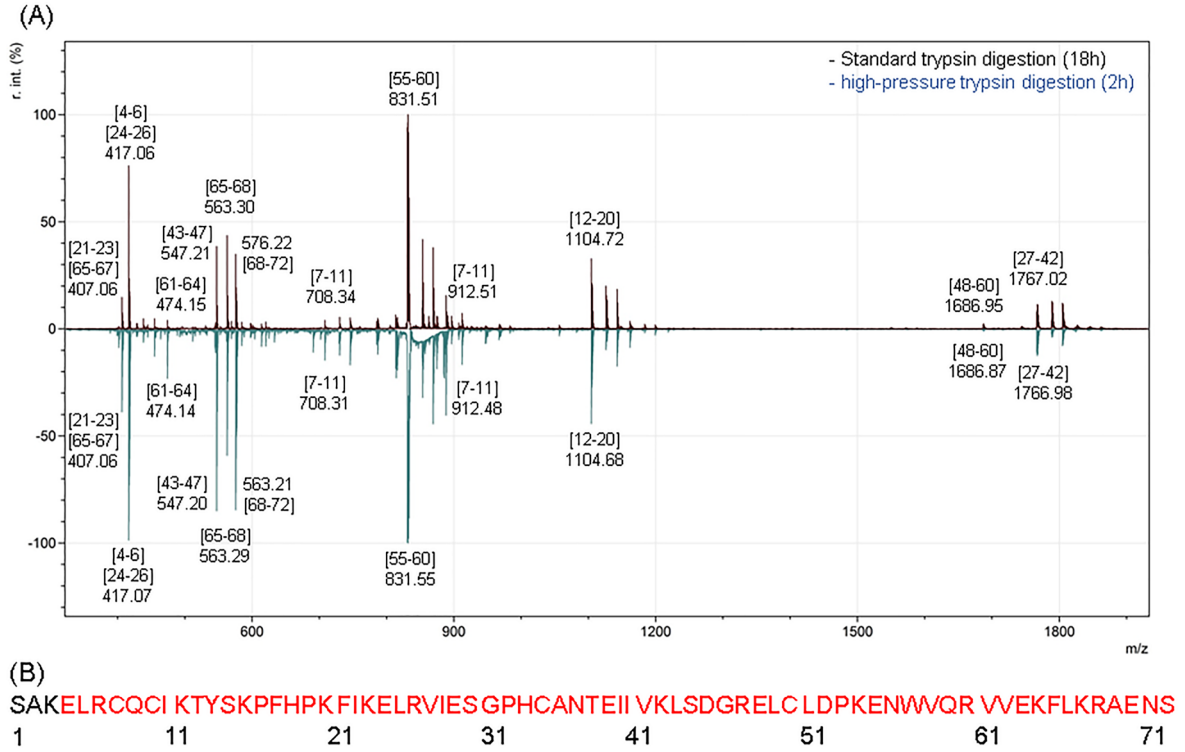

Figure 3. (A) Comparison of an IL8 digestion with trypsin under high pressure and standard conditions. Salt adduct ions (Na^+, K^+) are not annotated, accounting for most of the unannotated peaks. (B) Sequence coverage of peptides is marked in the IL8 sequence (red, 96%). Proteolytic digestion were carried out in triplicates, and digestion yields >95% (errors 12−15%) estimated by 1D SDS gel electrophesis.

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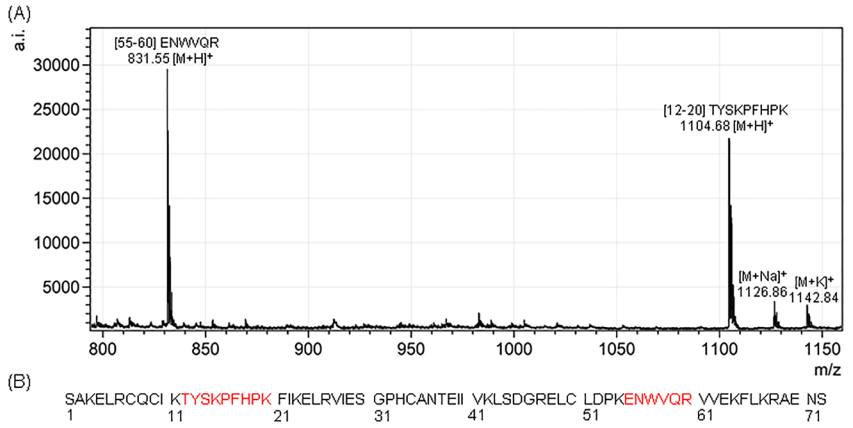

Figure 4. MALDI-MS of epitope peptides eluted upon epitope extraction of IL8 digested by trypsin under high pressure conditions from the immobilized monoclonal anti-IL8 antibody. (B) Highlighted epitope peptides in the sequence of IL8.

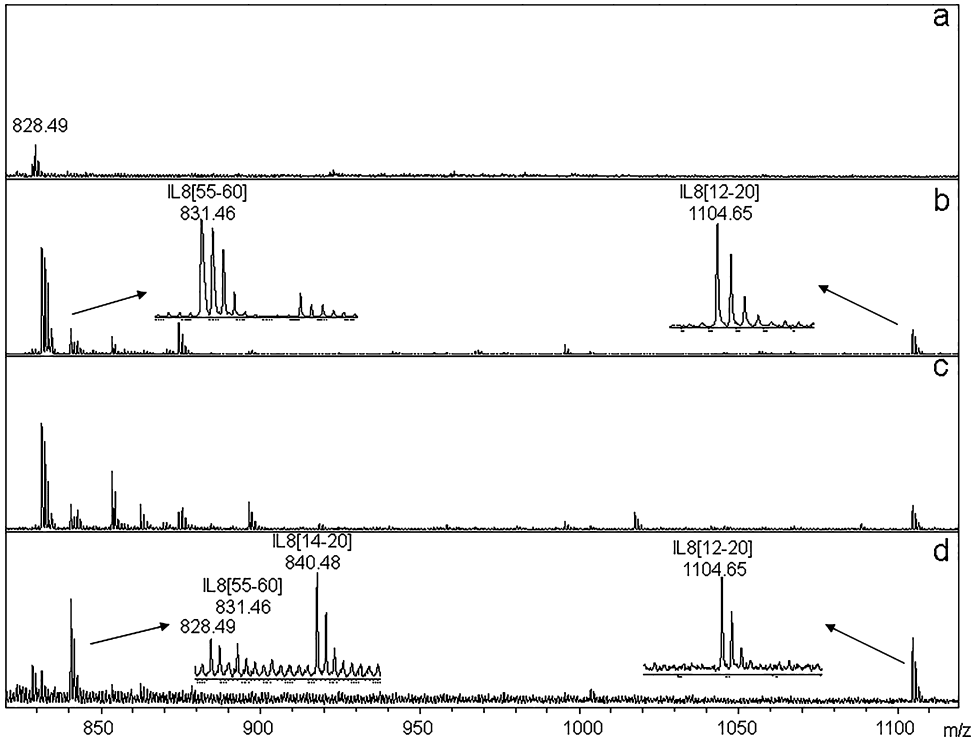

Figure 5. Direct identification of IL8 epitopes by elution and MALDI-MS from SPR chip. Monitoring of elution fractions by MALDI-MS was performed by washing form the SPR-chip upon epitope extraction with trypsin: (a), MALDI-MS of DHB matrix which gives a background signal at m/z 828.49; (b) MALDI-MS of elution fraction collected at 50 min; (c) MALDI-MS of elution fraction collected at 120 min; (d) MALD-MS of fraction collected upon injection of 0.1% TFA after 150 min.

the affinity column revealed two distinct epitope peptides, IL8 [12−20] (TYSKPFHPK) and IL8 [55−60] (ENWVQR), consistent with an assembled (discontinuous) epitope comprising two spatially adjacent sequences of IL8 (Figure 4). Due to the unknown concentration of the epitope peptides in the elution fraction a direct determination of a dissociation constant was not possible. However, a qualitative estimation of the affinity binding was obtained by injection of the elution fraction containing the two epitope peptides over the immobilized anti-IL8 antibody chip. The sensorgram of this injection showed two discrete binding events (Supplementary Figure S3) indicating specific binding of both epitope peptides to the anti-IL8 antibody. In the same manner, an IL8 digestion mixture with chymotrypsin did not provide specific epitope peptides in the elution fraction. Since chymotrypsin was found to cleave within the sequences of both identified epitope peptides (F17, W56), this result is well explained by the loss of binding affinity of the small chymotryptic peptides.

Direct Identification of Epitopes upon Elution from SPR Chip. In an additional, direct approach, identification of the epitope peptides of IL8 was obtained upon proteolytic epitope extraction with trypsin and MALDI-MS from the SPR chip containing the immobilized antibody. The tryptic IL8 digest was injected over the antibody, elution from the SPR chip was performed over 120 min, and fractions were directly analyzed by MALDI-MS. The mass spectrometric analyses of elution fractions revealed the epitope peptides

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[55−60] and [12−20] (Figure 5), in complete agreement with the epitope peptides identified from the sepharose microaffinity column (Figure 4). The last elution/washing step with 0.1% TFA showed an additional peptide at m/z 840.46 IL8[14−20] due to unspecific cleavage after Y13, that eluted under acidic conditions. These results illustrate the feasibility for direct epitope identification upon affinity-extraction and elution from SPR chip surfaces with high specificity and sensitivity.

Synthesis and Characterization of the Epitope Peptides. To validate the epitope peptides identified by proteolytic affinity-MS, the peptide sequences were synthesized by SPPS using standard Fmoc chemistry. The molecular masses of the HPLC-purified synthetic epitope peptides IL8[12−20] (m/z 1103.56) and IL8[55−60] (m/z 830.4) were verified by MALDI-MS (Figures S4 and S5). Subsequently, SPR determinations with the immobilized anti-IL8 antibody revealed K_D values for the epitope peptides in the μM range (Table 2; Figure S6).

Table 2. SPR Determination of Affinity Binding Constants of the Tryptic Epitope Peptides[a]

peptide	K_D (μM)	k_a (1/M·s))	k_d (1/s)
IL8[55-60]	63.3	36.1	2.29×10^{-3}
(error)	(3.41)	(0.24)	(0.11×10^{-3})
IL8[12-20]	ca. 80		
(error)	(ca. 5)		

[a]The anti-IL8 antibody was immobilized on a 16-mercaptohexadecanoic acid functionalized gold chip, and the running buffer was PBST (see Supplementary Figure 6).

Location of the Epitopes on IL8. Highlighting the sequences of the two epitope peptides on the structure of IL8 (PDB-ID: 1IL8) ascertains that they reside in close spatial proximity forming an assembled epitope. IL8[12−20] comprises most of the N-terminal loop (amino acids 10−18) located between two N-terminal cysteine residues and the first strand of an antiparallel β-sheet. Several residues of the epitope (such as the shielded K11, K15; Y14, F17) have been reported to interact with the N-terminus of CXCR1.[10−12,14,15,31−33] The second epitope peptide IL8[55−60] is located at the C-terminal end of the third strand of the antiparallel β-sheet and the N-terminal turn of the α-helix. It comprises the residue C50 that was shown to play a role in the interaction with the N-terminus of CXCR1.[10,12] The overlap of the epitope peptides with the chemokine-binding site 1 on CXCR1 well explains how the anti-IL8 antibody interferes with the IL8-receptor binding and inhibition of the CXCR1 activation.

CONCLUSIONS

In the present study, we have applied proteolytic epitope extraction−mass spectrometry to elucidate an assembled epitope of a monoclonal anti-human IL8 antibody that has been reported to inhibit the biological effect of IL8.[16−20] In the identification of the epitope, proteolytic digestion at high pressure was found to significantly accelerate and facilitate the digestion compared to standard digestion procedures at ambient pressure, while the specificity of the proteases was fully maintained. This provided the identification of a specific, assembled (discontinuous) epitope of IL8 to the anti-IL8 antibody, comprising the peptide sequences IL8[12−20] located in the N-terminal loop and IL8[55−60] at the transition of a β-sheet to an α-helix. Moreover, the epitope peptides were ascertained by epitope extraction and direct mass spectrometric analysis upon elution of the epitopes from the SPR chip (Figure 5). The epitope peptide sequences in the structure of IL8 overlap with the binding site 1 of IL8 to the CXCR1 receptor (Figure 6). Thus, the antibody blocks the capturing of the IL8 N-Loop by the CXCR1 receptor preventing the first step of the binding process, explaining conclusively the inhibition of IL8−CXCR1 interaction by the anti-IL8 antibody reported in previous studies.

The combination of proteolytic epitope excision− and epitope extraction−mass spectrometry and SPR is shown here as a powerful tool for epitope identification and affinity determination. It has been applied to the chemical structure identification and affinity determination of both linear as well as assembled, discontinuous epitopes,[24−27,30] in contrast to alternative methods such as HDX-MS that require an independent experimental setup for determination of affinity binding constants of epitopes. The identified epitope peptides are tested at present as competitive inhibitors neutralizing the IL8−CXCR1 interaction. In addition, future studies will be directed to elucidate the antibody paratope. Like the epitope peptides, the corresponding paratope peptides may constitute lead structures for the development of specific small-molecule inhibitors of the IL8−CXCR1 interaction.

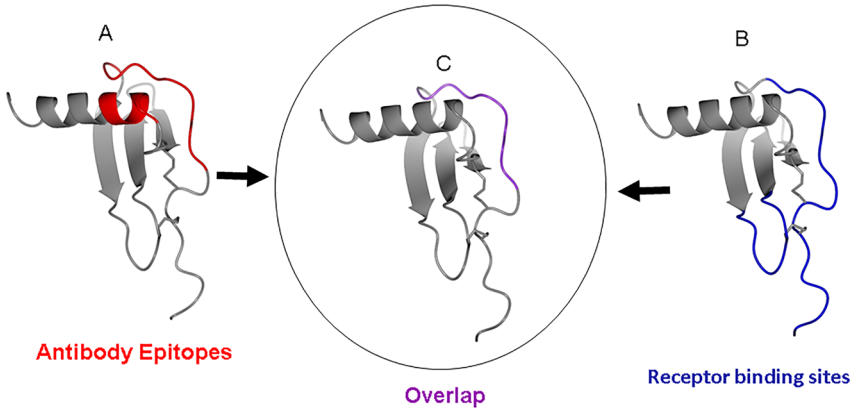

Figure 6. (A) Epitope peptides [12−20] and [55−60] are highlighted (red) in the crystal structure of IL8 (PDB 1IL8). (B) Binding sites of IL8 (blue) to the CXCR1 receptor. (C) Overlapping region of the antibody epitope and the receptor binding site on IL8.

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■ ASSOCIATED CONTENT

Supporting Information

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Supplementary tables and figures (PDF)

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Notes

The authors declare no competing financial interest.

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