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ARTICLE

Identification of the dimerisation interface of human interleukin-8 by IL-8-variants containing the photoactivatable amino acid benzoyl-phenylalanine

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Abstract The three-dimensional structure of human interleukin-8 (hIL-8) was determined by the use of NMR and X-ray methodology. At high concentrations interleukin-8 and many other chemokines form a non-covalent homodimer. Several studies have been performed to investigate the relevance of the dimer on receptor activation and led to contradictory results. In order to obtain a better understanding of the dimerisation process, covalently linked homo- and heterodimers were produced by photo-induced dimerisation of hIL-8 analogues that contain the photo-activatable amino acid *p*-benzoyl-phenylalanine (Bpa) at different positions. Whereas the N-terminal fragment (1–54) was expressed as recombinant thioester, the C-terminal fragments (55–77) that contain Bpa either at position 65 or 74 were obtained by solid-phase peptide synthesis. The segments were combined by expressed protein ligation and led to full length IL-8 variants containing the non-proteinogenic amino acid Bpa at single positions. IP_3 activity tests showed high biological activity for the CXCR1–GFP receptor for both variants comparable to that of the native ligand. The refolded and purified ligation-products were used for dimer formation by UV-irradiation. The analysis of the reaction mixture was performed by gel-electrophoresis and mass spectrometry and showed that dimer formation of IL-8 occurred in a position dependent manner. [Bpa^{74}]hIL-8 has a high tendency to form covalent dimers whereas no dimer formation was observed for the variant with Bpa at position 65. Accordingly one residue of the dimerisation interface could be identified.

Dedicated to Prof. K Arnold on the occasion of his 65th birthday.

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Introduction

The knowledge of the three-dimensional structure of a given protein as well as the formation of macromolecular protein complexes is the prerequisite for understanding the processes in living cells. Mainly two high-resolution methods are commonly used for the determination of such structures: NMR spectroscopy and X-ray structure analysis. However, both techniques comprise significant disadvantages that limit their application. Whereas the high protein concentration favors association and aggregation in NMR studies, X-ray structure analysis requires protein crystals. This however is often not easy to obtain and finding the best conditions for crystallisation is frequently the most difficult part.

Cross-linking in combination with mass spectrometry and theoretical methods can be used as a low resolution method to elucidate the structure of a protein (Sinz 2003) or can be applied to study the interaction of peptides/proteins with other proteins (Carter et al. 1999; Henry et al. 2002), nucleic acids (Steen and Jensen 2002) or lipids (Ridder et al. 2004).

In order to covalently link interaction partner on a specific external pulse photo-crosslinking is applied (Brunner 1993). This requires photo-reactive crosslinking molecules that can be activated by light. Most successfully applied groups include azido-, trifluoromethyldiazirine- or benzoyl–phenyl-groups, that generate reactive nitrenes, carbenes or diradicals after illumination,

respectively. In order to incorporate them into proteins either unselective labelling with bifunctional reagents or selective introduction into the sequence as modified amino acids can be applied. However, to investigate interactions at a molecular level, mostly the second approach has been used and 4-azido-Phe, 4-(3-trifluormethyldiazirin)-Phe and 4-benzoyl-Phe have been described (Brunner 1993; Weber and Beck-Sickinger 1997). Introduction of these derivatives usually is performed by solid phase peptide synthesis, however this approach limits the application to proteins <50 amino acids. To circumvent this size limitation native chemical ligation methodology has been developed (Dawson et al. 1994), in which a C-terminal peptide thioester reacts with a second peptide containing an N-terminal cysteine. Based on this methodology expressed protein ligation was designed for the introduction of modifications, like the introduction of photo-reactive amino acids, into large proteins (David et al. 2004; Muir 2003; Muir et al. 1998).

Here, we describe the application of expressed protein ligation to obtain photo-activatable analogues of human interleukin-8 (hIL-8). hIL-8 is a pro-inflammatory chemokine, which acts predominantly at neutrophile granulocytes. It is expressed as a 99-amino-acid precursor protein, the removal of a signal sequence and further processing leads to several active isoforms from 69 to 79 amino acids in length. All possess an N-terminal ELR-motive, which is essential for receptor binding and activity. Further characteristic elements are four conserved cysteine residues that form two disulfid-bridges and stabilise the overall protein structure (Clark-Lewis et al. 1994). Because the first two cysteines are separated by a further amino acid (glutamine) hIL-8 belongs to the CXC-chemokine family (Baggiolini et al. 1997). The three-dimensional structure of hIL-8 has been solved by NMR and X-ray crystal structure determination (Baldwin et al. 1991; Clore et al. 1990). It is characterised by a central β-sheet motive which is stabilised by a C-terminal α-helix.

hIL-8 forms non-covalent dimers at those concentrations that are needed for NMR-spectroscopy (Clore et al. 1990). The dissociation constant of this dimer was determined to be 18 μM (Burrows et al. 1994). The relevance of the dimers for function however is still discussed controversially. hIL-8 variants with reduced dimerisation potential suggest, that the monomer is as active as the dimer at CXCR1 and CXCR2 (Horcher et al. 1998), furthermore Fernando et al. (2004) claim that dissociation is essential for receptor binding. In order to study the dimerisation interface we produced two variants of hIL-8 (1–77) that contain the photo-activatable amino acid Bpa at position 65 and 74. By in vitro dimerisation and photo-crosslinking followed by mass spectrometry we could convincingly show, that position 74 is part of the dimerisation interface whereas position 65 contributes only to a minor extend. Position 74 does not contribute to receptor interaction whereas the loss of Arg65 leads to a four-fold loss in activity.

Material and methods

Synthesis of [Bpa^{65}]hIL-8 and [Bpa^{74}]hIL-8

The C-terminal fragments were synthesised by automated solid-phase peptide synthesis using a robot (Syro, MultiSynTech, Witten, Germany) on a Wang-resin preloaded with serine (0.63 mMol/g). The Fmoc/tBu-protected monomers (Novabiochem/MERCK, Schwalbach, Germany) were applied 0.5 M in tenfold excess. As activating agents 1-hydroxy-benzotriazole and diisopropyl-carbodiimide were used 0.5 M. The Fmoc-protected *p*-benzoyl-phenylalanine (Bachem, Basel, Switzerland) was introduced into the growing chain by manual coupling two times (0.25 M, fivefold excess, 3 h). After TFA-cleavage using 90% TFA and 10% scavenger (ethandithiol/thioansiole 3:7) the crude peptides were separated by preparative RP–HPLC using a gradient from 20 to 70% acetonitrile/water (0.1% trifluoroacetic acid) within 40 min. Retention time was observed with 24.1 min for [Bpa^{65}]hIL-8 (55–77) and with 24.5 min for [Bpa^{74}]hIL-8 (55–77). The identity was confirmed by MALDI-MS (Voyager II, Perseptive, Framingham, MA, USA). Molecular mass of [Bpa^{65}]hIL-8 (55–77) was determined with 2,882.1 Da (M_{theor} = 2,882.0 Da) and of [Bpa^{74}]hIL-8 (55–77) with 2,967.2 Da (M_{theor} = 2,967.2 Da). The N-terminal thioester-segment hIL-8 (1–54)-MESNA was obtained by expression of the intein-fusion protein in *Escherichia coli* and purification by the IMPACT method (New England Biolabs) as described previously (David et al. 2003). Ligation of the synthesised peptides containing the N-terminal cysteine 55 with hIL-8 (1–54)-MESNA-thioester was performed as reported in David et al. (2003). The reaction mixture was subjected to a refolding procedure to form the disulfide bridges and to remove the urea. This was performed by dialysis against 50 mM Tris-buffer (pH 8) containing 0.5 M NaCl, 10 mM cysteine, 1 mM cystine and urea in decreasing concentrations from 3 to 0 M. Educts were removed by RP–HPLC, purity was proven by analytical HPLC and identity was confirmed by MALDI-MS. Molecular mass of [Bpa^{65}]hIL-8 (1–77) was determined

with 9,013 Da (M_{theor} = 9,012.3 Da) and of [Bpa^{74}]hIL-8 (1–77) with 9,096 Da (M_{theor} = 9,097.4 Da). The ligation to yield [Bpa^{65}]hIL-8 (1–77) was 32.4%, after refolding and purification 5.3% (0.7 mg). [Bpa^{74}]hIL-8 (1–77) yielded in 25.4%, after refolding and purification 10.5% (0.95 mg) was obtained.

Determination of the biological activity

The biological activity was investigated in an inositol phosphate (IP_3) accumulation assay. 1.5×10^5 COS-7 cells/well were seeded in 12-well plates and transiently transfected with 0.4 μg vector DNA encoding the human CXCR1–EGFP-fusion protein. For co-transfection 0.1 μg of plasmid DNA coding for the chimeric G-protein $G\alpha_{\Delta 6qi4myr}$ and 1.5 μl of metafectene (Biontex, Munich, Germany) was used per well. One day after transfection cells were incubated with 2 μCi/ml myo-[^{3}H]-inositol (25.0 Ci/mmol; Perkin Elmer Life Sciences) and washed after 16 h with 1 ml culture media containing 10 mM LiCl. Cells were stimulated in media without FCS containing 10 mM LiCl in the absence or with increasing concentrations of agonist at 37°C. After 1 h the reaction was stopped by aspiration of the medium and cell lysis was performed with 300 μl 0.1 M NaOH. After adding 100 μl of 0.2 M formic acid and sample dilution intracellular IP_3-levels were determined by anion-exchange chromatography as described (Berridge 1983; Berridge et al. 1983). Each assay was performed three times as biological duplicate. Dose–response curves were calculated using Prism 3.02 (GraphPad, SanDiego, CA, USA).

Photochemical cross-linking

For photochemical cross-linking peptides were dissolved in 100 μl aqua dest. (100 μM) and irradiated with UV-light (Atlas Fluotest forte, 366 nm, 180 W) on ice for 1 h. Twenty microlitre samples were taken and analysed by SDS-PAGE using a 20% acrylamide-gel. To investigate the identity of the cross-linked products the mixture was directly used for MALDI-TOF mass spectrometry. To determine the dimer dissociation constant (K_d) of [Bpa^{74}]hIL-8 the peptide was dissolved in 10–100 μM in water and irradiated by UV-light at 362 nm. Twenty microlitre of the 100 μM-sample and equal protein content of all other probes were loaded onto a gel. After Coomassie blue-staining the gel was analysed by using a Molecular Imager® FX ProPlus (BioRad Laboratories, Hercules, CA, USA). Images were obtained by using a 531 nm laser, a 555 nm long pass filter and a pixel size of 50 μm^2. Densitometric analysis of the monomer- and dimer bands was performed with the Quantity One software (version 4.2.1., BioRad Laboratories, Hercules, CA, USA). For K_d-determination log [dimer] was plotted versus log $[(\%D)/0.04[100-(\%D)]^2]$ as described in Refs Gallagher and Huber (1997) and Manning et al. (1996), where $\%D$ is the percent of the protein that actually is a dimer at the various [dimer]. For such a plot a straight line with a slope = 1 should result. The resulting log [dimer]-value for log $[(\%D)/0.04[100-(\%D)]^2] = 0$ is equal to K_d. Applying this method K_d of [Bpa^{74}]hIL-8 was determined with 55 μM.

Results and discussion

Human Interleukin 8 (hIL-8) forms a non-covalent homodimer at higher concentrations. It was shown that the monomer is fully active (Horcher et al. 1998), however it is still unclear whether the dimer also can activate the receptor or whether it prevents receptor binding as suggested by Fernando et al. (2004). To further address this question and to determine the dimer interface covalent dimers were produced by photo-crosslinking of photo-reactive hIL-8 variants. These analogues were produced by incorporation of the photo-activatable amino acid benzoyl-phenylalanine (Bpa) into the C-terminal part of hIL-8. As the NMR-structure suggests the position 74 points to the dimer partner, whereas position 65 does not show any interaction (Clore et al. 1990), these two positions were chosen for the introduction of Bpa in order to investigate different sites of the molecule.

hIL-8 (55–77)-analogues were synthesised by robot assisted solid-phase peptide synthesis. Arginine 65 or alanine 74 was replaced by *p*-benzoyl-phenylalanine, which was introduced manually as Fmoc-protected building block. After cleavage of the peptides from the resin with trifluoroacetic acid (TFA) the fragments containing the N-terminal cysteine 55 were separated by HPLC and their identity was proven by MALDI-TOF mass spectrometry.

The purified peptides were used for native chemical ligation with hIL-8 (1–54)-MESNA-thioester (Fig. 1) in a 1:1 ratio and ligation reaction was proceeded for 48 h in denaturating conditions in the presence of 3 M urea. The ligated products were refolded, urea was removed by dialysis and educts were removed by preparative HPLC. Purity of the ligation products was investigated by analytical HPLC and identity was proven by MALDI-TOF mass spectrometry (Fig. 2).

The activity of both constructs was determined by using the IP_3-assay in COS-7 cells transfected with CXCR1–EGFP and $G\alpha_{\Delta 6qi4myr}$. Dose–response curves

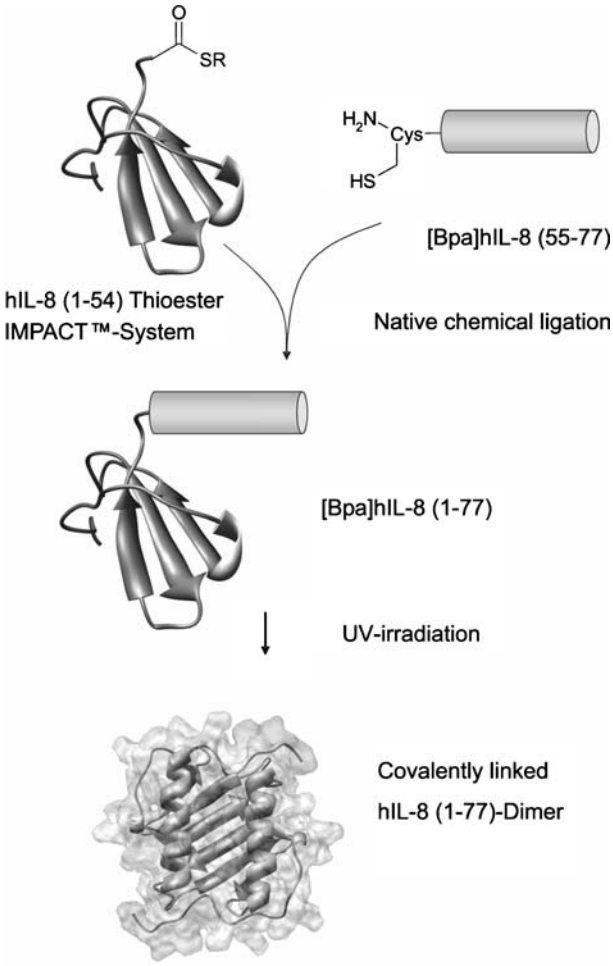

Fig. 1 Schematic view of the strategy to obtain photo-activatable protein variants. hIL-8 (1–54) was expressed as recombinant thioester by using the IMPACT™-system. The peptide-thioester was used for native chemical ligation with hIL-8 (55–77) with Bpa either at position 65 or 74. Covalently linked dimers were obtained by UV-irradiation (drawn after PDB-entry 1IL8 and 3IL8)

of the Bpa-analogues were recorded and compared with the native ligand (Fig. 3). Both analogues still were able to activate the EGFP-fused CXCR1-receptor in a dose-dependent manner. Whereas [Bpa^{74}]hIL-8 (EC_{50} 156.3 ± 11.5 nM) was about as active as hIL-8 (EC_{50} 271.3 ± 33.9 nM), [Bpa^{65}]hIL-8 was ca. fourfold less active (EC_{50} 973.7 ± 172.9 nM). The slightly increased activity of [Bpa^{74}]hIL-8 might be due to the increased hydrophobicity that occurs by replacing alanine by the large, aromatic and hydrophobic Bpa. Accordingly, hydrophobic interactions can be speculated to contribute to ligand receptor interaction. The fourfold decreased activity of [Bpa^{65}]hIL-8 probably is caused by the exchange of arginine residue at position 65 by alanine. Either a direct effect, caused by the loss of the side chain or an indirect deriving from a different ligand conformation can be speculated. The reduced activity in all assays compared to (Wu et al. 1993) can be explained by the C-terminal fusion of the receptor with the EGFP, which could lead to a weaker interaction of the receptor with G-proteins.

Photochemical cross-linking was performed by UV-irradiation of 100 μM samples of [Bpa^{65}]hIL-8, [Bpa^{74}]hIL-8 and a mixture of both in a 1:1-ratio. At these concentrations hIL-8 forms non-covalent dimers (Burrows et al. 1994) and the possibility of cross-linking to yield the covalently linked dimers is given. SDS-gel electrophoresis was performed to investigate the cross-linking rate (Fig. 4). The best yield could be observed when the crosslinker was placed at position 74. According to the 3D-structure obtained by X-ray crystallography or NMR this position points to the β-sheet of the partner in the dimer (Baldwin et al. 1991; Clore et al. 1990). A significantly lower yield was observed for the variant with Bpa at position 65. Here the Bpa-side-chain directs to the surrounding media and is

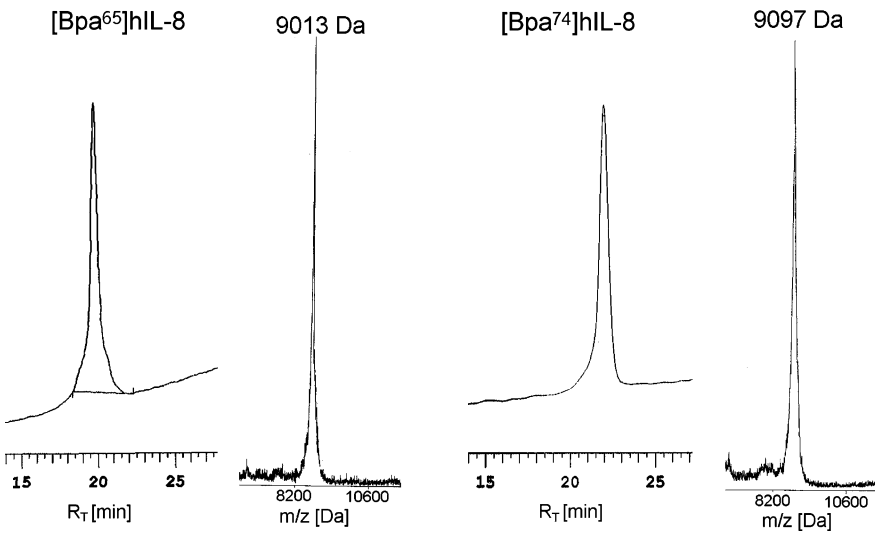

Fig. 2 Analytical HPLC and MALDI-TOF mass spectra of the ligation products [Bpa^{65}]hIL-8 (*left*) and [Bpa^{74}]hIL-8 right with a H_2O/acetonitril-gradient of 20–70% with 0.1% TFA within 40 min

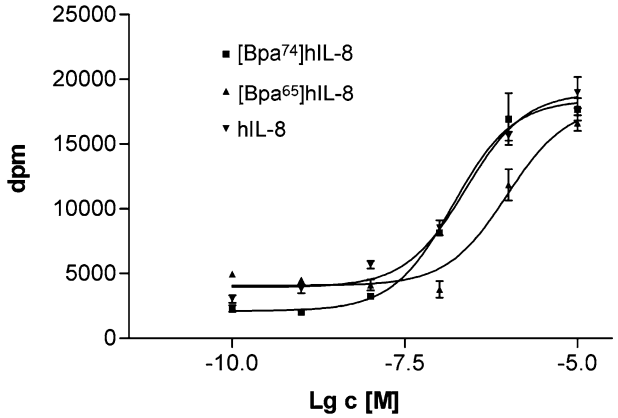

Fig. 3 IP_3-assay at COS-7 cells transfected with CXCR1–EGFP and $G\alpha_{\Delta 6qi4myr}$. Dose–response curves were recorded for [Bpa^{65}]hIL-8, [Bpa^{74}]hIL-8 and native hIL-8

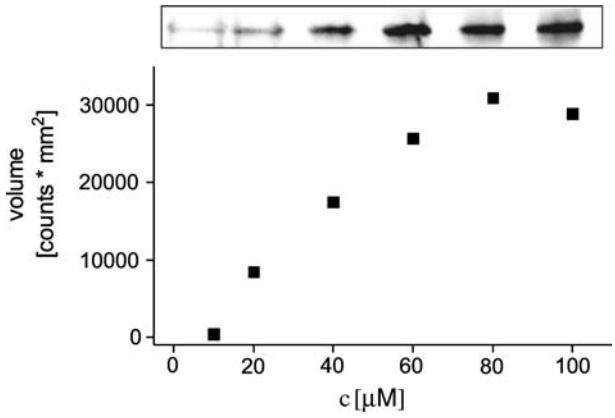

Fig. 5 Determination of the dissociation constant of [Bpa^{74}]hIL-8. [Bpa^{74}]hIL-8 in different concentrations was irradiated by UV-light. Respectively, the same protein-content was analysed by SDS-PAGE and the dimer bands were quantified

suggested to have no contact to the dimer partner. In the mixture of both variants the yield of the cross-linking product was comparable with [Bpa^{74}]hIL-8 suggesting that it mainly is conferred by the [Bpa^{74}]hIL-8 variant. The dimer dissociation constant of [Bpa^{74}]hIL-8 was determined with approximately 55 µM (Fig. 5). The lower dimer formation compared to Ref. Burrows et al. (1994) with 18 µM may be caused by the large benzoyl-phenylalanine in contrast to the naturally occurring alanine. The K_D of [Bpa^{65}]hIL-8 could not be determined because of the low amount of the formed dimer.

The identity of the cross-linking products was investigated by MALDI-TOF mass spectrometry (Table 1). The masses of all three products could be detected

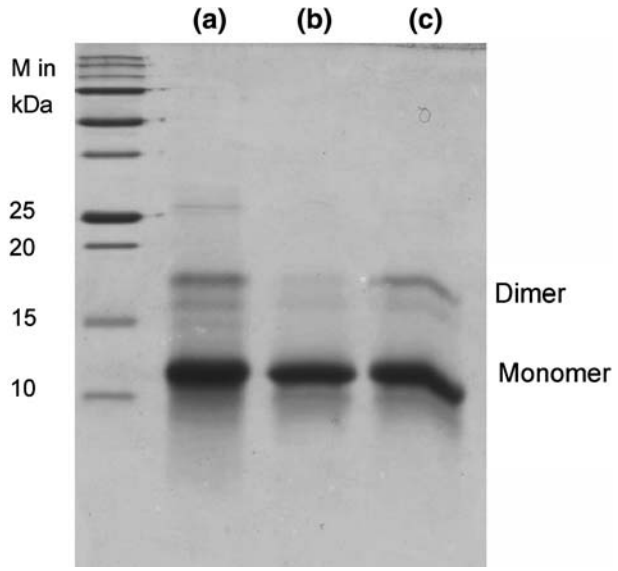

Fig. 4 SDS-PAGE of cross-linking of [Bpa^{74}]hIL-8 (**a**), [Bpa^{65}]hIL-8 (**b**) and a 1:1-mixture of both variants (**c**)

(Fig. 6) and measured masses were in good agreement with the calculated masses. Interestingly, in the 1:1 ratio only the heterodimers were found and no homodimers could be detected, which again supports the hypothesis that the dimerisation occurs by [Bpa^{74}]IL-8, whereas [Bpa^{65}]IL-8 does not contribute. The measured masses could be confirmed by SDS-PAGE. Here the masses of the dimers are in the range of 18 kDa as calculated from the protein standard. Also the ratio of the mass-intensities observed by mass spectrometry was comparable to the ratios monitored by SDS-PAGE (Fig. 4).

In conclusion photo-chemically cross-linked hIL-8 dimers could be generated. As expected, the yield of the linked product was highest with Bpa at position 74, where the side-chain points to the dimer partner. When the side-chain points to the surrounding media at position 65, only low product formation could be observed. Accordingly, we conclude that Arg65 does not play a role in dimer formation. This is in agreement with earlier studies, in which C-terminally truncated IL-8 variants do not dimerise although they still contain the Arg65 (Jin et al. 2005).

Accordingly, we could identify the first residue in the dimerisation interface of hIL-8 by experimental methods. The Bpa residue had only a low influence on the receptor binding when it was inserted at position 74 instead of alanine and even a slightly increase in activity was monitored. The replacement of Arg65 and thus the elimination of one positive charge, however, led to a fourfold decrease in activity. This suggests some contribution of the C-terminal helix in receptor binding. Next steps will include the production of higher quantities of the cross-linked dimers in order to study the dimer activity at both receptors CXCR1 and CXCR2.

Table 1 Molecular masses of BPA-containing hIL-8 monomers and of the obtained cross-linking products investigated by MALDI-TOF MS

	Mass monomer (Da)		Mass dimer (Da)	
	Calculated	Measured	Calculated	Measured
[Bpa^{65}]hIL-8	9,012	9,013	18,026	18,023
[Bpa^{74}]hIL-8	9,097	9,096	18,194	18,195
[Bpa^{65}]hIL-8 + [Bpa^{74}]hIL-8 1:1	18,109	18,100		

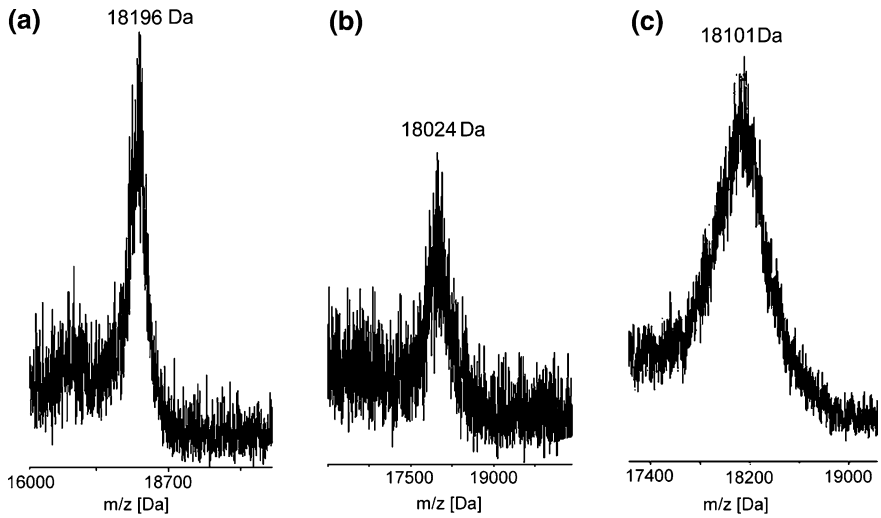

Fig. 6 MALDI-TOF mass spectra of the cross-linking mixtures containing [Bpa^{74}]hIL-8 (**a**), [Bpa^{65}]hIL-8 (**b**) and a 1:1-mixture of both variants (**c**)

Single chain IL-8-dimers were already shown to be active in receptor binding and in the chemotaxis of neutrophiles (Leong et al. 1997). This is in contrast to (Fernando et al. 2004), in which dimer dissociation is described to be necessary for CXCR1 binding.

Studies to identify the cross-linking site by protease digestion and mass spectrometry are currently in progress. Both variants furthermore will be used for cross-linking studies of hIL-8 with both receptors to identify the contact points of the C-terminal part of hIL-8.

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