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A truncated form of CKβ8-1 is a potent agonist for human formyl peptide-receptor-like 1 receptor

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1 Human formyl peptide-receptor-like-1 (FPRL-1) is a promiscuous G protein-coupled receptor (GPCR), and belongs to a chemoattractant receptor family protein. This receptor has been reported to interact with various host-derived peptides and lipids involved in inflammatory responses. We described here, a novel role for FPRL-1 as a high-affinity β-chemokine receptor for an N-terminally truncated form of the CKβ8 (CCL23/MPIF-1) splice variant CKβ8-1 (22–137 aa).

2 RT-PCR analysis of mRNA derived from human tissues and cells revealed a predominant expression of FPRL-1 in inflammatory cells, particularly in neutrophils.

3 Intracellular calcium mobilisation assay, used as screening tool, in recombinant Chinese hamster ovary (CHO-K1) and human embryonic kidney (HEK293s) cells coexpressing FPRL-1 and $G\alpha_{16}$, demonstrated FPRL-1 is a functional high-affinity receptor for CKβ8-1 (46–137 aa, sCKβ8-1), with pEC_{50} values of 9.13 and 8.85, respectively.

4 The FPRL-1 activation in CHO-K1 cells is mediated by $G\alpha_i/G\alpha_o$ proteins, as assessed by pertussis toxin sensitivity and inhibition of forskolin-induced cyclic AMP accumulation.

5 Binding experiments were performed with a radio-iodinated synthetic peptide, [^{125}I]-WKYMVm, a known potent FPRL-1 agonist. CHO-K1 cell membranes expressing FPRL-1 bound [^{125}I]-WKYMVm with a K_d value of 9.34. Many known FPRL-1 agonists were tested and sCKβ8-1 was the most effective nonsynthetic ligand in displacing the radiolabelled agonist, with a pIC_{50} of 7.97.

6 The functional significance of sCKβ8-1 interaction with FPRL-1 was further demonstrated by the activation of polymorphonuclear leukocytes (PMNs) calcium mobilisation and chemotaxis. These interactions were shown to be *via* FPRL-1 by specific blockade of PMNs activation in the presence of an FPRL-1 antibody.

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Abbreviations: cAMP, cyclic AMP; CHO-K1, Chinese hamster ovary; FLIPR™, Fluorescent Imaging Plate Reader; FPRL-1, formyl peptide-receptor-like-1; GPCRs, G protein-coupled receptors; HEK293 s, Human embryonic kidney (s, adapted from suspension); MPIF-1, myeloid progenitor inhibitor factor-1; PMNs, polymorpho-nuclear leukocytes; PTX, pertussis toxin; RT, reverse transcription

Introduction

Human formyl peptide-receptor-like-1 (FPRL-1) is a seven-transmembrane G protein-coupled receptor (GPCR), sharing 69% identity at the amino-acid level with formyl peptide receptor (FPR) and 83% identity with FPRL-2. FPR, FPRL-1 and FPRL-2 are located on chromosome 19q13.3 and are phylogenetically clustered with chemokine receptors (Murphy *et al*., 1992; Haviland *et al*., 1993; Fredriksson *et al*., 2003). FPRL-1 is expressed in astrocytes, neutrophils and monocytes (Durstin *et al*., 1994; Le *et al*., 2000). Bacterial chemotactic peptides, such as *N*-formyl-methionyl-leucyl-phenylalanine (fMLF), have been shown to activate FPR at picomolar concentrations and elicit the release of proinflammatory mediators from leukocytes, thereby leading to chemotaxis (Le *et al*., 2001). FPRL-1 has been reported as a low-affinity fMLF receptor (Le *et al*., 2001). This class of receptors has been proposed to play a role in host defence mechanisms due to their ability to interact with bacterial formylated peptides (Le *et al*., 2001; 2002). Whereas, FPRL-2 is expressed in monocytes but not in neutrophils, and is not activated by *N*-formylpeptides (Le *et al*., 2000). Thus far, no ligands have been identified for FPRL-2.

The first ligand reported to bind with high affinity to FPRL-1 was the lipid mediator lipoxin A_4 (LXA_4) eicosanoid and its isomer 15-epi LXA_4 (Fiore *et al*., 1994; Fiore & Serhan, 1995). Recently, a variety of agonists for FPRL-1 have been reported (Le *et al*., 2001; 2002), including the V3 region of the HIV-1 envelope gp120, glucocorticoid-induced annexin 1 (ANXA1)-derived peptide, and several amyloidogenic proinflammatory polypeptides, such as the acute-phase protein SAA, the 42 amino-acid form of β-amyloid ($A\beta_{42}$) and human prion

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peptide (hPrP, 106–126 aa). Moreover, synthetic peptides derived from random peptide libraries, such as MMK-1, W-peptide and its isoform (W-peptide (m)) (Le *et al*., 1999; Christophe *et al*., 2001), have been described as highly potent agonists for FPRL-1. The affinities displayed by these ligands, as determined by calcium flux, binding and chemotaxis, were between 1 pM and 5.0 μM (Le *et al*., 2002). It should be noted that, these ligands bind to the same receptor, despite not sharing any substantial homology or displaying any known structural similarities (Le *et al*., 2002).

Since human FPRL-1 is expressed in neutrophils and monocytes (Le *et al*., 2000), and is activated by a diverse class of ligands mostly involved in the release of proinflammatory mediators, we postulated that a chemokine could be a potent endogenous agonist for FPRL-1. We therefore evaluated a collection of inflammatory mediators including more than 77 recombinant chemokines and identified a novel N-terminally truncated form of β-chemokine CKβ8-1 (sCKβ8-1, 46–137 aa), as a high-affinity FPRL-1 agonist.

The chemokines are 8–14 kDa-secreted cytokines, and four subfamilies have been discovered including: CXC(α), CC(β), C(γ) and CX3C (Murphy *et al*., 2000). Myeloid progenitor inhibitor factor-1 (CCL23/MPIF-1) belongs to the second subfamily. Its cDNA encodes a signal sequence of 21 aa followed by a 99 aa (CKβ8) or 116 aa (CKβ8-1) mature form protein. Both mature forms have been identified as putative ligands for CCR1 receptor (Youn *et al*., 1998). Multiple chemokines may activate a single chemokine receptor, as CCR1 is activated by RANTES, MIP-1α, MP-1β, CKβ8 and CKβ8-1 (Murphy *et al*., 2000). However, little is known about the specificity of CKβ8-1 with other receptors.

In the present study, we report the detailed expression profile of FPRL-1, and the findings that this GPCR is activated by sCKβ8-1 at subnanomolar concentrations and is coupled to $G\alpha_i/G\alpha_o$ protein. Furthermore, we demonstrate that sCKβ8-1 induces calcium mobilisation and chemotaxis in PMNs *via* FPRL-1. These studies emphasise the possible proinflammatory role for FPRL-1 through its interaction with sCKβ8-1. To our knowledge this is the first description of a chemokine as a potent ligand for FPRL-1.

Methods

Cloning of cDNA encoding the human FPRL-1 receptor

The pIRES-neo2 expression vector (BD Biosciences, Palo Alto, CA, U.S.A.) was modified to increase the stability of mRNA cloned into the polylinker (Dr H. Weir, AstraZeneca R&D, Alderley Park, England) and was named pGEN-IRES-neo2. The human FPRL-1 coding sequence (Swissprot accession number P25090) was obtained from Dr. W. Koopmann (AstraZeneca R&D, Lund, Sweden) and subcloned into the pGEN-IRES-neo polylinker, as an *Eco*RI–*Xho*I fragment ligated into *Eco*RI–*Sal*I. A CCACC Kozak consensus sequence was added 5′ to the ATG start codon.

Quantitative reverse transcriptase-PCR analysis

Human tissue cDNAs or RNAs were purchased from Clontech (Palo Alto, CA, U.S.A.) and Invitrogen (Paisley, England). Primary cells were purified from human peripheral blood using standard methods. Total RNA was extracted using TRIzol and cDNA synthesis was using the Superscript system (Gibco BRL Life Technologies). Taqman quantitative PCR was performed on an ABI 7700 (CGRB, Oregon, U.S.A.) apparatus as manufacturer's recommendations, using FPRL-1 probe oligonucleotide 5′-CGGCATGACACGCACAGT-CACCACC-3′ and primer sequences 5′-GTGATCTG-GGTGGCTGGATT-3′ and 5′-AGGGCCAGGTTCAGGTA-ACA-3′.

Chemicals

Peptidic and nonpeptidic ligands were obtained from commercial sources and were dissolved either in dimethyl sulphoxide (DMSO), 1 : 1 DMSO/distilled water, or in 1 : 1 ethanol/DMSO. The compounds used for dose–response experiments were: MPIF-1/CKβ8/CCL23 (22–120 aa), sCKβ8 (46–120 aa) (N-terminally truncated form of CKβ8 or sCKβ8), CKβ8-1 (22–137 aa), sCKβ8-1 (46–137 aa), IL-8, RANTES (R&D Systems, Minneapolis, U.S.A.), human Prion protein (hPrP, aa106-126), amyloid β protein (Aβ_{42}, 1–42 aa) acute phase protein (SAA), V3 region of the HIV-1 envelope gp120 (HIV-1_{Bru} gp120, 414–434 aa or F-peptide), MMK-1 (Bachem, Torrance, CA, U.S.A.), W-peptide (WKYMVM) and its isoform (WKYMVm) (Phoenix Pharmaceuticals, Belmont, U.S.A.), Lipoxin A_4 (LXA_4) (Calbiochem, La Jolla, CA, U.S.A.). Other compounds used were forskolin, pertussis toxin (PTX) (Sigma-Aldrich, Oakville, ON, Canada) and Melanin Concentrating Hormone (MCH) (American Peptide Company, Sunnyvale, CA, U.S.A.). A 17-amino-acid synthetic peptide, named SHAAG peptide which corresponds to the alternatively splice variant region of CKβ8-1 ($_{47}$LWRRKIGPQMTLSHAAG$_{63}$), was synthesised using standard procedures.

Cell transfection

HEK293 s cells were transfected with FPRL-1 (or with other GPCRs) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.) following the manufacturer's conditions and by adapting the transfection protocol to a 96-well plate. CHO-K1 cells were transfected by electroporation as described (Groblewski *et al*., 1997). Following the electro-shock, 8×10^4 cells were seeded per well. HEK293 s and CHO-K1 cells stably expressing $G\alpha_{16}$ protein (Molecular Devices, Sunnyvale, CA, U.S.A.) were used to coexpress FPRL-1 or a control GPCR.

Measurement of intracellular Ca^{2+}

HEK293 s cells transiently expressing FPRL-1 or unrelated GPCRs (such as Melanin concentrating hormone receptor, MCH1R (Lembo *et al*., 1999)) were plated (8×10^4 cells per well) in 96-well, poly-D-lysine-coated black FLIPR plates (Becton Dickinson, ON, Canada), and loaded with 100 μl Dulbecco's modified medium (DMEM) + 10% foetal bovine serum containing the calcium-sensitive dye Fluo3-AM (TEF LABS, Austin, TX, U.S.A.) (4 μM final concentration), pluronic acid (20% final concentration) (Molecular Probes, OR, U.S.A.) and were incubated at 37°C for 1 h in a humidified chamber (5% CO_2/95% air). Following the incubation step, cells were washed five times in Hanks' balanced salt solution buffer (HBSS) supplemented with

20 mM HEPES + 0.1% BSA pH 7.4 (Wisent, Saint Bruno, QC, Canada). CHO-K1 cells were loaded with 100 μl HBSS buffer with 2.5 mM of Probenicid (Sigma-Aldrich, Oakville, ON, Canada) containing Fluo3-AM and pluronic acid as mentioned above. Mobilisation of intracellular Ca^{2+} was measured online using the Fluorescent Imaging Plate Reader (FLIPR™) (Molecular Devices) as published (Lembo *et al*., 1999). Ligands were tested at 1 μM (final concentration, except chemokines were at 66 nM, or different starting concentrations when indicated). $[Ca^{2+}]_i$ mobilisation in CHO-K1 and HEK293 s was measured by integrating the kinetic curve on intracellular Ca^{2+} concentration from 0 to 3 min using the FLIPR system following the addition of mentioned compounds, and plotted as a function of the concentration of the peptide used. To measure $[Ca^{2+}]_i$ in HEK293 s cells stably expressing CCR1, cells were plated at 100×10^4 cells per well. Before incubating with test ligands, 0.75 mM carbachol (final concentration) was added for 3 min to prime the cells (without this priming CCR1 calcium responses were not detected). All graphical data in this work were calculated by nonlinear regression method using GraphPrism software, version 3.02.

Pertussis toxin (PTX) treatment

CHO-K1 cells transiently expressing FPRL-1 were treated for 6 h with 100 ng ml^{-1} of PTX in HAM's F12 modified medium (1 ×) with 2 mM glutamine and 10% foetal bovine serum (Wisent, Saint Bruno, QC, Canada). After the incubation period, cells were loaded with Fluo3-AM dye and pluronic acid as described above, and washed four times in HBSS buffer. To test the viability of CHO-K1 cells after PTX treatment, MCH1R was used as a positive control (Lembo *et al*., 1999; Hawes *et al*., 2000).

Cyclic AMP (cAMP) assays

CHO-K1 cells were transfected as above with FPRL-1 (or with a control GPCR) and plated in a P10 culture dish. Two days post-transfection, cells at 70–80% confluency (6×10^5 ml^{-1}) were detached using a PBS-based cell dissociation buffer (Invitrogen, Carlsbad, CA, U.S.A.). Cyclic AMP measurement was done using AlphaScreen™ cAMP assay kit (PerkinElmer Life Sciences, Montréal, QC, Canada) following the manufacturer's conditions. To stimulate cAMP production, forskolin was added at 10 μM (final concentration). The concentrations of ligands used were 10 pM to 200 nM for chemokines, 1 pM to 20 nM for W-peptide and 0.5 nM to 10 μM for other compounds. The percentage inhibition was calculated as follows: percentage inhibition $= 100 \times (F\text{-}F\text{:}A)/F$, where F is the amount of cAMP in the presence of forskolin and $F\text{:}A$ is the amount of cAMP in the presence of forskolin plus agonist.

Monoclonal FPRL-1 antibody

A monoclonal FPRL-1 antibody was prepared by genetic immunisation (Genovac AG, Freiburg, Germany). Monoclonal antibodies were screened for anti-FPRL-1 activity by flow cytometry and for receptor blocking activity using the FLIPR assay. For blocking studies, cells transiently or stably expressing FPRL-1 were incubated at 37°C for 1 h with antibody (6C7-3) at a concentration of 5 μg ml^{-1} (1 : 200 dilution) during the Fluo3-AM dye loading. After the incubation period, cells were washed and $[Ca^{2+}]_i$ was measured by FLIPR system.

Isolation of PMNs from human donor blood and calcium flux assays

PMNs were prepared from healthy human donor blood (approval by a local Ethics Committee), collected in 10 U ml^{-1} of heparin, by centrifugation through Polymorphprep (Robbins Scientific, Solihull, England). Cells were washed with PBS containing 0.2% (w v^{-1}) glucose and red blood cells were removed by two rounds of hypotonic lysis in ice-cold 0.2% (w v^{-1}) NaCl for 1 min. For calcium flux assays, PMNs were resuspended in assay buffer (137 mM NaCl, 11 mM Glucose, 10 mM HEPES, 2.7 mM KCl, 0.4 mM $K_2H_2PO_4$, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.1% BSA pH 7.4) containing 5 μM Fluo-3AM. Cells were incubated for 90 min at room temperature with gentle mixing. Dye-loaded cells were washed once and resuspended at 4×10^6 cells ml^{-1}. Calcium flux assays were performed using a FLIPR system, in 96-well FLIPR plates containing 2×10^5 cells per well.

Chemotaxis assay in PMNs

Migration assays were performed in 96-well ChemoTx™ microplates (Neuroprobe, Gaithersburg, MD, U.S.A.) in assay buffer (as mentioned above). Test compounds were added to the lower chamber, and 2×10^5 cells per well were placed on to the upper chamber. Following 2 h incubation at 37°C, filters were removed from the chambers, washed, fixed and migrated cells were stained with Alamar Blue™ (Serotec, Kidlington, England). Data are expressed as a percentage of cells migrating.

Radioligand binding assay

A volume of 250 μl of membrane preparation (0.6 mg ml^{-1}, with maximum binding (B_{max}) of 1.8 pmol mg^{-1} membrane protein) of CHO-K1 cells stably expressing FPRL-1 (PerkinElmer Life Sciences, Montréal, QC, Canada) were diluted in 7.5 ml of incubation buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5% BSA). The incubation time was 90 min at room temperature in the presence of 150 μl of diluted membrane preparation, 10 μl DMSO or unlabelled ligand from 10^{-5} to 10^{-15} M diluted in DMSO, and 10 μl of labelled ligand ([^{125}I]-WKYMVm at 0.05 nM (PerkinElmer Life Sciences)) diluted in incubation buffer. After incubation, unbound tracer was removed by filtration over GF/C harvest plates (Millipore, Nepean, ON, Canada), presoaked in 50 mM Tris-HCl, pH 7.4, then washed nine times with 500 μl of the same buffer (icecold). Bound label was counted using a TopCount® Microplate Scintillation and Luminescence Counter (PerkinElmer Life Sciences).

Results

Expression profile of FPRL-1

Preliminary studies on the distribution of FPRL-1 mRNA by Northern blot analyses demonstrated abundant levels in lung,

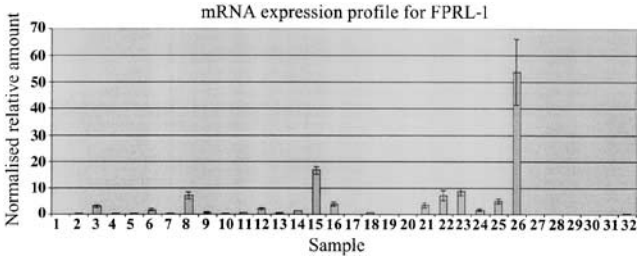

Figure 1 Quantitative reverse transcriptase-PCR analysis of human FPRL-1. RNA samples are derived from various human tissues and cells. FPRL-1 transcripts are detected in bone marrow, synovial tissues, lung, and in various blood cells, such as neutrophils and dendritic cells. Samples are: 1 brain, 2 heart, 3 lung, 4 liver, 5 trachea, 6 placenta, 7 colon, 8 bone marrow, 9 testis, 10 kidney, 11 thymus, 12 spleen, 13 skin, 14 adipose tissue, 15 immature dendritic cells (DC), 16 mature DC, 17 keratinocytes, 18 naïve T cells, 19 activated T cells 1, 20 activated T cells 2, 21 and 22 osteo-arthritis (OA) synovium, 23 rheumatoid-arthritis (RA) synovium, 24 and 25 peripheral blood (PB) monocytes, 26 PB neutrophils, 27 $CD4^+$ T cells, 28 $CD8^+$ T cell, 29 B cells, 30 Type 1 humoral (Th1) cells, 31 Type 2 humoral (Th2) cells, 32 eosinophils. Results are normalised to 18S ribosomal RNA and are presented as relative values. Results are from duplicate PCR reactions.

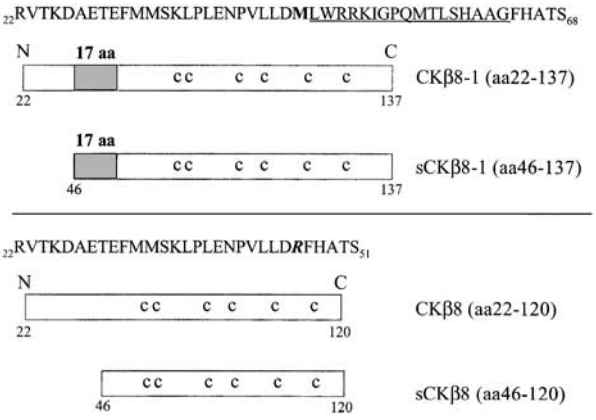

Figure 2 A schematic representation of full-length and truncated forms of CKβ8 and CKβ8-1 used in this study. The signal peptide is from 1 to 21 and is not depicted. The grey box represents the region of 17 amino acid (SHAAG peptide) absent in CKβ8, the corresponding sequence is represented above and underlined. sCKβ8-1 (46–137 aa) starts from methionine (shown in bold) at position 46, sCKβ8 (46–120 aa) starts from arginine (shown in bold and italic) at position 46. c: conserved cysteine residues.

placenta and tissues known to have a relatively high degree of phagocytic cell infiltrates, and neutrophils (Fiore *et al*., 1994; Le *et al*., 2000). A more detailed expression profile for FPRL-1 was performed, using quantitative-PCR, across different human tissues and blood cells (Figure 1). Of the various tissues tested, the highest relative mRNA levels were observed in bone marrow, synovial tissues and lung. From different blood cells tested, neutrophils and dendritic cells (immature, myeloid) appear to express the highest levels of FPRL-1 mRNA.

A truncated form of CKβ8-1 induces mobilisation of intracellular Ca^{2+} in FPRL-1 expressing cells

We initiated our search for additional FPRL-1 ligands by using a comprehensive collection of peptidic and nonpeptidic ligands obtained from commercial sources. The cellular assay used was based on the measurement of intracellular calcium ion release in cells coexpressing $G\alpha_{16}$ protein, a G-protein α subunit belonging to the Gq class, and FPRL-1. The overexpression of $G\alpha_{16}$ efficiently switches receptor signalling from $G\alpha_i/G\alpha_o$ or $G\alpha_s$ to the PLCβ pathway (Offermanns & Simon, 1995).

Of all the compounds tested (>1300), including more than 77 chemokines, an N-terminally truncated form of CKβ8-1, sCKβ8-1 (Figure 2), and W-peptide, a known FPRL-1 agonist, were the two most potent compounds to elicit a dose-dependent increase in the mobilisation of intracellular calcium response in CHO-K1 cells coexpressing $G\alpha_{16}$ protein and FPRL-1 (Figure 3a and Table 1). Compounds depicted in Figure 3a did not elicit responses in nontransfected CHO-K1 cells expressing either $G\alpha_{16}$ protein or other unrelated G-protein-coupled receptors (data not shown), suggesting the specificity of sCKβ8-1 for FPRL-1. Similar pEC_{50} values were obtained in $G\alpha_{16}$ and FPRL-1 transfected-HEK293s cells (Table 1). Moreover, in the absence of $G\alpha_{16}$, similar calcium mobilisation responses were observed with sCKβ8-1 and W-peptide in CHO-K1 cells transiently expressing FPRL-1 (data not shown). Interestingly, the short form of CKβ8 (46–120 aa), and long form of CKβ8 (22–120 aa), and of CKβ8-1 (22–137 aa) displayed low potency (pEC_{50} <5.7) at FPRL-1 or were inactive (Figure 3b and Table 1). These results suggested that the structural determinants of CKβ8-1 specificity for FPRL-1 might be the 17-amino-acid peptide at the N-terminus, since the remaining sequence of the molecule is identical to CKβ8. To explore this hypothesis, we synthesised the 17-amino acid stretch peptide (Figure 2), named SHAAG peptide ($_{47}$LWRRKIGPQMTLSHAAG$_{63}$) and determined its potency in cells coexpressing $G\alpha_{16}$ and FPRL-1. The SHAAG peptide was ~60–200 times less potent at FPRL-1 as compared to sCKβ8-1, but ~120 times more potent than long form CKβ8-1 (Figure 3b and Table 1). In CHO-K1 and HEK293s cells, coexpressing $G\alpha_{16}$ and FPRL-1, other known FPRL-1 ligands (i.e. $A\beta_{42}$, SAA, and hPrP) were ~200 to over 1000-fold less potent at FPRL-1 than sCKβ8-1, and are in agreement with published results (Le *et al*., 2002), whereas, the LXA_4 observed potency for FPRL-1 was low (pEC_{50} <6). To eliminate the possibility that the low potency displayed by the full-length recombinant CKβ8-1 at FPRL-1 is not due to a misfolding during synthesis and/or degradation during purification process, we measured $[Ca^{2+}]_i$ release in cells stably expressing CCR1, and confirmed the biological activity of the samples used (Figure 3c). RANTES, a CCR1 agonist, produced a pEC_{50} value of 9.57 ± 0.06 ($n=4$), which is in agreement with published results (Chou *et al*., 2002). The rank order of potency of CKβ8, CKβ8-1 and of the N-terminally truncated forms at inducing calcium flux *via* CCR1 was as follows: sCKβ8 (46–120 aa) > CKβ8 (22–120 aa) > sCKβ8-1 (46–137 aa) >CKβ8-1 (22–137 aa) (Figure 3c). Hence, the potency of the long form CKβ8-1 is ~200–300-fold lower at FPRL-1 than, at the CCR1 receptor.

FPRL-1 is a $G\alpha_i/G\alpha_o$-coupled receptor

To determine which Gα protein was involved in the stimulation of PLCβ by the activated human FPRL-1, CHO-K1 cells transiently expressing FPRL-1, in the absence of $G\alpha_{16}$ protein, were pretreated with PTX. This pretreatment abolished the calcium responses mediated by W-peptide and sCKβ8-1,

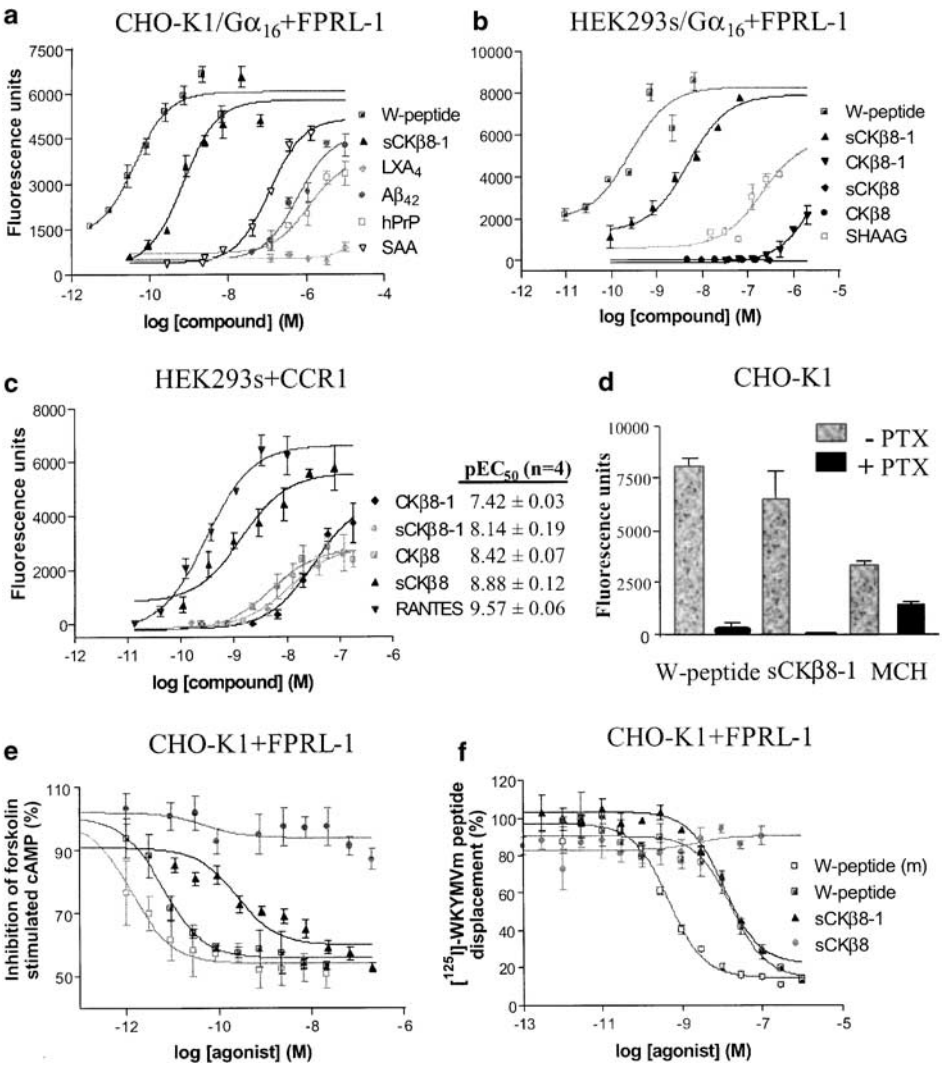

Figure 3 sCKβ8-1 activates and binds to FPRL-1 with high potency, and is coupled to $G\alpha_i/G\alpha_o$ protein. (a) Dose–response curves of the $[Ca^{2+}]_i$ changes evoked in the presence of W-peptide, sCKβ8-1, LXA_4, $A\beta_{42}$, hPrP, SAA, in CHO-K1 cells coexpressing $G\alpha_{16}$ protein and FPRL-1. (b) $[Ca^{2+}]_i$ mobilisation in HEK293s cells coexpressing $G\alpha_{16}$ and FPRL-1, in the presence of full-length CKβ8 (22–120 aa), CKβ8-1 (aa22-137), sCKβ8 (46–120 aa), sCKβ8-1 (46–137 aa) and the 17-aa SHAAG peptide. Representative data (from $n=3$) are shown. (c) CKβ8-1 (22–137 aa) is active on CCR1 stably expressed in HEK293s cells ($n=4$), pEC_{50} values are summarised. (d) $[Ca^{2+}]_i$ mobilisation was measured after 1 h PTX (100 ng ml^{-1}) treatment, in CHO-K1 cells transiently expressing FPRL-1. PTX blocked (>95%) calcium mobilisation induced by sCKβ8-1 (66 nM) or W-peptide (6.6 nM). In CHO-K1 cells transiently expressing MCH1R, PTX partially inhibited MCH-induced (1 μM) calcium mobilisation response. (e) Dose-dependent inhibition of forskolin-stimulated cAMP accumulation in CHO-K1 cells transiently expressing FPRL-1 with sCKβ8-1. Data are from a representative experiment (from $n=3$–4) and normalised to the amount of cAMP produced in forskolin-stimulated cells (set to 100%). (f) Dose-dependent displacement of [^{125}I]-WKYMVm by sCKβ8-1. Membrane preparations from CHO-K1 cells stably expressing FPRL-1 were incubated with [^{125}I]-WKYMVm (0.05 nM) in the absence or presence of indicated unlabelled ligands (from 10^{-5} to 10^{-15} M). Each point was determined in triplicate, and data are from a representative experiment ($n=3$). Results are presented as a percentage of displaced [^{125}I]-WKYMVm. pEC_{50} and pIC_{50} values are given as mean$\pm$s.e.m. Each point is determined in triplicate.

suggesting the involvement of $G\alpha_i/G\alpha_o$ protein and not $G\alpha_q$ in this pathway (Figure 3d) ($n=3$). As a control, CHO-K1 cells transiently expressing MCH1R were treated with PTX and incubated with melanin-concentrating hormone (MCH). In accordance to a proposed dual coupling ($G\alpha_i/G\alpha_q$) mechanism for this receptor (Hawes *et al*., 2000), PTX treatment partially ($\sim$50%) inhibited MCH-induced calcium mobilisation response (Figure 3d).

To further demonstrate the involvement of $G\alpha_i/G\alpha_o$ protein in FPRL-1 signalling pathway, the inhibition of forskolin-stimulated cAMP accumulation in CHO-K1 cells was assessed. sCKβ8-1 alone, failed to inhibit basal cAMP levels (data not shown) but did inhibit, in a dose-dependent manner, the forskolin-stimulated cAMP accumulation ($n=4$) (Figure 3e and Table 1). Previous studies have shown that both forms of the synthetic W-peptide (WKYMVM and WKYMVm) elicited a release of intracellular calcium in cells expressing FPRL-1 with EC_{50} values of 2 nM and 25 pM, respectively (Christophe *et al*., 2001). The pIC_{50} values for W-peptide (10.38 ± 0.38) ($n=4$) and its isoform (11.87; 12.19) for inhibition of forskolin-stimulated cAMP accumulation are in accordance with published data (Christophe *et al*., 2001). Nontransfected

Table 1 pEC_{50} and pIC_{50} values of sCKβ8-1 and known FPRL-1 ligands in various functional assays

Compound	*Intracellular Ca^{2+} mobilisation pEC_{50} ($n=3$)*		*Adenylyl cyclase pIC_{50}*	*[^{125}I]-W-peptide (WKYMVm) displacement, pIC_{50} (n = 3)*
	CHO-K1 cells	*HEK293s cells*	*CHO-K1 cells*	*CHO-K1 cells*
sCKβ8-1 (aa46-137)	9.13±0.02	8.85±0.07	9.02±0.20 ($n=4$)	7.97±0.04
SCKβ8 (aa46-120)	<5.0	Inactive	Inactive ($n=2$)	Inactive
CKβ8-1 (aa22-137)	<5.7	<5.7	n.t.	n.t.
CKβ8 (aa22-120)	<5.0	Inactive	n.t.	n.t.
SHAAG peptide	6.74±0.23	7.15±0.23	n.t.	n.t.
Amyloid β protein (Aβ_{42})	6.09±0.25	<6.0	6.76; 5.90 ($n=2$)	Inactive
Serum amyloid A protein (SAA)	6.88±0.07	<6.0	6.38; 6.48 ($n=2$)	<5.52
Lipoxin A_4 (LXA_4)	<6.0	<6.0	<5.7 ($n=2$)	Inactive
Human prion protein (hPrP)	<6.0	<6.0	Inactive ($n=2$)	Inactive
W-peptide (WKYMVM)	10.68±0.25	9.56±0.18	10.38±0.38	7.67±0.06
W-peptide (WKYMVm)	n.t.	n.t.	11.87; 12.19 ($n=2$)	9.34±0.08 (K_d)

The pEC_{50} or pIC_{50} values are given as mean±s.e.m., and were calculated as −log of the EC_{50} or −log of the IC_{50} values (50% of the maximal compound effect). n.t.: not tested. The K_d value for WKYMVm was obtained from saturation binding experiments.

CHO-K1 cells, or CHO-K1 cells expressing an unrelated GPCR were treated with the same range of agonist concentrations and exhibited no inhibition of cAMP accumulation (data not shown). Aβ_{42} and SAA, also dose-dependently inhibited forskolin-stimulated cAMP accumulation with pIC_{50} <6.7, while sCKβ8 and other ligands were found to be weakly active (pIC_{50} <5.7) or inactive (Table 1). Similar pIC_{50} values were obtained for sCKβ8-1 and W-peptide in CHO-K1 cells stably expressing FPRL-1 (not shown).

Efficient displacement of [^{125}I]-W-peptide by sCKβ8-1

To characterise the binding properties of sCKβ8-1, membranes prepared from CHO-K1 cells stably expressing FPRL-1 were incubated with the selective FPRL-1 ligand [^{125}I]-WKYMVm (Christophe *et al*., 2001). The binding was specific and saturable for FPRL-1 using W-peptide (data not shown). Concentrations of unlabelled ligands used to displace [^{125}I]-WKYMVm, were from 0.001 pM to 100 nM for chemokines (sCKβ8-1, sCKβ8), W-peptide and its isoform, and from 1 pM to 10 μM for Aβ_{42}, SAA, LXA_4 and hPrP (106-126). The observed K_d value for WKYMVm was 9.34±0.08, and sCKβ8-1 was found to be the most effective, nonsynthetic, agonist at competitively displacing [^{125}I]-WKYMVm (Figure 3f and Table 1), this was followed by SAA with a pIC_{50} value of <5.52. In agreement with the low potency values observed in the calcium mobilisation assay, other tested compounds did not displace the labelled ligand. Collectively, the data presented clearly demonstrate the ability of sCKβ8-1 to bind and activate FPRL-1 receptor with high efficacy and potency.

sCKβ8-1 induces calcium flux and chemotaxis in polymorphonuclear leukocytes (PMNs)

Neutrophils play a pivotal role in the innate immune response to infection (Ye & Boulay, 1997). Since these cells express FPRL-1, we evaluated the effect of sCKβ8-1 on PMNs calcium mobilisation. In neutrophils, $[Ca^{2+}]_i$ increase by PLCβ activation was shown to signal chemotaxis, whereas, high cellular concentrations of cAMP to block that migration (Lang *et al*., 2003). This is in accordance with FPRL-1 signalling ($G\alpha_i/G\alpha_q$) pathway (Figures 3d, e). As shown in Figure 4a, sCKβ8-1 elicited a dose-dependent increase in the mobilisation of $[Ca^{2+}]_i$. The rank order of potency for the various FPRL-1 ligands in PMNs was as follows: W-peptide >sCKβ8-1≥MMK-1. Interleukin-8 (IL-8), known to activate CXCR1 and CXCR2 receptors, induced a dose-dependent calcium response indicating the integrity of the PMNs preparation (Doroshenko *et al*., 2002).

The physiological relevance of sCKβ8-1 as a ligand for FPRL-1 was assessed by PMNs chemotaxis experiments. sCKβ8-1, MMK-1 and W-peptide (WKYMVm) induced the migration of PMNs at concentrations ranging from 1 pM to 20 μM. The maximum percentage of cell migration produced by sCKβ8-1 was reached at 1 μM, 12 μM with MMK-1 and 100 nM with W-peptide (Figure 4b).

The cell migration data demonstrates the ability of sCKβ8-1 to activate human PMNs and suggests that this activity is mediated *via* FPRL-1 receptor endogenously expressed in these cells. To demonstrate the specificity of sCKβ8-1 for FPRL-1, human PMNs were pre-treated in the presence or absence of monoclonal FPRL-1 antibody, and calcium mobilisation in response to sCKβ8-1 was measured. In PMNs, antibody pre-treatment reduced the $[Ca^{2+}]_i$ mobilisation by 80–90% when incubated with sCKβ8-1 (Figure 4c, dashed lines). Similar responses were obtained in HEK293s cells stably coexpressing $G\alpha_{16}$ and FPRL-1 (Figure 4d). Interestingly, the antibody pretreatment had no effect on ATP-mediated calcium response in these cells (Figure 4e), thus demonstrating the specific blockage of FPRL-1-mediated calcium mobilisation. Collectively, the data confirm the effect produced by sCKβ8-1 in human PMNs is mediated by FPRL-1.

Discussion

In the present study, we have identified a truncated form of the chemokine CKβ8-1 (sCKβ8-1) as a potent agonist for human FPRL-1, capable of inducing the release of intracellular calcium and inhibiting forskolin-stimulated cAMP accumulation in a PTX-sensitive manner. Moreover, sCKβ8-1 mediated a calcium flux and chemotactic activity in human PMNs *via* FPRL-1 suggesting a physiological role for this β-chemokine.

Lipoxins are members of the eicosanoid family of bioactive lipid mediators generated during cell – cell interactions (Maddox *et al*., 1997), and Lipoxin A_4 was the first reported

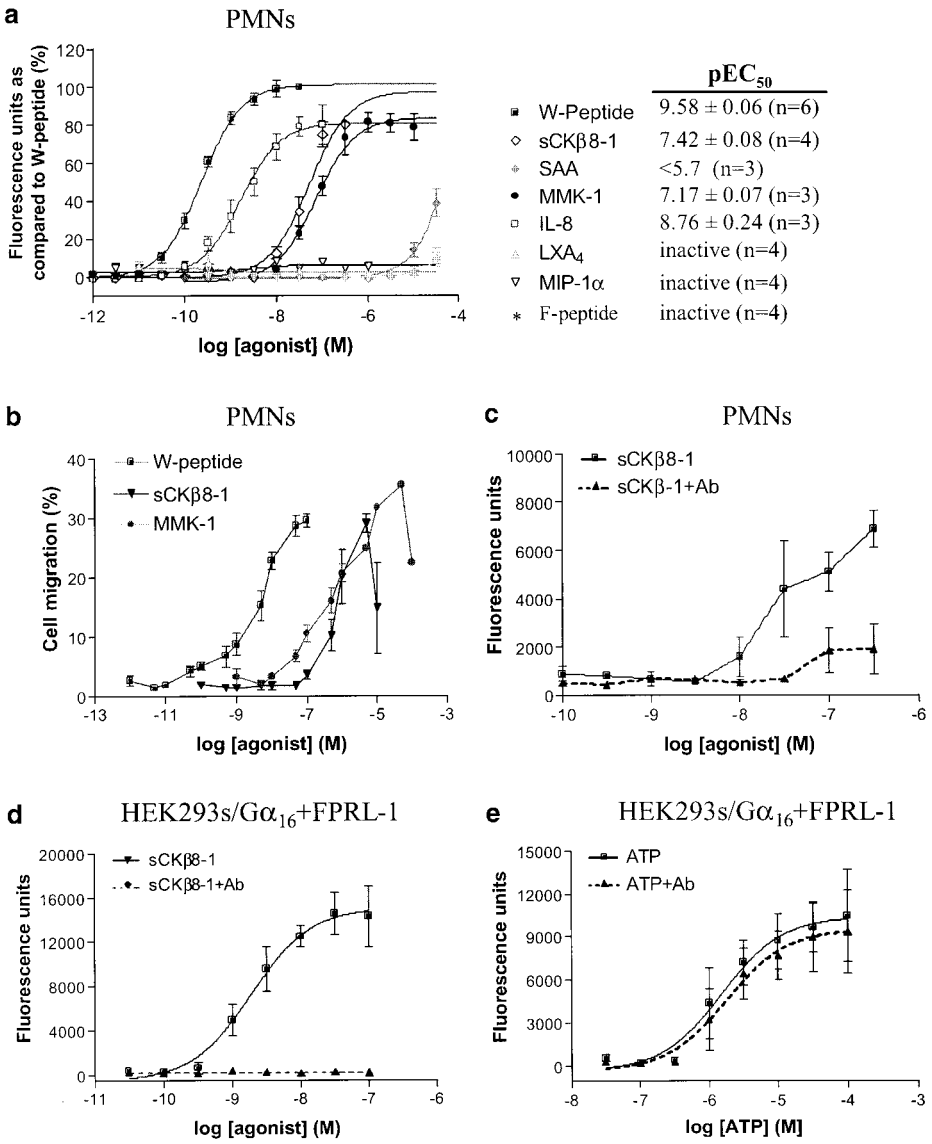

Figure 4 sCKβ8-1 induces calcium flux and chemotaxis in human PMNs through FPRL-1. (a) $[Ca^{2+}]_i$ mobilisation in PMNs was measured in response to ligands shown. Data was normalised to the maximum response of W-peptide (WKYMVm) in each donor ($n=3$–6), and pEC_{50} values are given as mean$\pm$s.e.m. (b) Chemotaxis. Human PMNs were exposed to sCKβ8-1, MMK-1 and W-peptide (WKYMVm) at indicated concentrations. W-peptide and sCKβ8-1 were tested on four donors, and MMK-1 on two donors. Chemotaxis is expressed as the percentage of total cells migrating from the upper chamber through the filter. (c) Human PMNs were preincubated with (dashed lines), or without (solid lines) monoclonal FPRL-1 antibody, and calcium mobilisation in response to sCKβ8-1 was measured. (d) $[Ca^{2+}]_i$ mobilisation response in HEK293s cells stably coexpressing FPRL-1 and Gα_{16}, preincubated with FPRL-1 antibody (or not), and activated in the presence of sCKβ8-1 or (e) ATP, at indicated concentrations. In PMNs, the absolute potency of all agonists was found to be variable between donors, therefore the results shown are a representative experiment from one donor (each point was determined in triplicate, except for (c) in duplicate).

FPRL-1 agonist (Fiore *et al*., 1994; Fiore & Serhan, 1995). The binding of [^{3}H]-LXA_4 to FPRL-1 has been studied on neutrophils and on membranes expressing FPRL-1 ($K_d=6.1$ nM) (Fiore *et al*., 1994; Fiore & Serhan, 1995; Maddox *et al*., 1997). The activation of FPRL-1 by LXA_4 was shown to release arachidonate *via* phospholipase A2 and phospholipase D pathways (Fiore *et al*., 1994; Fiore & Serhan, 1995). In various functional assays, we observed that potencies for some putative endogenous ligands were considerably lower than sCKβ8-1. However, the pEC_{50} values we obtained with these endogenous ligands are in close agreement with published data (Le *et al*., 2002). In our experimental conditions, LXA_4 had no effect on the phospholipase C signalling pathway, nor did it inhibit the forskolin-stimulated cAMP accumulation. Moreover, LXA_4 did not competitively displace [^{125}I]-W-peptide on CHO-K1 cells expressing FPRL-1, nor induce calcium mobilisation in PMNs, at concentrations up to 10 μM. The discrepancy between our data and the reported potency values for LXA_4 (Fiore *et al*., 1994; Fiore & Serhan, 1995; Le *et al*., 2002) may be explained by different experimental conditions: (i) intracellular calcium flux measurement activated by phospholipase C pathway compared to arachidonate release measurement, and (ii) a quite selective radiolabelled FPRL-1 agonist (W-peptide

isoform) (Christophe *et al*., 2001) for displacement studies, as compared to [3 H]-LXA_4 displacement (Fiore *et al*., 1994; Fiore & Serhan, 1995; Chiang *et al*., 2000). In a recent study using neutrophils, which express FPR and FPRL-1, LXA_4 failed to generate any agonist-induced biological responses mediated by W-peptide, such as NADPH-oxidase activation and increase of the CR3 granules, an indicator of neutrophil cells secretory machinery (Christophe *et al*., 2002). Hence, the authors concluded that LXA_4 most likely activated and signalled its effects *via* a receptor different from FPRL-1. It is also possible to conceive that LXA_4 may be recognising other entities expressed on cells, and this interaction may somehow depend or be synergised with the expression of FPRL-1.

The mRNA expression profile for FPRL-1 revealed that the receptor is predominantly distributed in lung, bone marrow, synovial tissues, and in various cell types such as monocytes and neutrophils. The FPRL-1 mRNA expression pattern in inflammatory tissues and cells suggested the possibility this receptor could be activated by a chemokine. CKβ8 and CKβ8-1 mRNAs are abundantly present in pancreas, and at lower extent in skeletal muscle and liver (Youn *et al*., 1998). Immunocytochemistry studies demonstrated the presence of CKβ8 (and most probably CKβ8-1) in preosteoblasts (Votta *et al*., 2000). Interestingly, CKβ8 displays chemotactic activity for osteoclast precursors isolated from human osteoclastoma tissues, monocytes and resting lymphocytes, suggesting a possible role for FPRL-1 in proinflammatory and immune reactions (Forssmann *et al*., 1997). In this regard, we have demonstrated that sCKβ8-1 activates human PMNs and induces chemotaxis. A possible link between high pancreatic CKβ8-1 expression (Youn *et al*., 1998) and FPRL-1 activation may be inflammatory diseases, such as the acute pancreatitis, mediated through the overexpression and secretion of the pancreatitis-associated protein (PAP). In rat, PAP injection led to neutrophil recruitment and inflammatory reactions in lung (Folch-Puy *et al*., 2003). Moreover, in pancreas, the β cells and periacinar myofibroblasts were shown to produce and secrete biologically active chemokines (i.e. MCP-1, RANTES, IL-8, MIP-1α) (Piemonti *et al*., 2002). In this context, since FPRL-1 is expressed in the lung, the possible role of PAP and other proinflammatory stimuli on the expression and secretion of CKβ8-1 from pancreas remains to be investigated.

Youn and colleagues have demonstrated the ability of long-form CKβ8 and CKβ8-1 to induce a rapid release of intracellular calcium *via* CCR1 in transfected HOS cells, and to trigger chemoattraction in neutrophils (Youn *et al*., 1998). Surprisingly, they found that MIP-1α, a known CCR1 and CCR5 agonist, induced robust Ca^{2+} mobilisation in neutrophils but did not provoke chemotaxis (McColl *et al*., 1993). In the present work however, MIP-1α did not mobilise $[Ca^{2+}]_i$ in PMNs, suggesting variable CCR1 and/or CCR5 expression levels. This notion is supported by reports suggesting the variable expression or existence of CCRs (i.e. CCR1) in resting human neutrophils (Cheng *et al*., 2001). We discovered that the sCKβ8-1-driven calcium flux in PMNs was blocked using a monoclonal FPRL-1 antibody, indicating that in PMNs the sCKβ8-1 response is mediated *via* FPRL-1.

In this study, we demonstrated the potent activity of a truncated form of CKβ8-1 for FPRL1. Albeit, we have not demonstrated the processing of sCKβ8-1 however, in the literature post-translational modifications including NH_2-terminal truncation of chemokines have been shown to affect their biochemical and biological characteristics (Proost *et al*., 1998). Hemofiltrate CC chemokine 1 (HCC-1), lacking the first eight amino acids was isolated from tumor cell lines (Vakili *et al*., 2001). The full-length HCC-1 was shown to be a weak agonist for CCR1 and was inactive at CCR5, however, HCC-1[9–74 aa] was characterised as a potent agonist for CCR1, CCR3, CCR5 (Vakili *et al*., 2001). Similar observations have been made for IL-8 and MIP-1β where the alternatively processed forms elicited different potencies on several chemokine receptors (Hebert *et al*., 1990). As a final note, the expression of Lkn-1 and CKβ8 in insect cells, two chemokines closely related to CKβ8-1 (Youn *et al*., 1998), resulted in the synthesis and secretion of N-terminally processed variants lacking the first 24 amino acids (sCKβ8) (Macphee *et al*., 1998; Lee *et al*., 2002). These deletions increased the potency $\sim$100-fold for CCR1 (Macphee *et al*., 1998; Berkhout *et al*., 2000; Lee *et al*., 2002). Interestingly, we have confirmed that the truncated forms of CKβ8 and CKβ8-1 have enhanced potencies at CCR1, and the full-length CKβ8-1 displayed a low potency at activating human FPRL-1. However, sCKβ8-1 was $\sim$2000 times more potent for this receptor. An integral part of the specificity of sCKβ8-1 at activating FPRL-1 is dependent on the 17-amino-acid region (SHAAG peptide), which is absent in the alternatively spliced variant CKβ8. The data, together with previously published results on Lkn-1, CKβ8 and other CC chemokines (i.e. HCC1, MCP-1, MCP-2, MIP-1β), suggest that the processing of the N-terminus of some members of β-chemokines, including CKβ8-1, may represent a novel mechanism to increase the diversity of inflammatory effects inherent to these ligands.

In conclusion, we have identified the N-terminally truncated form of CKβ8-1, as a highly potent ligand of human FPRL-1. Given their broad chemotactic specificities, β-chemokines may play a central role in development and maintenance of the leukocyte infiltration found in many diseases, such as allergic inflammation, arthritis, nephritis and experimental autoimmune encephalomyelitis (Ye & Boulay, 1997). Our discovery could have interesting implications for the development of anti-inflammatory therapies by developing selective antagonists to FPRL-1.

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