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Characterization of the molecular interactions of interleukin-8 (CXCL8), growth related oncogen α (CXCL1) and a non-peptide antagonist (SB 225002) with the human CXCR2

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Abstract

Neutrophil recruitment to inflammatory sites is mediated by two related receptors: CXC chemokine receptors 1 (CXCR1) and 2 (CXCR2). Both receptors share two ligands, interleukin-8 (CXCL8) and GCP-2 (CXCL6), whereas several chemokines, including growth related oncogen α (CXCL1) and a non-peptide antagonist (SB 225002) are specific for CXCR2. The objective of this study was to map the different amino acids involved in the binding and activation/inhibition of human CXCR2. This was performed by exchanging non-conserved amino acids of CXCR2 with their counterparts in CXCR1. The mutants generated showed that: (a) for CXCL8 binding, the N-terminus of CXCR1 and the second extra-cellular loop of CXCR2 are determinant, the N-terminus of CXCR2 is not sufficient and the transmembrane domain seven is probably involved; (b) for CXCL1, the N-terminus of CXCR2 is necessary but not sufficient for binding. The activation study indicated that amino acids critical for activation are not necessarily involved in binding process. Finally, the mechanism of binding of a non-peptide antagonist on CXCR2 was investigated: it occurred through epitopes (a) which were disseminated within the receptor, (b) which differed according to the use of CXCL8 or CXCL1 as a competitor and (c) which did not necessarily overlap with agonist binding sites. We also showed that inhibition of binding and inhibition of activation involved different amino acids.

Keywords: G protein-coupled receptor; Binding; Activation; CXCL8; CXCL1; SB 225002

1. Introduction

During the inflammatory response, the chemokine interleukin-8 (CXCL8, according to the new nomenclature [1]) attracts neutrophils to injured sites through the activation of two closely related GPCRs: CXCR1 and CXCR2. Similar to CXCL8, another more recently described chemokine, granulocyte chemotactic protein 2 (CXCL6), is also able to stimulate both CXCR1 and CXCR2 [2]. Other neutrophil attracting chemokines that mostly activate CXCR2 have also been described, including CXCL1, epithelial neutrophil-activating peptide 78 (ENA-78 or CXCL5) and neutrophil activating peptide-2 (NAP-2 or CXCL7) [3–5]. The most studied of these chemokines, CXCL8 and CXCL1, bind and activate a shared receptor (CXCR2) which is widely distributed on neutrophils. However, the resulting functional response appears to be unique to each of these chemokines [6,7]. These differential effects could not solely be explained by the co-activation of CXCR1 by CXCL8 in these cells, as the CXCL8 or CXCL1 responses in CXCR2 transfected cells were not identical [8].

Several studies have attempted to define the binding and activation sites of CXCL8 and CXCL1 on CXCR2: (a) by alanine scanning mutagenesis [9], (b) by exchanging human CXCR2 regions with their rabbit equivalents [10], (c) or by their counterpart in human CXCR1 [11,12], or (d) by inhibiting the binding of natural ligand by synthetic peptides [13]. The results are often conflicting

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Abbreviations: aa, amino acid; CXCL1/Gro-α, growth related oncogen α; CXCL8/IL-8, interleukin-8; EC_{50}, effective concentration 50%; EC, extra-cellular loop; GPCR, G protein-coupled receptor; tm, transmembrane domain; IC_{50}, inhibitory concentration 50%.

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and difficult to interpret because of the versatility of these various models and the marked modifications introduced into the wild-type protein. Nevertheless, these studies are of interest in guiding the development of antagonists.

In 1998, SB 225002 (*N*-(2-hydroxy-4-nitrophenyl)-*N*′-(2-bromophenyl)urea), the first non-peptide antagonist of CXCR2, was described [14]. This compound inhibited the binding of both CXCL8 and CXCL1 on recombinant and native CXCR2 and also blocked CXCL8 and CXCL1-induced chemotaxis and margination of human and rabbit neutrophils without having any affinity or activity on CXCR1. Based on these results, White *et al.* suggested that CXCL8 attracts neutrophils preferentially through the activation of CXCR2. SB 225002 was first developed as a potential treatment of inflammatory diseases, including chronic obstructive pulmonary disease and rheumatoid arthritis. More recently, bronchopulmonary dysplasia and retrovirus infections have been proposed as new potential therapeutic targets for CXCR2 antagonists [15,16].

The aim of this study was to pinpoint the amino acids involved in the binding and activation of human CXCR2 upon interaction with CXCL8, CXCL1 and the non-peptide antagonist, SB 225002. For such a purpose, non-conserved amino acids of CXCR2 were exchanged with their counterparts as found in CXCR1, which shares 73% amino acid sequence identity with CXCR2 and weakly binds CXCL1 and SB 225002.

2. Materials and methods

2.1. Generation of cell lines stably expressing wild-type and mutant human CXCL8 receptors

Wild-type CXCR1 and CXCR2 receptors were first cloned from HL60 cells in pcDNA3 (Invitrogen Corporation). They were then used as a template for the site-directed mutagenesis with QuickChange Site directed Mutagenesis Kit (Stratagen). The designation and sequence composition of mutant receptors are as follows: (a) for chimeras: N1-2, CXCR1 (aa 1–42) and CXCR2 (aa 47–360); N2-1, CXCR2 (aa 1–46) and CXCR1 (aa 43–355); EC2-1-2, CXCR2 (aa 1–84), CXCR1 (aa 181–202) and CXCR2 (aa 201–360); (b) for mutants: FXVXI-50-VXIXA means that the CXCR2 sequence ‘‘FVVII’’ starting 50 amino acids after the first methionine was mutated in ‘‘VVIIA’’, its corresponding sequence in CXCR1. According to this example, the following mutants were synthesized: FXVXI-50-VXIXA, SI-168-GC, LL-174-MN, AXXVL-177-SXXFF, N-203-D, N-206-K, QS-216-HT, LI-225-FV, T-285-S, HXD-291-NXG, I-304-F and L-312-I (Fig. 1). After mutation and verification of the mutant DNA sequences the plasmids were used to transfect human Jurkat and murine BaF-3 cells by DMRIE-C Reagent (Life Technologies, Invitrogen Corporation). A mock transfection by pcDNA3 was performed in parallel. The cell cultures were grown in

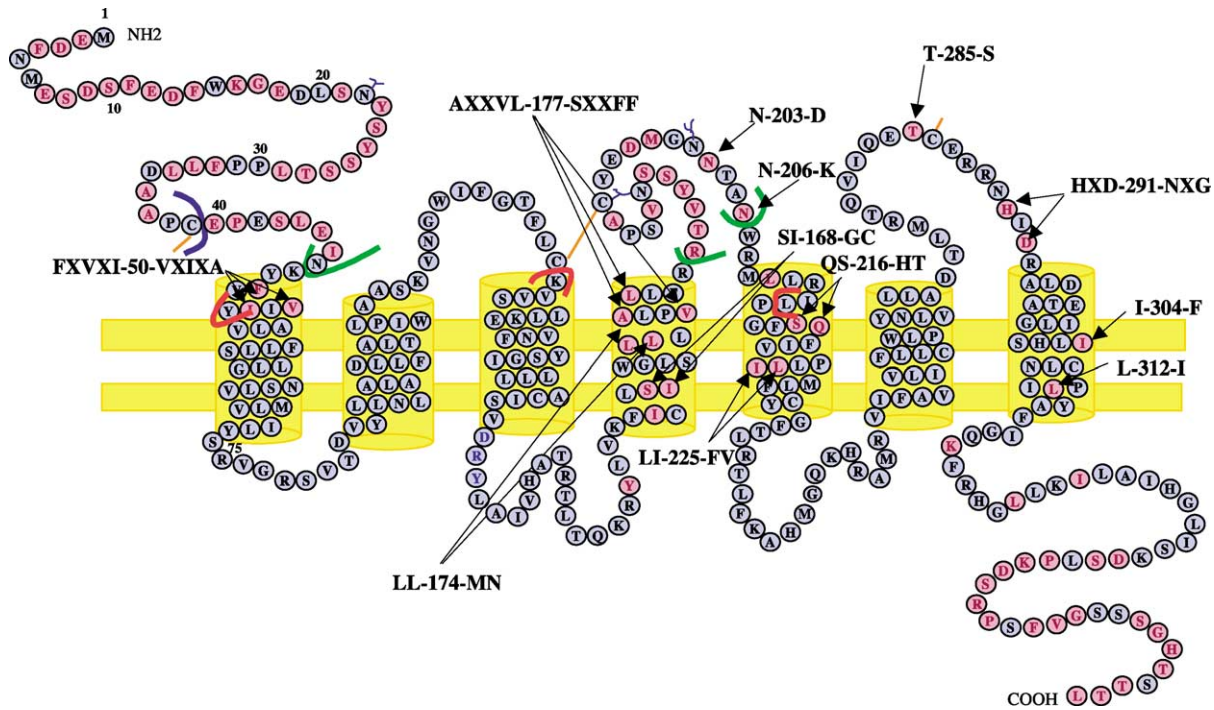

Fig. 1. Schematic illustration of the secondary structure of the CXCR2 (Swiss-Prot #P25025) and localization of mutated residues. CXCR2 amino acids homologous to those of CXCR1 are represented in blue. When they have been mutated together, divergent amino acid (pink) are linked by arrows and the localization of the first mutated amino acids is indicated (FXVXI-50-VXIXA, SI-168-GC, LL-174-MN, AXXVL-177-SXXFF, N-203-D, N-206-K, QS-216-HT, LI-225-FV, T-285-S, HXD-281-NXG, I-304-F and L-312-I). Green lines delimit chimeras used in this study (N1-2, N2-1 and EC2-1-2), red and blue lines delimit chimeras used in previous studies, respectively ‘‘AB1’’ or ‘‘45A’’ and ‘‘4442’’ [11,12,22].

appropriate medium (RPMI, 1% (v/v) non-essential amino acids, 1% (v/v) Glutamax (Life Technologies, Invitrogen Corporation), 10% (v/v) fetal bovine serum (PAN Biotech) for Jurkat cells, completed with 2 nM murine interleukin-3 (Peprotech, TEBU International) for BaF-3 cells) during 48 hr before adding the selective agent G418 (Life Technologies, Invitrogen Corporation). Receptor expression was tested by fluorescence active cell sorter analysis using specific antibodies and by [^{125}I]-CXCL8 or [^{125}I]-CXCL1 binding assays. Positive cells were sorted by magnetic separation MS+ Separation Columns (Miltenyi Biotec) using specific antibodies.

2.2. Flow cytometry analysis

The expression of wild-type and mutant receptors was assayed by staining the cells with CXCR1-fluorescein isothiocyanate-conjugated or CXCR2 *R*-phycoerythrin labeled mouse anti-human monoclonal antibodies (PharMingen). Briefly, 10^6 cells were washed three times in PBS (Life Technologies, Invitrogen Corporation) supplemented with 0.5% BSA, incubated at room temperature with mouse IgG during 15 min and then at 4° with chemokine receptor antibody (1:50) during 45 min. The cells were then washed three times before analysis. To determine the percentage of positive cells, a marker was set at a position giving a maximum of 1% positive cells in mock-transfected cells.

2.3. [^{125}I]-CXCL8 or [^{125}I]-CXCL1 binding analysis

To determine ligand binding selectivity, 10^6 Jurkat transfected cells were incubated at room temperature during 90 min in duplicate in 250 μL of medium Earle Balanced Salt Solution (Life Technologies, Invitrogen Corporation) supplemented with 2.2 g L^{-1} $NaHCO_3$, 25 mM HEPES, 0.1% (w/v) gelatin, 0.1% (w/v) NaN_3 and 0.008% (w/v) CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) with 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 (from 10^{-5} to 10^{-9} M). The assay was terminated by filtration of the cells through Whatman GF/C filters pre-soaked in polyethyleneimide 1% (w/v) and BSA 1% (w/v). Non-specific binding was determined in the presence of 100 nM unlabeled ligand. Maximum binding was determined in the absence of unlabeled ligand. The data were curve-fitted with the use of Prism software (Graphpad Software).

2.4. Intracellular [Ca^{2+}] measurements

BaF-3 transfected cells were washed in Hank's buffered saline solution supplemented with 2 mM Ca^{2+}, 1 mM Mg^{2+} and 10 mM HEPES. Cells were then suspended (10^7 mL^{-1}) in the same buffer containing 5 μM Fura (Molecular Probes, Interchim) and incubated for 45 min at 37° in the dark. The cells were then diluted in the previous buffer (1/5), incubated for 15 min at 37° in the dark, washed twice in the same buffer and suspended at a final concentration of 4×10^6 mL^{-1}. One hundred microliter of cell suspension was placed at room temperature in a 96-well plate. Fluorescence data acquisition was performed with the Fluorescence Drug Screening System, and data analyzed with AquaCostmos software (Hamamatsu-Photonics K.K.). The data shown are expressed as arbitrary fluorescence units normalized to the initial baseline level and maximal calcium release after the addition of Calcium Ionophore A23187 (Sigma Chemical Co.) (maximum response–basal response). EC_{50} and IC_{50} were determined by non-linear regression using GraphPad Prism software.

2.5. Drugs

CXCL1 and CXCL8 were from Peprotech (TEBU international). SB 225002 was synthesized at Laboratoires Fournier. [^{125}I]-CXCL1 (2000 Ci mmol^{-1}) was obtained from Amersham Pharmacia Biotech and [^{125}I]-CXCL8 (2200 Ci mmol^{-1}) was purchased from Perkin-Elmer.

3. Results and discussion

3.1. Construction and expression of CXCR2 mutants and chimeras

To identify the amino acids involved in the binding to and activation of CXCR2, various mutants and chimeras were generated. Although they are structurally related and share two ligands (e.g. CXCL8 and CXCL6), human CXCR1 and CXCR2 show differences in their binding characteristics. The most divergent regions of the two receptors are clustered in the N-terminal end, the second EC and the C-terminal extremity (Fig. 1). As no significant binding changes were observed when the C-terminal extremity was truncated, we focused our attention on the other diverging regions [17]. The N-terminal segment and second EC were too unrelated to be investigated by systematic single amino acids mutagenesis. Therefore, three different chimeras (N1-2, N2-1 and EC2-1-2, limits of chimeras are represented by green lines on Fig. 1) were generated to determine their relevance in the interaction between ligands and receptor. In order to accurately change the extra-cellular part of the protein, we performed PCR synthesis rather than the restriction fragment ligation technique. This technique allowed to remove extra-cellular regions of receptor protein without affecting transmembrane domains. Isolated variant amino acids were mutated to their CXCR1 equivalent by site-directed mutagenesis (Fig. 1). Two mutants N-203-D and N-206-K were produced in order to clarify the role of the second EC known to play an important role in the structure–function relationship of CXCR2 [12,13] as well as of several other GPCRs

[18]. In addition, 10 other mutants were generated. Level of expression of mutated receptors was monitored by flow cytometry analysis, and improved by enrichment of CXCR2-expressing cells using magnetic separation. The expression of three CXCR2 mutants (T-285-S, SI-168-GC and AXXVL-177-SXXFF) was neither detected by flow cytometry, nor by binding experiments with CXCL8 or CXCL1. All other constructs or wild-type receptors showed a percentage of positive labeled cells between 85.7 and 99.0%, and a mean of fluorescence between 10.9 and 38.2 (data not shown). Expression of receptors has been checked regularly and cells showing a decrease of expression have been replaced.

3.2. Binding competition using [^{125}I]-CXCL8 as a ligand

All the mutants showed a binding affinity for CXCL8 similar to wild-type CXCR2, except mutants I-304-F and L-312-I which showed a significant loss of affinity of 13.6 and 4.5-fold, respectively (Table 1). Two chimeras, namely N2-1 and EC2-1-2, did not present binding of radiolabeled CXCL8 whereas N1-2, containing the N-terminal region of CXCR1, did. As expected CXCL1 was not able to compete with [^{125}I]-CXCL8 on CXCR1 [19,20]. It also failed to compete with [^{125}I]-CXCL8 on chimera N1-2. Similarly, CXCL1 was not able to displace [^{125}I]-CXCL8 bound on HXD-291-NXG (mutation in the third EC (Fig. 1)). However, on all other mutants, including I-304-F and L-312-I, CXCL1 competed for CXCL8. No conclusion could be drawn from chimera N2-1 or EC2-1-2 since they did not bind radiolabeled CXCL8 (Table 1).

As observed in L1-2 and HEK293 cells and in spite of a difference in the chimera constructions, we can conclude that: (a) the chimera N1-2, corresponding to "AB1" of Ahuja *et al.* [12], and "45A" of Wu *et al.* [11] (Fig. 1, delimited by red lines) bound with high affinity CXCL8 without competing for cold CXCL1 and this binding cannot be competed by cold CXCL1, (b) chimera N2-1 (BA1 in [12]) did not bind labeled [^{125}I]-CXCL8. Chimera EC2-1-2 was not described in previous studies since no appropriate enzyme restriction sites exist in the vicinity of the second EC. Ahuja *et al.* included in their chimeras the exchange of the tm IV which contains several sequence differences between CXCR1 and CXCR2 [12]. Taken together, these data (Table 1) show that (a) the N-terminal extremity of CXCR1, but not CXCR2, and the second EC of CXCR2, but not CXCR1, are determinant for CXCL8 binding (b) the N-terminal region of CXCR2 is not sufficient, in contrast to the CXCR1 one, to provide CXCL8 binding (Fig. 2). Although, peptides corresponding to a different region of CXCR2 failed to show inhibition of CXCL8 binding [13], the first part of the second EC should be involved. However, neither the mutants N-203-D nor N-206-K revealed a significant loss of CXCL8 binding. These results are consistent with the study by Luo *et al.* which showed that Tyr188, Ser189 and Asn191, located in this first part of the second EC are key residues in the CXCL8 binding. His291, located in the third EC, was also identified as essential, in agreement with our results [21]. Some amino acids described by other authors to be important in the binding and activation of CXCR2, were not tested in our study because they are also present in CXCR1. Finally, the tm VII could also be involved in CXCL8 binding since its binding affinity for two mutants I-304-F and L-312-I was reduced (Table 1 and Fig. 2). This was unexpected because tm VII, as well as the third EC, is seldom involved in structure/conformation studies of GPCRs [18].

Table 1
Ligand binding selectivity of wild-type and mutant or chimeric receptors

Constructs	CXC chemokine binding affinity (EC_{50}, nM)					
	[^{125}I]-CXCL8			[^{125}I]-CXCL1		
	CXCL8	CXCL1	SB 225002	CXCL8	CXCL1	SB 225002
CXCR1	1.4 ± 0.7	ND				
CXCR2	1.2 ± 0.8	1.0 ± 0.5	9.9 ± 5.1	0.7 ± 0.1	0.9 ± 0.3	87.9 ± 23.0
N1-2	0.4 ± 0.09	ND	331.0	NS	NS	NS
N2-1	NS	NS	NS	NS	NS	NS
EC2-1-2	NS	NS	NS	1.1 ± 0.7	1.1 ± 0.6	84.5 ± 24.2
FXVXI-50-VXIXA	0.7 ± 0.3	2.4 ± 0.5	48.6 ± 29.8	2.1 ± 1.3	1.0 ± 0.5	88.3 ± 10.7
LL-174-MN	1.7 ± 1.2	0.6 ± 0.3	108.3 ± 0.5	0.6 ± 0.3	1.9 ± 1.1	17.2 ± 2.1
N-203-D	0.1 ± 0.05	0.3 ± 0.1	24.0 ± 10.5	0.5 ± 0.1	0.6 ± 0.2	29.2 ± 8.0
N-206-K	0.2 ± 0.09	1.0 ± 0.5	65.8 ± 28.5	0.9 ± 0.1	0.5 ± 0.2	81.4 ± 10.1
QS-216-HT	0.1 ± 0.03	0.3 ± 0.05	30.6 ± 4.8	0.3 ± 0.2	0.8 ± 0.3	130.1 ± 53.2
LI-225-FV	0.3 ± 0.1	1.3 ± 0.2	ND	0.9 ± 0.2	0.4 ± 0.02	42.4 ± 9.8
HXD-291-NXG	1.6 ± 1.0	ND	ND	7.1 ± 0.1	0.4 ± 0.2	42.5 ± 15.8
I-304-F	16.3 ± 9.4	0.2 ± 0.01	10.9 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	10.9 ± 2.1
L-312-I	5.4 ± 0.9	0.8 ± 0.8	ND	NS	NS	NS

The ligand binding selectivity of each receptor was determined on Jurkat cells exposed to 0.15 nM radiolabeled chemokine and varying concentration of cold chemokine or non-peptide antagonist as described in Section 2. Values shown correspond to the mean of three independent experiments in duplicate ± SEM. NS: no specific binding of the radiolabeled ligand; ND: no displacement of the radiolabeled chemokine by the cold ligand.

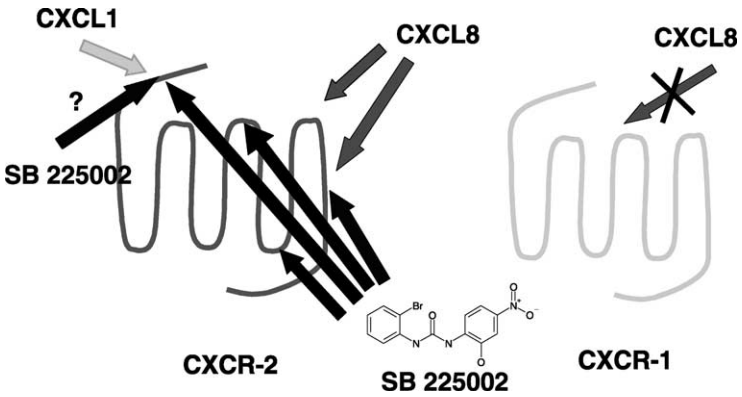

Fig. 2. Localization of the amino acids involved in the inhibition of unlabeled ligands by [^{125}I]-ligand binding to the mutant receptors. Regions involved in CXCL8, CXCL1 and SB 225002 binding are indicated by dark grey, grey and black arrows, respectively.

3.3. Binding competition using [^{125}I]-CXCL1 as a ligand

None of the mutants revealed any significant change in the ability of cold CXCL1 to compete with radiolabeled CXCL1 (Table 1). Neither chimera N1-2 nor N2-1 permitted a binding of [^{125}I]-CXCL1. Competition binding of CXCL8 against radiolabeled CXCL1 to mutants FXVXI-50-VXIXA, LL-174-MN, N-203-D, N-206-K, QS-216-HT, LI-225-FV and I-304-F and chimera EC2-1-2 did not shift the EC_{50}. Nevertheless, CXCL8 failed to compete with [^{125}I]-CXCL1 binding on mutant L-312-I situated in the tm VII. Surprisingly, mutant HXD-291-NXG, which previously displayed no competition of CXCL1 with [^{125}I]-CXCL8, presented a 10-fold shift of CXCL8 binding ability against [^{125}I]-CXCL1.

Data obtained with chimera N1-2 predicted that the N-terminus of CXCR2 is responsible for CXCL1 binding, since as previously mentioned, [^{125}I]-CXCL8 binding could not be competed with cold CXCL1. This result was further supported by the lack of binding of [^{125}I]-CXCL1. In contrast to a previous report [12], the N-terminal extremity of CXCR2 was not sufficient to confer CXCL1 binding ability since chimera N2-1 did not bind CXCL1. As none of the other mutants showed a decrease in CXCL1 binding, we were unable to identify which other part of the receptor was important for CXCL1 binding, although it is likely that the N-terminal extremity plays a central role in this event (Fig. 2). A recent publication of chimeras between the N-terminal extremities of CXCR2 and CXCR4 showed that, in the absence of the N-terminus of CXCR2, cell activation by CXCL1 remained possible, whereas CXCL8 failed to induce an intracellular calcium release, suggesting that the N-terminus of CXCR2 was not involved in CXCL1-induced activation [22]. Nevertheless the N-terminal change in their chimera "4222" (substitution of the amino-terminus of CXCR2, by the amino-terminus of CXCR4) was done at the level of Cys39, eight highly variable amino acids before our N1-2 chimera. These eight acids amino acids might be key residues in CXCL1 binding.

Some of the CXCL8 losses in affinity observed with radiolabeled CXCL8 were not confirmed with CXCL1, and vice versa. The most relevant example is mutant HXD-291-NXG, on which CXCL1 did not displace [^{125}I]-CXCL8 while CXCL8 weakly competed with [^{125}I]-CXCL1. These results confirm that CXCL8 and CXCL1 binding sites are clearly distinct, as previously described by Ahuja *et al.* [12].

3.4. Mapping of a non-peptide antagonist selective for CXCR2

In order to provide a molecular basis to pharmacological properties of SB 225002, we probed the binding pattern of this non-peptide CXCR2 antagonist [14]. SB 225002 was 9-fold more potent to displace CXCL8 than CXCL1 in Jurkat T cells transfected with wild-type human CXCR2 yielding IC_{50} of 9.9 and 87.9 nM, respectively (Table 1). These data are consistent with those from White *et al.* [14] who reported a binding affinity of 22 nM vs. [^{125}I]-CXCL8 whereas [^{125}I]-CXCL1 was not tested.

Against [^{125}I]-CXCL8, competition binding assay of SB 225002 to chimera N1-2 and mutants, N-203-D and N-206-K, located in the second EC showed a significant reduction of affinity of 33.4, 24.2 and 6.6-fold, respectively. SB 225002 did not bind to three mutants bearing mutations in the third EC (HXD-291-NXG), the fifth, and the seventh tm (LI-225-FV, L-312-I) (Table 1 and Fig. 2). These results were expected since we mutated a protein (CXCR2) which permits SB 225002 binding in a protein (CXCR1) which does not bind SB 225002. Four conclusions arise from this set of experiment: (a) the amino acids involved in the binding of SB 225002 are disseminated throughout the receptor; (b) amino acids involved in the binding competition of SB 225002 against CXCL8 are clearly distinct from those involved against CXCL1 (see mutants LI-225-FV and HXD-291-NXG); (c) as observed with CXCL1 the N-terminal extremity of CXCR2 plays an important role in SB 225002 binding; and (d) the binding sites of the antagonist are not necessarily overlapping binding sites of the agonists (Fig. 2). This study is the first to provide an

explanation for the molecular interactions of a non-peptide antagonist of CXCR2 with its receptor. The postulated binding sites are potentially hundred of Å apart, whilst the bulk size of SB 225002 is in the range of 10 Å. We suggest two explanations for this observation: more than one molecule of SB 225002 interacts with the receptor at the same time or, the folding of the receptor and more precisely of the EC allows this interaction. Given the number, the location of the amino acids involved in their binding and the bulk size of the SB 225002 molecules, it is more likely that a change in protein conformation is responsible of the loss of binding of this non-peptide antagonist. In this respect, the mutation that affect the binding of SB 225002, could play a role either directly or indirectly in the modification of this tertiary structure change.

Mutations as aforementioned did not affect the binding of SB 225002 in competition binding studies using [^{125}I]-CXCL1. In addition, CXCL1 did not bind to chimeras N1-2 and N2-1 so it was not possible to conclude regarding binding interactions between SB 225002 and CXCL1.

Interpretation of the data from the present study is limited due to the lack of radiolabeled SB 225002. Indeed, the affinity of this antagonist on different mutants and chimeras was measured indirectly through displacement of natural ligands which themselves display modifications in their binding properties to mutated receptors. Nevertheless, receptor mutagenesis is the most common technique to investigate the molecular mechanism of interaction of a non-peptide antagonist with a receptor. It has been successfully used for the study of different GPCRs: the human β2 adrenergic receptor the human NK-1 (substance P) receptor [23], the human B2 receptor [24] and more recently for CXCR4 [25].

3.5. Calcium mobilization in CXCR2 mutants and chimeras

The next objective was to investigate whether results from binding experiments were correlated with functional responses. For such a purpose, cytosolic free calcium change was used as a functional readout of cell activation. Experiments in 96-well plates using a fluorescent reader camera, provided highly reproducible results (Fig. 3) since all the stimulations and observations were done simultaneously. The results are summarized in Fig. 3A. Mutants and chimeras that were not able to bind agonists were excluded from this assay. Cell lines stably expressing wild-type CXCR2 or its mutants N-203-D and N-206-K respond strongly to CXCL8 and CXCL1 giving EC_{50} of about 15 nM which was quite similar to our wild-type CXCR2 and in agreement with published data for wild-type CXCR2 [14]. CXCL8 and CXCL1 were weak agonists to two other mutants tested (N1-2 and I-304-F). For these two mutants an EC_{50} could not be determined since a calcium release was first observed with concentrations of agonists as high as 1 μM (Fig. 3A). Higher concentration stimulations were not possible for technical reasons. These results cannot be explained by a lack of binding efficiency since both receptors present binding characteristics similar to the wild-type, except for a reduction of affinity for CXCL8 on mutant I-304-F (10-fold EC_{50} increase). The data from the chimera N1-2 are consistent with the results published by Doranz *et al.* who were not able to observe intracellular calcium release after stimulation of their chimera [22]. In contrast, Ahuja *et al.* and Wu *et al.*, which showed that their corresponding chimeras elicit calcium response or chemotactic responses, proposed that N-terminal part plays a role in the activation of the receptor [11,12]. In the present study and that of Doranz *et al.*, the N-terminal ends are slightly shorter than those of Ahuja *et al.* and Wu *et al.* They have included the first part of the tm I in their shift of the N-terminal extremity of the CXCR2 by the CXCR1 one. The amino acids situated in the higher part of the tm I are therefore probably necessary to produce an efficient binding of CXCL8 and CXCL1 to CXCR2. Finally, although the ligand binding activity was fully preserved, LI-225-FV did not respond to high agonist concentration (1 μM) [13]. Mutations can generate new intramolecular constrains that keep the receptor in a silent conformation. Our study did not allow to conclude this point, however, different hypothesis could be considered. (a) The amino acids involved in the activation of the receptor could be prevalent in the coupling with the G-protein. In this respect, most of these amino acids are located in the extra-cellular or transmembrane regions of the protein and are unlikely to interact directly with the G protein. Nevertheless, they can alter the receptor coupling throughout indirect conformational changes. (b) A change in the processing of the protein, e.g. a flaw of dimerization might have altered the coupling. In this regard, homodimerizations of chemokine receptors have been shown to modulate their activity [26]. Such a mechanism has not been characterized yet for CXCR2. There is no general correlation between agonist stimulation and receptor oligomerization. A constitutive oligomerization increased by receptor stimulation is more commonly observed, yet constitutive oligomerization is not always observed or necessarily regulated by receptor occupancy (for review, see [23,27]). Nevertheless, we cannot rule out that oligomerization played a role in the regulation of CXCR2 by SB 225002, and signal transduction was consequently affected by its modification.

The action of SB 225002 was also investigated in mutants giving a calcium response to agonists (Fig. 3B). In cells stably transfected with CXCR2, White *et al.* reported that SB 225002 dose-dependently inhibited calcium mobilization induced by both CXCL8 and CXCL1 with an IC_{50} values of 40 and of 20 nM, respectively [14]. In our cellular model, similar results were obtained with IC_{50} values of 25 and 61 nM for CXCL8 and CXCL1, respectively (Fig. 3). The IC_{50} of SB 225002 against the calcium response in mutant I-304-F was similar to that obtained

(A)

Construct	Agonist Potency EC_{50} (nM)		SB 225002 Antagonist Potency IC_{50} (nM)	
	CXCL8	CXCL1	CXCL8	CXCL1
CXCR2	5.9	17.0	25	61
N1-2	>100	>100	130	25
N-203-D	13.5	28.5	300	490
N-206-K	3.8	15.7	650	39 0
LI-225-FV	NR	NR		
I-304-F	>100	>100	19	21

(B)

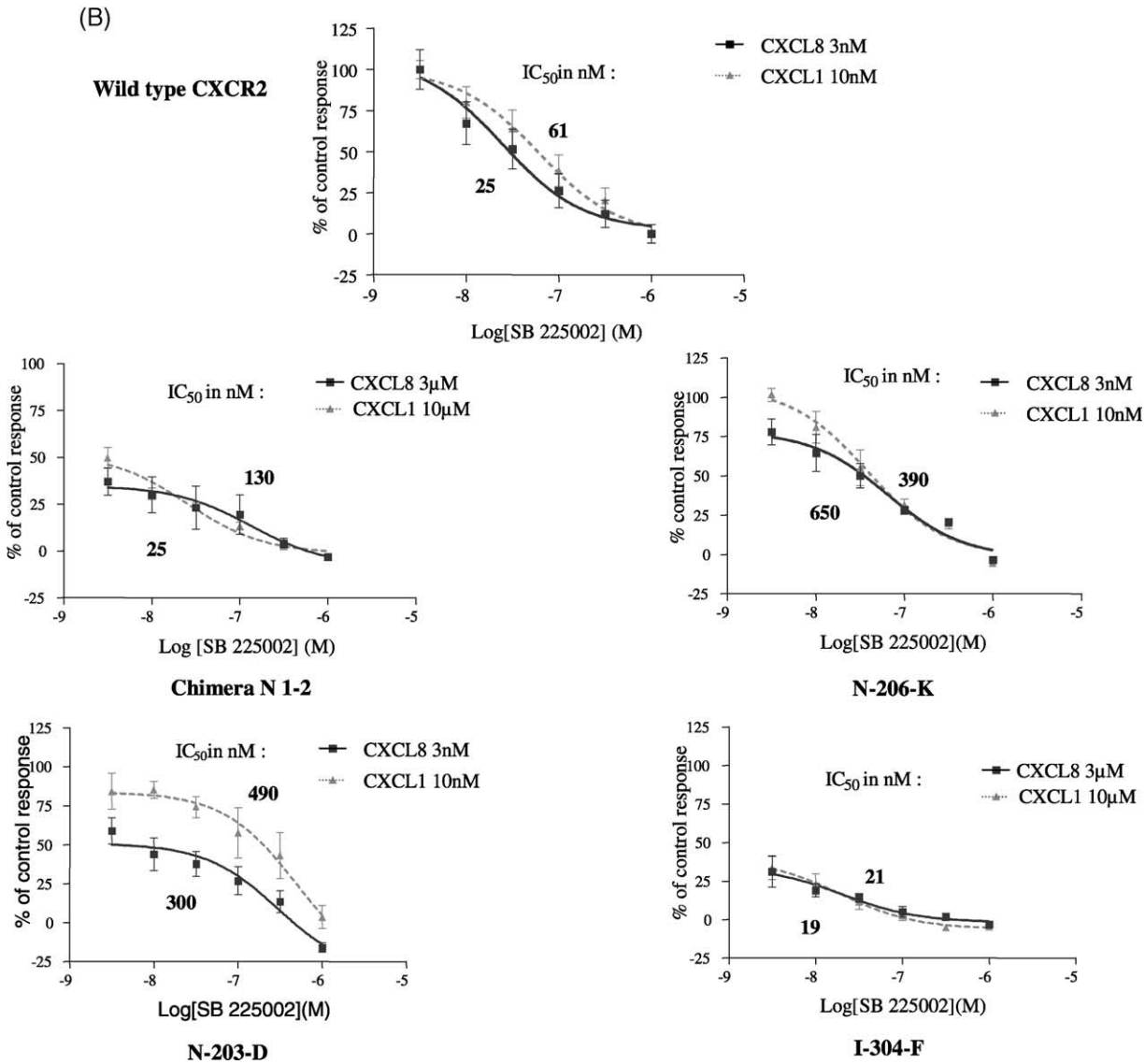

Fig. 3. Inhibition by SB 225002 of agonist-induced intracellular calcium release in BaF-3 cells. (A) Transfected BaF-3 cells were tested for their ability to release intracellular calcium in response to various concentrations of CXCL8 or CXCL1 (3–1000 nM) and to evaluate the ability of SB 225002 to inhibit it. The data are expressed as arbitrary fluorescence units normalized to the initial baseline level and maximum calcium release (cf. Section 2). EC_{50} were determined by non-linear regression using GraphPad Prism software. The values are the means from three independent experiments in duplicate. NR: no response for agonist concentration $\leq$1 μM, >100: response observed with too high dose stimulation for determination of an EC_{50}. (B) Inhibition of calcium mobilization in BaF-3 cells, stably transfected with the indicated protein, stimulated with an EC_{50} concentration of CXCL8 or CXCL 1 (values are indicated on each panel) after a 2 min pre-treatment with the indicated concentration of SB 225002. Data represent means ± SEM of three independent experiments (each in duplicate). Responses are expressed in percent of the maximal calcium release induced by Ionophore A23187 (cf. Section 2).

using the wild-type CXCR2, whereas an 8-fold reduction in the antagonist activity was found with both mutants N-203-D and N-206-K. Even at the lowest concentration (3×10^{-8} M), the calcium released from the stimulation of the chimera N1-2 and the mutant I-304-F, was greatly reduced when compared to the wild-type CXCR2. This observation is of particular interest in respect to I-304-F which did not exhibit any alteration in its ability to bind SB 225002 regardless the use of CXCL8 and CXCL1 as a ligand. Although the mutation I-304-F affected the capacity of CXCR2 to release calcium upon stimulation, it did not affect the inhibitory effect of SB 225002. However, given the weak responses induced by the binding of either CXCL8 or CXCL1, it is difficult to draw a conclusion from experiments using N1-2 and I-304-F. These results support the view that inhibition of intracellular calcium release by SB 225002 was clearly linked to different amino acids than those likely involved in its binding, since it is possible to observe: (1) strong binding affinity with weak inhibition capacity (N-203-D or N-206-K for CXCL1), (2) weak binding with strong efficiency in inhibition of activation of the cell (N1-2 with CXCL8) or (3) weak binding and activation inhibition (N-203-D with CXCL8). The second EC participated in the efficient binding (i.e. binding and signaling) of SB 225002. In contrast, the N-terminus of CXCR2 was probably not involved in its activation: SB 225002 had a weak affinity for chimera N1-2 (IC_{50}: 331 nM) but its inhibition of signal transduction, was almost as efficient as the inhibition observed in wild-type CXCR2, (IC_{50} against CXCL8 and CXCL1: 25 and 61 nM vs. 130 and 25 nM, respectively). Further studies aiming at screening drugs against CXCR2 and modeling drug–receptor interactions should pay a particular attention to the second EC.

3.6. Concluding remarks

Several structure–activity studies on GPCR (human β2 adrenergic receptor, human NK-1 receptor, human B_2 receptor, endothelin A or B receptors, neurotensin receptor-1) underlined the predominant role of transmembrane domains for the binding and action of non-peptide antagonists. These studies, in conjunction with molecular modeling, proposed a tentative binding pocket for non-peptide antagonists within the seven transmembrane domains. Nevertheless, the position of amino acids identified to be important in the binding of non-peptide antagonists to CXCR4 are quite similar to our results that implicate the second EC [25]. Thus, we postulate that ECs of chemokine receptors have a more crucial role in receptor binding and activation than in other GPCRs.

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