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Cloning and characterization of guinea pig interleukin-8 receptor

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Abstract

CXC-chemokine receptors 1 and 2 and their ligands (CXCL1, 2, 3, 5, 6, 7, and 8) induce the selective recruitment of neutrophils during inflammation. Such receptors have not been characterized yet in guinea pig, an animal inflammation model of interest. We report the identification, cloning, and characterization of a CXCL8 receptor in guinea pig. Human CXCL8 produced *in vivo* neutrophilia, chemotaxis and intracellular calcium release of guinea pig neutrophils. The expression of this receptor at their neutrophil surface was investigated. The cDNA encoding a functional CXCL8 receptor was cloned from guinea pig neutrophils and sequenced. It was synthesized using RT-PCR, with oligonucleotide primers derived from well conserved regions of published CXCL8 receptors. This sequence presented an open reading frame coding for 352 amino acids and shares, at the amino acid level, 70 and 69% identity with human and rabbit CXCR2, respectively. The receptor was mainly expressed in neutrophils but it was also present in kidney, lung, spleen and, to a less extent, in heart. Cloned receptor transfected cells showed that this receptor displayed high affinity for human CXCL8, slightly lower than the affinity observed with guinea pig neutrophils. CXC chemokines from both rabbit and human were shown to induce inositol phosphate accumulation in these transfected cells. Receptor binding and activation characteristics together with sequence homology suggested that we identified a guinea pig equivalent of the human CXCR2 receptor.
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Keywords: Guinea pig; Cloning; Chemokine; G-protein coupled receptor; CXCL1; CXCL8

1. Introduction

Interleukin-8 (IL-8 or CXCL8 [1]) is a well characterized chemotactic cytokine (chemokine). Chemokines are a family of over 40 small cytokines of about 8 kDa, which have first been isolated by their ability to induce chemotactism of leukocytes. They are implicated in a wide range of pathologies: inflammation, viral infection, cardiovascular diseases, etc. [2–4]. Chemokines have been classified in four sub-families depending on the number and spacing of the first conserved cysteine residues in the NH_2 terminus region: CXC (to which belongs CXCL8), CC, XC, and CX_3C. These different sub-families act on different receptors as well as different cell types. The chemokine receptors belong to the G-protein coupled receptors superfamily and show a global conserved conformation: seven transmembrane domains, an extracellular NH_2 terminal region, an intracellular COOH terminal region and three extracellular loops as well as three intracellular loops. Nineteen human chemokine receptors (6 CXC, 11 CC, 1 XC, and 1 CX_3C) have been cloned so far. CXC chemokines, like CXCL8 preferentially attract and activate neutrophils. CXCL8 has been shown to be implicated in chronic inflammatory diseases including psoriasis, rheumatoid arthritis, chronic obstructive pulmonary disease (COPD) and acute respiratory distress syndrome (ARDS). CXCL8 activates human neutrophils by binding with high affinity to two receptors, CXCR1 and CXCR2. It shares both its receptors with granulocyte chemotactic peptide 2 (CXCL6) and CXCR2, with growth-related oncogen-α, -β, -γ and epithelial cell-derived neutrophil attractant-78 (CXCL1, 2, 3, and 5, respectively). Their binding on neutrophils results in cellular shape change, transient

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Abbreviations: ARDS, acute respiratory distress syndrome; COPD, chronic obstructive pulmonary disease; CXCL1, growth-related oncogen-α; CXCL8, interleukin-8; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; HEPES, 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; HBSS, Hank's Balanced Salt Solution; HSA, human serum albumin; RT, reverse transcription.

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increase in intracellular calcium and up regulation of adhesion surface proteins. Experiments have shown that blocking antibodies against CXCR2 or non-peptide antagonist of CXCR2 have promising therapeutic potential in the treatment of chronic inflammatory diseases [5–7]. Mouse and rat have a quite distant CXCL8 system; they possess a chemokine, KC, showing intermediate biological properties and amino acid sequence similarity between CXCL8 and CXCL1 [8]. It seems that rodents have a unique receptor for these chemokines with homology both with human CXCR1 and CXCR2. Neutrophils from mice lacking the murine CXCL8 receptor homologue gene fail to migrate in response to the CXC chemokines [9] and calcium desensitization experiments suggest a similar situation in rats [10]. The guinea pig has been extensively used as an *in vivo* model of skin inflammation [11] and pulmonary airway hyper-reactivity [12]. Receptors for CXCL8 have not been characterized in guinea pig so far. We have, therefore, identified and characterized such receptor(s).

2. Materials and methods

Studies were performed with Dunkee Hartley guinea pigs (IFA-CREDO). Care of guinea pigs was according to institutional guidelines. They were housed in conventional conditions, under 12 h/12 h dark/light cycles. Water and food were available *ad libitum*. Experiments were conducted in accordance with the ethical animal experimentation committee at Laboratoire Fournier.

2.1. Inhibition of neutrophils sequestration in vivo

The right carotid artery and the left jugular vein of guinea pigs were cannulated under general anesthesia (ketamine 90 mg/kg, xylasine 15 mg/kg), to allow for the intravenous infusion of human CXCL8 and blood sampling. Neutropenia and neutrophilia were induced by an infusion of three doses of human recombinant CXCL8 (15, 50, and 100 ng/kg/min). Blood samples were always obtained from carotid artery at selected intervals, over 3 hr after infusion of human CXCL8. The white blood cell ratio was counted with a Coulter counter (ZBI/Coultroniso) and the percentage of polymorphonuclear leukocyte change was normalized in reference to the baseline value.

2.2. Isolation of guinea pig neutrophils

While animals were under general anesthesia (ketamine 90 mg/kg, xylasine 15 mg/kg), peritoneal neutrophils were harvested by peritoneal lavage with 5 mL of PBS (Life Technologies, Invitrogen Corporation), 12 hr after intraperitoneal injection of 100 ml/kg of 1% casein solution (w/v) in PBS. After red blood cell lysis, neutrophils were counted in a hemocytometer.

2.3. Neutrophils chemotaxis assay

Guinea pig neutrophils were washed in HBSS. Cells were suspended at a concentration of 5×10^5 cells in 50 μL migration buffer (HBSS, supplemented with 0.1% HSA (w/v)) and were loaded on the top filter of a Boyden micro-chamber (NeuroProbe). Migrated cells, after 90 min at 37° through 5 μm pore size polycarbonate membranes (Nuclepore), were stained with Hemacolor solutions (Merck) and counted. fMLP was used as a positive control. Data were expressed as chemotactic indexes, which represent the ratio of the number of cells (observed at a 500× magnification) migrated in response to the studied chemoattractant with the number of cells that spontaneously migrated in presence of the dilution buffer.

2.4. Measurement of cytosolic calcium in purified guinea pig neutrophils

Neutrophils were washed in HBSS supplemented with 2 mM Ca^{2+}, 1 mM Mg^{2+}, and 10 mM HEPES; cells were suspended (10^7 mL^{-1}) in the same buffer containing 5 μM of Fura2-acetoxymethylester (Molecular Probes, Interchim) and incubated for 45 min at 37° in the dark. The cells were then diluted in the same buffer (with a ratio of 1:5), cells effluxes were allowed to occur for 15 min at 37° in the dark. Then, the cells were washed twice in the same buffer and suspended at a final concentration of 10^6 mL^{-1}. Intracellular Ca^{2+} release was measured in a continuously stirred cuvette at a volume of 1 mL and room temperature. Fluorescence data acquisition was performed in a LS50B spectrofluorometer (Perkin-Elmer) with excitation wavelengths of 340 and 380 nm and an emission wavelength of 450 nm, according to Grynkiewicz *et al.* [13].

2.5. cDNA synthesis

Total RNAs were isolated from guinea pig blood leukocytes using SV RNA isolation system (Promega). Blood was taken from the carotid artery and collected on EDTA (10 mM). Red blood cells were lysed by SV RNA Red Blood Cell Lysis (Promega). Leukocytes were isolated by centrifugation for 10 min at 800 *g* and washed in HBSS. Using oligo(dT) primer (50 pmol/μL), cDNA was synthesized from 1 μg of total RNA using Super Script II Reverse Transcriptase (Life Technologies, Invitrogen Corporation).

2.6. Cloning the open reading frame of a guinea pig CXCL8 receptor

Two oligonucleotides were designed from conserved regions between published mammalian CXCL8 receptors. The alignment was done with CLUSTAL W software (http://bioweb.pasteur.fr/seqanal/tmp/clustalw) using GenBank sequences : rabbit CXCR1 (M74240), rat CXCR1

(U70988), human CXCR2 (M73969), rabbit CXCR2 (L24445) and mouse CXCR1/2 (L23637). The oligonucleotides were used as primers for the amplification of the internal region of a putative guinea pig CXCL8 receptor gene. The oligonucleotide sequences were as follows: *sense gpA*: 5′-GTC TAC CTG CTG AAC CTG GCC AT-3′, *antisense gpB*: 5′-CGG TGC TTC TGC CCC ATG TGG GCC T-3′. The PCR was done with Platinum Taq DNA polymerase (Invitrogen Corporation). Cycling parameters were: 95° for 1 min; 35 cycles of 95° for 1 min; 60° for 30 s; and 72° for 30 s; and 72° for 10 min. The amplified cDNA was isolated on agarose, subcloned in pGemT easy vector (Promega), and sequenced using the Sequitherm EXCEL II kit-LC (Epicentre Technologies) and an automated DNA sequencer (model 4000, LI-COR). The whole sequence of the new identified receptor was isolated using 3′ and 5′RACE System for Rapid Amplification of cDNA Ends (Life Technologies, Invitrogen Corporation) according to manufacturer instructions with a Gene Specific Primer (GSP3′ for 3′RACE-PCR and GSP5′, its complementary sequence, for 5′RACE-PCR), matching with the middle of the sequence previously amplified with gpA and gpB. This sequence was more specific for a putative receptor than gpA and gpB, which were designed from conserved sequences: GSP3′: 5′-GTA GCA CTG TCT ACC TGC TCG, GSP5′: 5′-CGA GCA GGT AGA CAG TGC TAC-3′. Finally, the amplified sequences allowed for design of two primers located at both extremities of the coding sequence of a putative gene in order to amplify the whole sequence: sense GP5: 5′-G**GA ATT CC**A TCC TGG CAA GAC ATA AGC TTA CCT G-3′ and antisense GP3: 5′-GC**T CTA GA**A AAG GAG AAA CCC AAG AGC CTA CAA C-3′. Nucleotides in boldface indicate restriction enzyme sequences, respectively *Eco*RI and *Xba*I, used to directionally insert the amplified sequence in the vector pcDNA-3.1 (Invitrogen Corporation). After insertion in this vector, the sequence of different clones were sequenced as previously described.

2.7. Analysis of receptor tissue expression

To determine the tissue specific expression of the receptor, total RNAs were isolated, in presence of DNAse, from different frozen tissues: lung, heart, liver, spleen, kidney, brain, bone marrow, and skeletal muscle (SV Total RNA Isolation System, Promega). The complementary DNAs (cDNAs) produced by reverse transcription (RT) of RNA were quantified by real-time PCR performed on iCycler (Bio Rad) with a One-Step Quantitative SYBR Green RT-PCR kit (Qiagen). Briefly, RT-PCR was processed on 200 ng of RNA with 300 nM of primers designed with Beacon Designer 2.0 Software (Biosoft International). According to manufacturer instructions, the thermal cycling conditions consisted of a RT stage (50°, 30 min), an initial activation of HotStart Taq DNA polymerase (95°, 15 min) immediately followed by 40 cycles of PCR amplification (denaturation: 94°, 15 s; annealing: 60°, 30 s; extension: 72°, 60 s). The quantity of RNA amplified in each samples has been normalized in reference to a control band of a housekeeping gene (18*S* mitochondrial rRNA). Quantifications have been performed according to comparative C_T method, with C_T, the cycle number at which an increase above background fluorescence could be reliably detected and where the amount of RNA was evaluated by normalization to an endogenous reference by $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_T = \Delta C_{T,q} - \Delta C_{T,cb}$, where $\Delta C_{T,q}$ is the difference in threshold cycles for target and reference, and $\Delta C_{T,cb}$ is the difference in threshold cycles for the internal calibrator and its reference, calibrator is CXCL8 receptor expression in blood neutrophils.

2.8. Cell transfection

COS-7 cells (ATCC: CRL1651) were cultured in Dulbecco's modified essential medium, containing 4.5 g/L glucose and supplemented with 10% (v/v) fetal bovine serum (Hyclone), 1% (v/v) non-essential amino acids, 1% (v/v) glutamate, 1% (v/v) sodium pyruvate (Life Technologies, Invitrogen Corporation). 2×10^5 cells were transfected with 1 μg plasmid using jetPEI (Polytransfection) according to the manufacturer's recommended procedure with a DNA/transfection agent ratio of 5.

2.9. [^{125}I]CXCL8 binding assay

To determine the ligand binding selectivity, 10^6 freshly prepared neutrophils were incubated in duplicate in 250 μL PBS containing 0.1% (w/v) BSA (Sigma) and a final concentration of 0.15 nM of ^{125}I ligand and varying concentrations of unlabeled chemokines (from 10^{-7} to 10^{-12} M) or SB 225002, a selective CXCR2 non-peptide antagonist (from 10^{-4} to 10^{-9} M) [14]. The assay was terminated by filtration on Whatman GF/C filters pre-soaked in polyethyleneimide 1% (w/v) and BSA 1% (w/v). Non-specific and maximal binding were determined in the presence and in the absence of 100 nM unlabeled ligand, respectively. The data were curve-fitted with Prism software (GraphPad Software Inc.).

2.10. Assay of phosphoinositide hydrolysis

COS-7 cells were seeded at 2.5×10^5 cell/mL in 12-well plates and transfected as previously described. Twenty-four hours later, the medium was replaced with 0.5 mL of serum free M199 medium (Gibco, Invitrogen Corporation) containing 1 μCi/mL of myo[^{3}H]inositol and cells were incubated 24 hr at 37°. Then, they were washed twice with a buffer containing 136 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 5 mM HEPES, and 11 mM glucose, pH 7.4. Loaded cells were incubated during 15 min at 37° with PI buffer containing 10 mM LiCl. Stimulation of phosphoinositide hydrolysis

was induced by 3×10^{-8} M agonist for 90 min and the reaction was stopped by addition of ice cold 5% $HClO_4$ (v/v) 50 mg/L phytic acid. The wells were then rinsed with chilled $HClO_4$ 2.5% (v/v). After neutralization with 5% K_2CO_3 (w/v) and 0.025 mL HEPES (1 M, pH 7.4), the supernatant was incubated during 2 hr on ice and then centrifuged 10 min at 4° and 3000 *g*. Eight hundred microliters of the clear supernatant were applied on anion exchange columns (Dowex AG1-X8) and the inositol phosphate eluted by 1 M ammonium formate (adapted from [15]). Radioactivity was determined by liquid scintillation spectrometry.

2.11. Compound and chemokines

CXCL1 and CXCL8 were from Peprotech (TEBU International). fMLP was purchased from Sigma-Aldrich. SB 225002 was synthesized at Laboratoires Fournier. [^{125}I]CXCL1 (2000 Ci/mmol) was obtained from Amersham Pharmacia Biotech and [^{125}I]CXCL8 (2200 Ci/mmol) was purchased from Perkin-Elmer. Human CXCL6 and rabbit CXCL1 were provided by J. Van Damme (Laboratory of Molecular Immunology, Rega Institute for Medical Research) and TR. Martin (Medical Research Service, Seattle Veterans' Affairs Medical Center), respectively.

2.12. Data analysis

Statistical significance was determined by Student's unpaired *t*-test ($P < 0.05$ was considered statistically significant).

3. Results

3.1. In vivo sequestration of neutrophils

Neutrophils exhibit the capacity to migrate in response to CXC chemokine. We used this characteristic in order to investigate the presence of one or several CXC-chemokine receptors, able to interact with human CXCL8, at the surface of guinea pig blood neutrophils. A change in the number of neutrophils in the blood of guinea pig was observed after infusion at the three doses of human CXCL8 tested (15, 50, and 100 ng/kg/min) (Fig. 1A). A slight decrease in circulating neutrophil number occurred immediately after administration and was prolonged for 5–10 min. This transient neutropenia was followed by a neutrophilia. Although the observed variation of neutrophil blood concentrations were small, human CXCL8 infused at 15, 50, and 100 ng/kg/min, increased the percentage of polymorphonuclear leukocytes by 190, 180, and 250%, respectively, compared with the basal level. The maximum effect was reached between 120 and 180 min following the beginning of the infusion.

3.2. In vitro chemotaxis of neutrophils

We then determined, *in vitro*, the chemotactic response of guinea pig neutrophils to CXCL8 and CXCL1 compared with fMLP, which was used as a positive control (Fig. 1B). There was a significant increase of migration to CXCL8 and, to a less extent, CXCL1. The migration indexes are of same magnitude as those observed with fMLP.

3.3. In vitro calcium release

In order to characterize more precisely the second messenger pathway coupled to the activation of the receptor activated by CXCL8, we measured the calcium released from isolated guinea pig neutrophils.

Stimulation of neutrophils with 10 nM of CXCL8 induced a release of intracellular calcium and these neutrophils became unresponsive to subsequent stimulation with 10 nM CXCL1. Stimulation of neutrophils with CXCL1 (10 nM) also produced a transient increase in intracellular calcium concentration but did not cause a loss of sensitivity to a further stimulation with CXCL8 (Fig. 1C, stimulation with a higher chemokine doses did not show better response (data not shown)).

3.4. Identification of a guinea pig sequence encoding a CXCL8 receptor like protein

Based on the previous results, suggesting the presence of a receptor for CXCL8 at the surface of guinea pig neutrophils, we tried to isolate the cDNA encoding this receptor. Oligonucleotides primers were designed based on the most highly conserved region of selected known CXCL8 receptors. The sequences of the primers correspond to the DNA sequence of the second transmembrane domains and third intracellular domain and were respectively called gpA and gpB. Using cDNA reverse transcribed from total RNA extracted from guinea pig total leukocytes and the primer couples, a sequence of expected size (467 bp) was amplified. The amplified segment was sequenced and, compared with the known sequences of CXC receptors. It shares high homologies with the cloned sequences of CXCR2 (Fig. 2A). This sequence was then used as a template to generate the entire sequence using 5′ and 3′RACE-PCR (cf Section 2). The full length cDNA coding sequence of 1056 bp was isolated and corresponded to a potential open reading frame of 352 amino acids (GenBank reference: AY237539). Multiple alignment of the guinea pig sequence was performed by Vector NTI 8.0 (InforMax). The different homologies and the predicted phylogeny are represented in Fig. 2A and B, respectively. The guinea pig receptor shares 70 and 69% identity with the human and rabbit CXCR2, respectively. As no other amplification was possible and as in some species such as mouse, CXCL8 receptor has intermediate properties between CXCR1 and CXCR2, it was of interest to study

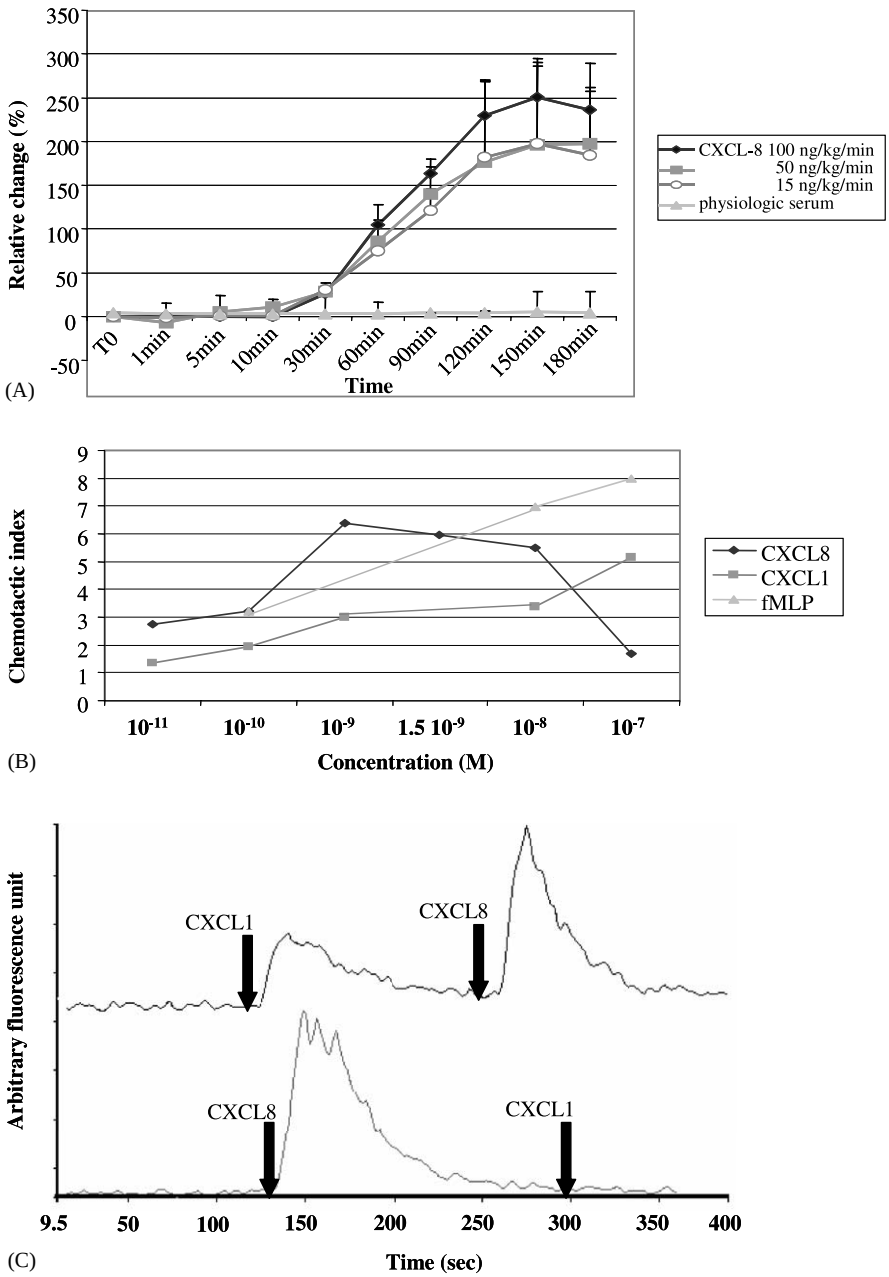

Fig. 1. (A) Effect of hCXCL8 on circulating neutrophils level. Total neutrophils were measured in blood samples obtained from control animals after intravenous perfusion of saline (▲), 15 ng (⬭), 50 ng (■) and 100 ng (◆) of hCXCL8. Data represent means ± SEM obtained from at least three guinea pigs. Time scale origin coincides with the start of the perfusion. (B) Guinea pig neutrophil chemotactic responses to hCXCL8, hCXCL1, and fMLP. Chemotactic responses were assayed as described in Section 2. A 1 value index corresponds to the migration observed in the absence of chemokine. Migration in absence of chemokine. Data are presented as mean ± SEM of five independent experiments. (C) Calcium mobilization response to CXCL8 or CXCL1 by purified neutrophils. Neutrophils were purified as described in Section 2. They were loaded by Fura2. Fluorescence was monitored before and after addition of chemokines (10^{-8} M CXCL8, 10^{-8} M CXCL1). Stimulations are represented by arrows. This figure is representative of four independent experiments.

the physiologic characteristics of the cloned guinea pig receptor.

3.5. Expression of the putative gpCXCR2 in normal tissues

As chemokine receptors are mainly expressed on circulating blood cells, we performed cloning experiments with neutrophils, cells known to be CXC-chemokines target. Nevertheless, the expression of CXCR1 and CXCR2 is found in other tissues. In order to determine the tissue distribution of this receptor, we analyzed mRNA expression of the gpCXCR2 in various tissues: heart, bone marrow, kidney, skeletal muscle, liver, brain, spleen, lung, and neutrophils. The level of expression was quantified by real-time RT-PCR. Quantifications were done in reference

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      CXCR2_gp          (1) -MENFIWDYNSSDYFG-------HYSTDLSQISSESSPCYPESLEINTYVVVIIY
CXCR1_human(P25024)     (1) -----MSNITDPQMWDFDDLN----FTGMPPADEDYSPCMLETETLNKYVVIIAY
CXCR2_human(P25025)     (1) -----MESDSFEDFWKGEDLSNYSYSSTLPPFLLDAAPCEPESLEINKYFVVIIY
CXCR2_mouse(P35343)     (1) MGEFKVDKFNIEDFFSG-DLDIFNYSSGMPSILPDAVPCHSENLEINSYAVVVIY
CXCR1_rat(P70612)       (1) MAEAEYFIWIAPEGDFEEEFGN---ITRMLPTGEYFSPCKR-VPMTNRQAVVVFY
CXCR2_rat(P35407)       (1) MGEIRVDNFSLEDFFSG-DIDSYNYSSDPPFTLSDAAPCPSANLDINRYAVVVIY

CXCR2_gp          (48) ALVSLLSLLGNSLVMMVVLHSRSTCSVTDVYLLNLAIADLLFALTLPFWAASKMI
CXCR1_human       (47) ALVFLLSLLGNSLVMLVILYSRVGRSVTDVYLLNLALADLLFALTLPIWAASKVN
CXCR2_human       (51) ALVFLLSLLGNSLVMLVILYSRVGRSVTDVYLLNLALADLLFALTLPIWAASKVN
CXCR1/2_mouse     (55) VLVTLLSLVGNSLVMLVILYNRSTCSVTDVYLLNLAIADLFFALTLPVWAASKVN
CXCR1_rat         (52) ALVFLLSLLGNSLVMLVILYRRRTRSVTDVYVLNLAIADLLFSLTLPFLAVSKWK
CXCR2_rat         (55) VLVTLLSLVGNSLVMLVILYNRSTCSVTDVYLLNLAIADLFFALTLPVWAASKVN

CXCR2_gp         (103) GWIFGTFMCKIISFLKEVNFYSSILLLACISV**DRYLAIV**HATRTVIQKRHLVKFV
CXCR1_human      (102) GWIFGTFLCKVVSLLKEVNFYSGILLLACISVDRYLAIVHATRTLTQKRHLVKFV
CXCR2_human      (106) GWIFGTFLCKVVSLLKEVNFYSGILLLACISVDRYLAIVHATRTLTQKRYLVKFI
CXCR1/2_mouse    (110) GWTFGSTLCKIFSYVKEVTFYSSVLLLACISMDRYLAIVHATSTLIQKRHLVKFV
CXCR1_rat        (107) GWIFGTPLCKMVSLLKEVNFFSGILLLACISVDRYLAIVHATRTLTRKRYLVKFV
CXCR2_rat        (110) GWIFGSFLCKVFSFLQEITFYSSVLLLACISMDRYLAIVHATSTLIQKRHLVKFV

CXCR2_gp         (158) CLAVWAVSLFLSLPMLLFRSTVYLLDLPLVCYEDIGSSTTRWRLVLRILPQFWGF
CXCR1_human      (157) CLGCWGLSMNLSLPFFLFRQAYHPNNSSPVCYEVLGNDTAKWRMVLRILPHTFGF
CXCR2_human      (161) CLSIWGLSLLLALPVLLFRRTVYSSNVSPACYEDMGNNTANWRMLLRILPQSFGF
CXCR1/2_mouse    (165) CIAMWLLSVILALPILILRNPVKVNLSTLVCYEDVGNNTSRLRVVLRILPQTFGF
CXCR1_rat        (162) CMGTWGLSLVLSLPFAIFRQAYKPYRSGTVCYEVLGEATADLRITLRGLSHIFGF
CXCR2_rat        (165) CITMWFLSLVLSLPIFILRTTVKANPSTVVCYENIGNNTSKWRVVLRILPQTYGF

CXCR2_gp         (213) ILPLLIMLFCYGCTLRTLFKAQMGQKHRAMRVIFAVVLVFLLCWLPYNLVLLADT
CXCR1_human      (212) IVPLFVMLFCYGFTLRTLFKAHMGQKHRAMRVIFAVVLIFLLCWLPYNLVLLADT
CXCR2_human      (216) IVPLLIMLFCYGFTLRTLFKAHMGQKHRAMRVIFAVVLIFLLCWLPYNLVLLADT
CXCR1/2_mouse    (220) LVPLLIMLFCYGFTLRTLFKAHMGQKHRAMRVIFAVVLVFLLCWLPYNLVLFTDT
CXCR1_rat        (217) LLPLFIMLVCYGLTLRTLFKAHMRQKRRAMWVIFAVVLVFLLCCLPYNLVLLSDT
CXCR2_rat        (220) LLPLLIMLFCYGFTLRTLFKAHMGQKHRAMRVIFAVVLVFLLCWLPYNIVLFTDT

CXCR2_gp         (268) LMRTGLIEETCERRDHIDRALEATEILGFLHSCLNPIIYAFIGQKFRYGLLKIMA
CXCR1_human      (267) LMRTQVIQETCERRNNIGRALDATEILGFLHSCLNPIIYAFIGQNFRHGFLKILA
CXCR2_human      (271) LMRTQVIQETCERRNHIDRALDATEILGILHSCLNPLIYAFIGQKFRHGLLKILA
CXCR1/2_mouse    (275) LMRTKLIKETCERRDDIDKALNATEILGFLHSCLNPIIYAFIGQKFRHGLLKIMA
CXCR1_rat        (272) LLGAHLIQDTCERRNNIDQALYITEILGFSHSCLNPVIYAFVGQSFRHEFLKILA
CXCR2_rat        (275) LMRTKLIKETCERQNEINKALEATEILGFLHSCLNPIIYAFIGQKFRHGLLKIMA

CXCR2_gp         (323) AYGLISKEFLPKEARPSFAGSSSGNTSTTL
CXCR1_human      (322) MHGLVSKEFLARHRVTSYTSSSVNVSSNL-
CXCR2_human      (326) IHGLISKDSLPKDSRPSFVGSSSGHTSTTL
CXCR1/2_mouse    (330) TYGLVSKEFLAKEGRPSFVSSSSANTSTTL
CXCR1_rat        (327) N--LVHKEVLTHHSASFRTSLTTIY-----
CXCR2_rat        (330) NYGLVSKEFLAKEGRPSFVGSSSANTSTTL
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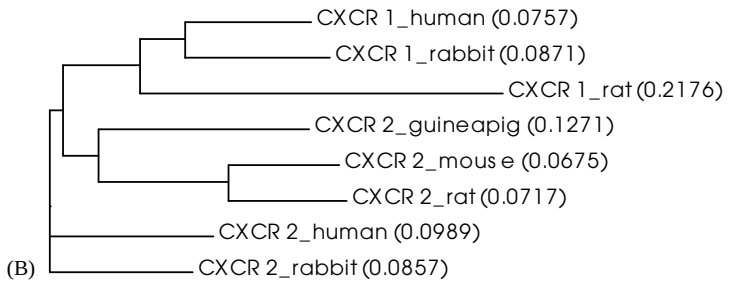
(A)



(B)

Fig. 2. (A) Homology between different CXCL8 receptors. Conservative amino acids are wrapped in dark grey and identical amino acids in grey, DRYLAIV sequence is indicated by bold letters, transmembrane domains are underlined. (B) Evolutionary tree. The tree was established using the Neighbor joining method, values indicate the distance between all pairs of analyzed sequences. All the data were analyzed by Vector NTI 8.0 (InforMax).

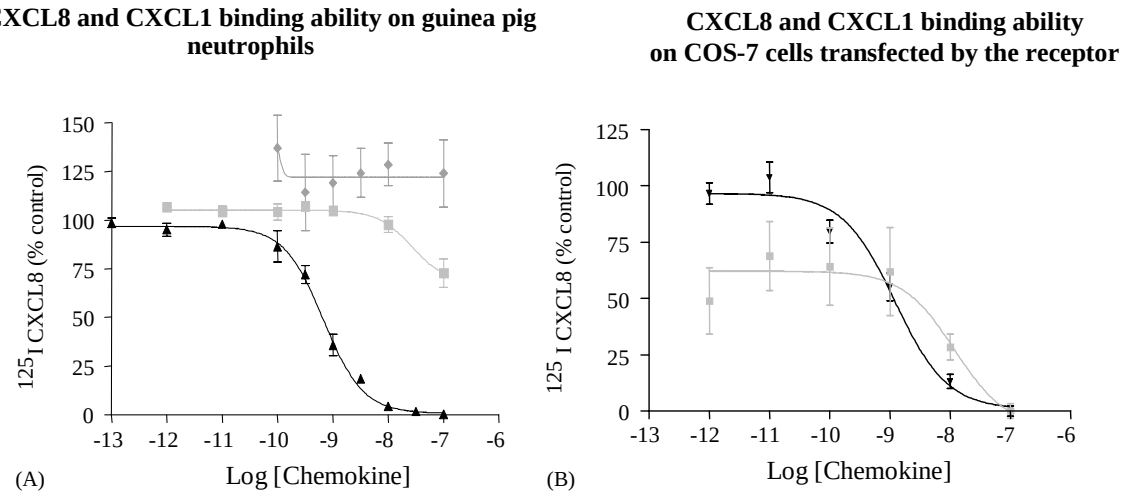


Fig. 3. Competition for [^{125}I]CXCL8. (A) Guinea pig neutrophils, (B) COS-7 transfected cells. Receptor binding assays were performed as described in Section 2. Each data point was determined in duplicate. Figure is representative of three independent experiments.

to the level of expression of the receptor in blood neutrophils, where the highest level of expression was found. We confirmed that the receptor was weakly expressed in the kidney, the lung, the spleen and, to a less extent, in the heart. It was predominantly expressed in neutrophils: gpCXCR2 is, respectively, 36, 118, 123, and 666 times less expressed in these tissues than in blood neutrophils. Other organs tested (brain, liver, skeletal muscle, and bone marrow) showed non-significant levels of expression.

3.6. [^{125}I]CXCL8 binding assays

To determine whether the binding profile of the receptor expressed in the guinea pig neutrophils could be confirmed by the transfection of our cloned receptor in COS-7 cells, we characterized the binding specificity of both transfected cells and neutrophils (Fig. 3). As guinea pig radiolabelled chemokines were not available, radiolabelled human CXCL8 was employed in the competition experiments. CXCL8 binds the native receptor expressed in neutrophils and the cloned receptor transfected in COS-7 cells with affinity that is equivalent to that observed on human neutrophils or on the human CXCR2 transfected COS-7 cells [16–18]. The competition binding curves for CXCL1 on neutrophils and transfected COS-7 cells were shifted 44.3- and 9.8-fold to the right, respectively, as compared with the binding curves observed with CXCL8 for competition against [^{125}I]CXCL8. Other competition experiments against [^{125}I]CXCL8, including human CXCL5 and the CXCR2 selective non-peptide antagonist SB 225002 were all unsuccessful.

3.7. Inositol phosphate accumulation

As the CXCR2 from different species were known to couple to phospholipase C, the guinea pig receptor was tested for its ability to induce phosphoinositide hydrolysis in transiently transfected cells. The cloned gpCXCR2, transfected in COS-7 cells, was tested, as described in Section 2, with various chemokines. Transfected cells accumulated significantly [^{3}H]inositol phosphate when stimulated with human CXCL8, CXCL1, and rabbit CXCL1, whereas no response was observed with human CXCL6 or CXCL5 (Fig. 4).

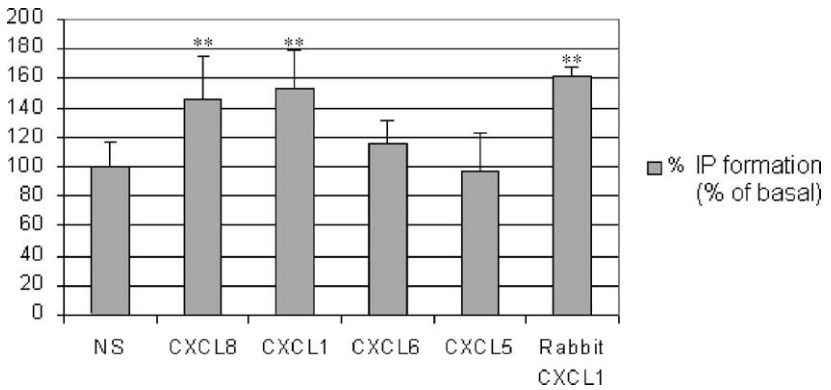


Fig. 4. Accumulation of [^{3}H]inositol phosphate in COS-7 cells after stimulation with 3×10^{-8} M of various chemokines. Three chemokines produce a significant increase (**: $P < 0.01$) of accumulated inositol phosphate: human CXCL1 and CXCL8 and rabbit CXCL1.

4. Discussion

In this study, we investigated the expression of a receptor for CXC chemokines at the surface of guinea pig neutrophils. Blood perfusion of chemotactic factors, such as CXCL1 or CXCL8, were shown to induce first a transient leukopenia followed by a neutrophilia [19,20]. For CXC chemokines this phenomenon mainly implicates neutrophils. Neutropenia results from either a transient increase in adherence to the endothelium or from the trapping of cells in the microcirculation. This decrease in circulating cells was over-compensated by mobilization of neutrophils from bone marrow, which was at the origin of the neutrophilia. We showed that human CXCL8 induced a transient neutropenia followed by neutrophilia in guinea pigs. Thus, neutrophils from guinea pigs expressed a receptor that allowed a functional stimulation by human CXCL8. Guinea pig neutrophils also showed a chemotactic response to human CXCL8, CXCL1, and fMLP in *in vitro* assays. The chemotaxis responses were less pronounced in comparison with published data, but human chemokines could be less potent than those of guinea pig on the cells from the same species [21]. These results demonstrate that guinea pig neutrophils expressed functional chemokine receptors responding to CXCL1 and CXCL8. In order to characterize this or these receptor(s), we performed the cloning of a putative CXC guinea pig receptor, with the hypothesis that this receptor exhibits some similarities with the known CXCL8 receptors. The protein encoded by this sequence, cloned from neutrophils, showed the three main characteristics of the chemokines receptors: a highly conserved DRYLAIV sequence (in bold letters in Fig. 2A), seven hydrophobic sequences, which are supposed to be transmembrane domains and a S/T rich COOH terminal domain that could allow the phosphorylation of the receptor. Moreover, it was highly homologous with others mammalian CXCR1 or CXCR2 (Fig. 2A). Sequence homology strongly suggested that we cloned a guinea pig CXCR2 like receptor. Indeed, this receptor is closely related to the rodent CXCR2 (67% of homology) and seems to be less phylogenetically related to the various CXCR1.

The profile of RNA expression, with a main expression in neutrophils, spleen, lung, and kidney, was in agreement with the previously published expression pattern of two rat genes orthologous to human CXCL8 receptors and with the expression pattern of guinea pig CXCL1 [22,23].

In order to fully characterize the CXCL8 receptor expressed in guinea pig neutrophils, we initially studied its binding characteristics. Competition binding experiments against [^{125}I]CXCL8 on guinea pig neutrophils showed a high affinity for human CXCL8 and weak or no affinities for other tested ligands. Using human [^{125}I]CXCL1 as a binding competitor (data not shown), no binding of the radiolabelled ligand was observed, suggesting that human CXCL1 had weak or no affinity for the receptor(s) expressed on the guinea pig neutrophils using the present experimental conditions. These results were not too surprising given that the human agonist was used and that the iodination could have altered the characteristics of CXCL1. We generated transient transfectants expressing the cloned receptor to determine whether human ligands would allow binding to these cells. Receptor transfected cells bound human CXCL1 and CXCL8 with affinity in agreement with the affinity obtained with guinea pig neutrophils.

Then, we studied the functional biological responses, which are often more sensitive than binding experiments. As indicated by degranulation and chemotaxis experiments with human CXCL8 acting on guinea pig neutrophils [24,25], we demonstrated that human CXCL8 had the capability to induce an intracellular calcium release and chemotaxis in neutrophils. Moreover, an intracellular calcium release and a chemotactic response were also observed on guinea pig neutrophils after stimulation with human CXCL1. However, human CXCL1 was not able to bind to rabbit CXCR2, and human CXCL8 was a poor agonist for mouse CXCR1/2 [18,26]. As neutrophils had been used, it was not possible to exclude the possibility of the presence of another receptor. Therefore, we performed an *in vitro* assay on transfected cells. Accumulation of inositol phosphate in the transfected cells showed that human CXCL1 and CXCL8 were the most efficient of the tested chemokines. Rabbit CXCL1 showed either a good signal induction (rabbit CXCR2 presents the most close sequence with our receptor, with 69% homology), whereas other chemokines did not show a significant response. These results further showed that we cloned a guinea pig CXCR2 like protein and that both human CXCL1 and CXCL8 were able to bind and activate this receptor.

All together, the binding and functional assays on neutrophils suggested that guinea pig could possess two receptors for CXCL8: (a) maximum concentration of CXCL1 (10^{-7} M) displaced 25% of the total [^{125}I]CXCL8 to displace all [^{125}I]CXCL8 binding on the cloned receptor expressed in COS-7 cells (Fig. 3), (b) the results obtained in intracellular calcium release experiments could be interpreted by the presence, at the cell surface, of a second receptor insensitive to CXCL1. Prior exposure of the cells to CXCL8 suppressed the ability of CXCL1 to mediate Ca^{2+} release, but CXCL1 pre-treatment failed to inhibit a response to CXCL8. Similar results were obtained with human or rabbit neutrophils, which present two receptors for CXCL8 [27,28]. However, calcium results could also be interpreted in a different way: guinea pig neutrophils could express a unique receptor less sensitive to human CXCL1. This could be explained by interspecies variations: human and guinea pig CXCL8 are more closely related than are human CXCL1 and guinea pig KC [8]. Despite several attempts, we failed to characterize the cDNA of another CXCR1/2 like protein. Therefore, it

was not possible to conclude whether a second receptor existed in guinea pig. Mice express one main CXC-chemokine receptor that binds KC (murine CXCL1), macrophage inflammatory protein-2 (murine CXCL2), ENA-78 (CXCL5) and GCP-2 (CXCL6). Due to the weak homology of their chemokine system with the human system, mice are not optimal for *in vivo* studies. In other respects, despite several attempts, no murine CXCL8 equivalent has been cloned [8]. KC acting on the mouse and rat CXCR2 homologue receptors are only distantly related to human CXCL1 and CXCL8 (mouse and rat KC show with human CXCL8 and CXCL1 55 and 47%, 48 and 58% amino acids sequence similarity, respectively). In contrast to the human situation in which ligands for CXCR1 and CXCR2 induce neutrophil migration, in mice, neutrophil migration is also induced by the CC chemokine MIP1-alpha [29]. In addition to a KC like chemokine, guinea pigs have a CXCL8 like chemokine (showing 58 and 68% amino acids sequence identity with the human CXCL1 and CXCL8, respectively [8]). Guinea pig is a model of interest, as it is the only rodent to present both chemokines and it is likely to have, like humans, two CXC receptors on neutrophils. The question of whether a CXCR1 like receptor is expressed at the cell surface still remains. Nevertheless, given the characteristics of the receptor that we cloned, guinea pig is a putative useful model for the study of the implication of CXCL1 and 8 in various pathologies. Indeed, guinea pig models exist for ARDS (pulmonary edema) [30], cystic fibrosis, bronchiectasis, chronic bronchitis, COPD (airway neutrophilia) [25,31], otitis media [32] and skin inflammation [33].

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