CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling

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The stromal cell-derived factor-1/CXCL12 chemokine engages the CXCR4 and CXCR7 receptors and regulates homeostatic and pathologic processes, including organogenesis, leukocyte homeostasis, and tumorigenesis. Both receptors are widely expressed in mammalian cells, but how they cooperate to respond to CXCL12 is not well understood. Here, we show that CXCR7 per se does not trigger $G_{\alpha i}$ protein–dependent signaling, although energy transfer assays indicate that it constitutively interacts with $G_{\alpha i}$ proteins and undergoes CXCL12-mediated conformational changes. Moreover, when CXCR4 and CXCR7 are coexpressed, we show that receptor heterodimers form as efficiently as receptor homodimers, thus opening the possibility that CXCR4/CXCR7 heterodimer formation has consequences on CXCL12-mediated signals. Indeed, expression of CXCR7 induces conformational rearrangements within preassembled CXCR4/$G_{\alpha i}$ protein complexes and impairs CXCR4-promoted $G_{\alpha i}$-protein activation and calcium responses. Varying CXCR7 expression levels and blocking CXCL12/CXCR7 interactions in primary T cells suggest that CXCR4/CXCR7 heterodimers form in primary lymphocytes and regulate CXCL12-promoted chemotaxis. Taken together, these results identify CXCR4/CXCR7 heterodimers as distinct functional units with novel properties, which can contribute to the functional plasticity of CXCL12. (Blood. 2009; 113:6085-6093)

Introduction

The chemokine stromal cell-derived factor-1/CXCL12 (SDF-1/CXCL12) is expressed in hematopoietic and nonhematopoietic tissues and was originally identified from bone marrow (BM) stromal cells as a pre-B cell growth factor. CXCL12 is essential for heart, gonad, nervous system, and blood vessel development, and mice with targeted deletion of the *Cxcl12* gene die perinatally.[1-3] In postnatal life, CXCL12 regulates the BM homing and retention of $CD34^+$ progenitor cells, the transendothelial migration of leukocytes, and their lymphoid and peripheral trafficking.[4,5] CXCL12 is also involved in pathologic processes, including development of primary epithelial tumors, where it regulates proliferation and survival of tumor cells, tumor angiogenesis, and metastasis. CXCL12 receptors, that is, CXC chemokine receptors 4 (CXCR4) and 7 (CXCR7), display a wide expression pattern in mammalian tissues. They are coexpressed in T- and B-cell subsets, endothelial cells, spinal ganglia, descending neurons, and human renal progenitor cells and in some tumor cells, primary human tumors, and tumor-associated endothelial cells (ie, breast, lung, and prostate).[6-12]

Both receptors can differentially contribute to CXCL12-mediated responses, as recently illustrated for the CXCL12-dependent homing of human renal progenitor cells with CXCR7 being involved in cell survival and cell adhesion to endothelium and CXCR4 in chemotaxis.[6] Characterization of CXCR7-deficient mice pointed to a dedicated role for CXCR7 in fetal endothelial biology, cardiac development, and B-cell localization.[12,13] CXCR7 also displays the propensity to modulate CXCR4 functions. This is suggested from the observation that expression of CXCR7 in mature B cells inversely correlates with the activity of CXCR4.[8] In zebrafish, recent works identified CXCR7 as a critical regulator of CXCR4-mediated migration of primordial germ cells[14] and the lateral-line primordium,[15] by CXCL12 scavenging that sharpens the chemokine concentration in the extracellular environment.[14,16] In T lymphocytes, CXCR7 is critical for CXCR4 to mediate CXCL12-dependent integrin activation.[17] Such a modulation was hypothesized to involve physical interaction of both receptors. In line with this, a recent study suggested that expression of CXCR4 and CXCR7 in HEK-293T cells correlates with heterodimer formation and a higher responsiveness to CXCL12 (ie, Ca^{2+} flux).[12] However, the molecular mechanisms underlying this process remain unknown.

Our earlier results suggested that CXCR7 is coexpressed with CXCR4 in primary human T lymphocytes and is implicated in the regulation of CXCL12-promoted cell migration.[10] In the present study, we characterize that ectopically expressed CXCR7 and CXCR4 form heterodimers as efficiently as homodimers, thus indicating that the relative receptor expression levels contribute to the occurrence of heterodimers. Using Bioluminescence Resonance Energy Transfer (BRET), we further demonstrate that CXCR7 expression induces conformational rearrangements within preexisting CXCR4-$G_{\alpha i}$ protein complexes. This phenomenon may account for our data showing that CXCR7 alters CXCR4-mediated $G_{\alpha i}$ protein activation and calcium responses. Finally, RNA interference targeting *CXCR7* and blockade of CXCL12/CXCR7 interaction indicate that heterodimers may form in primary T cells and contribute to chemotaxis toward CXCL12.

Submitted December 24, 2008; accepted April 8, 2009. Prepublished online as *Blood* First Edition paper, April 20, 2009; DOI 10.1182/blood-2008-12-196618.

The online version of this article contains a data supplement.

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Methods

Materials and cDNA constructs

MIP-1β/CCL4 and SDF-1/CXCL12 were provided by F. Baleux. The human SMARTpool scrambled control (SCR, siCONTROL nontargeting no. 1) and *CXCR7* siRNA duplexes were obtained from Dharmacon RNA Technologies (Lafayette, CO). The CXCR7 receptors tagged at the C-tail with *Renilla* luciferase (Rluc; CXCR7-Rluc) or Yellow Fluorescent Protein (YFP; CXCR7-YFP) were obtained by inserting in-frame the human *CXCR7* cDNA into the pcDNA3/CMV-Rluc or the pcDNA3/CMV/GFP Topaze vectors (a gift from Dr R. Jockers, Institut Cochin, Paris, France). CXCR4-Rluc, CXCR4-YFP, CXCR4-N119K-YFP, and the lentiviral vectors encoding human CCR5, CCR5-GFP, CXCR4, CXCR7, or CXCR7-GFP have been described.[10,18-20] Plasmids encoding Gβ1 and Gγ2, $G_{\alpha i1}$-Rluc,[21] and $G_{\alpha i1}$-60-Rluc[22] were provided by Drs R. Jockers, J. P. Pin (Institut de Génomique Fonctionnelle, Montpellier, France), and M. Bouvier (Université de Montréal, Montréal, QC), respectively. Guanosine 5′-0-(γ-thio) triphosphate (GTPγS) and pertussis toxin (PTX) were from Sigma-Aldrich (St Louis, MO).

Cell culture and transfection

HEK-293T cells were grown in culture medium (Dulbecco modified Eagle medium supplemented with 10% (vol/vol) fetal bovine serum, 4.5 g/L glucose, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 1 mM glutamine, and 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; all reagents are from Invitrogen, Cergy Pontoise, France). The CHO-K1 cell line stably expressing apoaequorin, $G_{\alpha 16}$ and CXCR4 (CHO-K1-CXCR4), was provided by Prof M. Parmentier (Université libre de Bruxelles, Bruxelles, Belgium). Human peripheral blood lymphocytes were isolated from heparin-treated blood samples of healthy blood donors, cultured, and activated, as described.[23] Transient expressions were achieved using Amaxa Nucleofector technology (Amaxa Biosystems, Gaithersburg, MD), the transfection reagent FuGene 6 (Roche Diagnostics, Basel, Switzerland), or the calcium phosphate precipitation method. When specified in the legends to the figures, stable expression of receptors was realized using a lentiviral-based strategy, as described.[23]

Flow cytometric analysis

Cell-surface expression of receptors was determined as described.[10] Staining was performed using the phycoerythrin-conjugated anti–human CXCR4 monoclonal antibodies (mAbs) 12G5 (BD Biosciences, San Jose, CA), allophycocyanin (clone RPAT4)–, and phycoerythrin (clone Leu-3a)–conjugated anti–human CD4 mAbs (BD Biosciences) or the anti–human RDC1/CXCR7 mAb 9C4 (a gift from Dr M. Thelen, Institute for Research in Biomedicine, Bellinzona, Switzerland). Analysis was carried out on a BD Biosciences FACSCalibur.

Functional assays

Intracellular calcium mobilization was analyzed using an aequorin-based assay, as described.[24] Briefly, apoaequorin-expressing cells were transfected or transduced to express receptors, incubated for 3 hours in the dark in the presence of 5 μM Coelenterazine h (Interchim, Montluçon, France), and then diluted 5-fold before use. Cells (25 000 cells/well) were added to variable concentrations of chemokines, and luminescence was measured for 30 seconds using the Mithras LB940 reader (Berthold Biotechnologies, Bad Wildbad, Germany). Functional parameters (half-maximal effective concentration [EC_{50}] and maximal response [E_{max}] values) were determined by nonlinear regression using a sigmoidal dose-response model with variable slope (GraphPad Software, San Diego, CA). [^{35}S]-GTP-γS binding assays and chemotaxis of T lymphocytes were carried out as previously described.[19,23,25]

BRET assay, luminescence, and fluorescence measurements

BRET assays, luminescence, and fluorescence measurements were performed as described previously.[18,26] Briefly, 48 hours after transfection, HEK-293T cells (1-2 × 10^5) were distributed in a 96-well microplate and incubated with 5 μM Coelenterazine h before being stimulated or not for the indicated times by 1 μM CXCL12 using the injection system of the lumi/fluorimeter Mithras LB940. For dose-response experiments (Figures S1, S4, available on the *Blood* website; see the Supplemental Materials link at the top of the online article), CXCL12 was added at the indicated concentration before adding Coelenterazine h. BRET values were immediately collected at 1-second intervals for 5 minutes using Mithras LB940, which allows the sequential integration of luminescence signals detected with 2 filters settings (Rluc filter, 485 ± 20 nm; and YFP filter, 530 ± 25 nm) as described previously.[18,26] Data were collected using the MicroWin2000 software, and BRET signal was expressed in milliBRET units (mBU), 1 mBU corresponding to the BRET ratio multiplied by 1000.

Statistical analysis

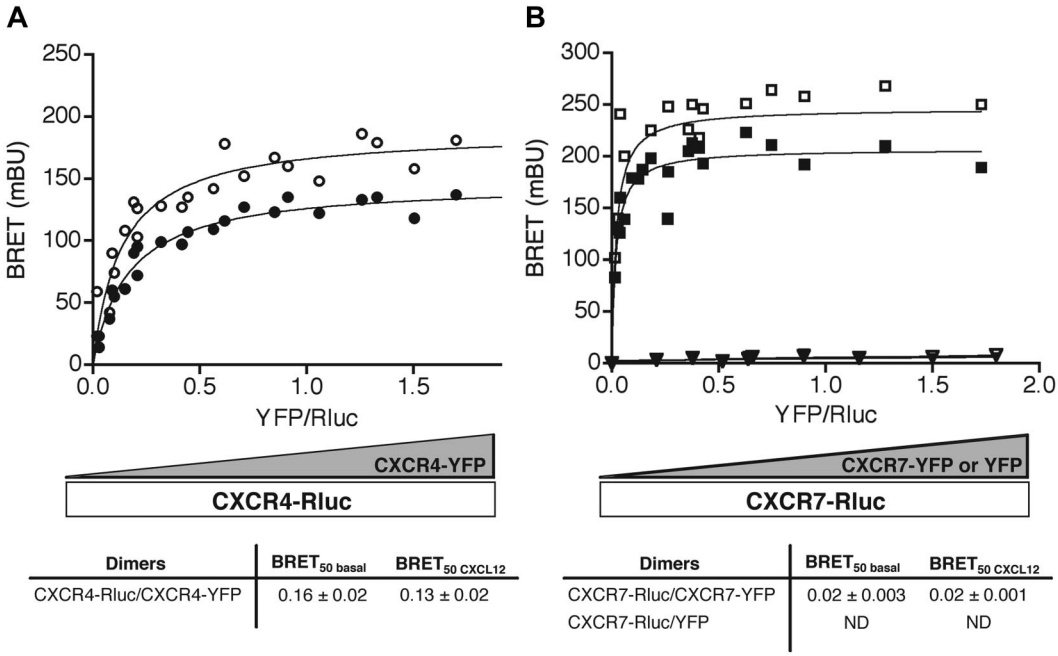
Results were analyzed by PRISM (GraphPad Software). Data are expressed as mean plus or minus SEM. Student *t* test was applied for statistical analysis.

Results

Homodimerization and heterodimerization of CXCR7

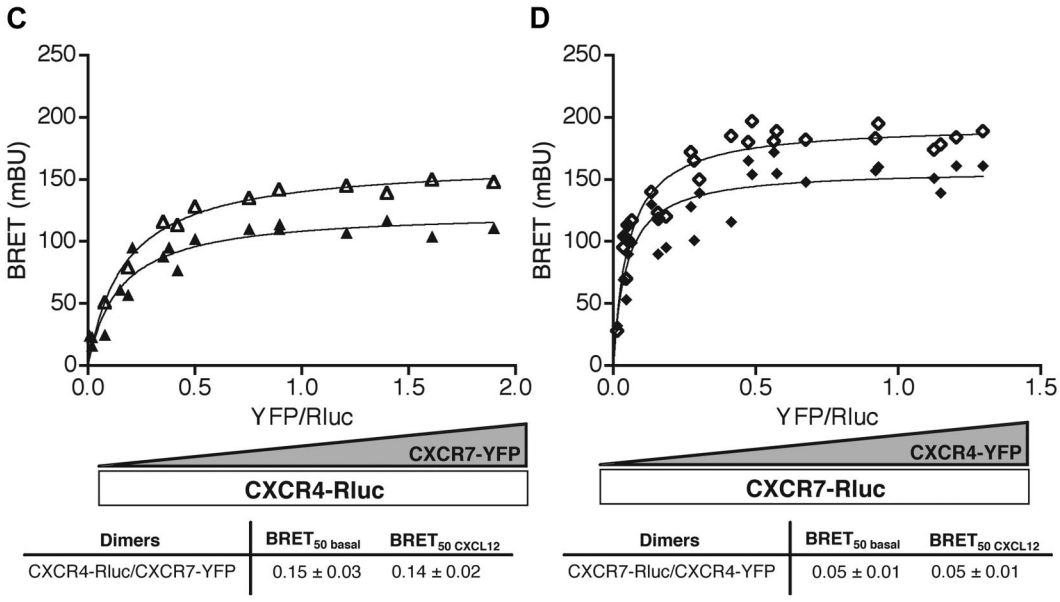
We have characterized the relative propensities of CXCR7 and CXCR4 to homodimerize and heterodimerize using a BRET-based titration assay.[18,27,28] CXCR4 or CXCR7 was fused to the energy donor Rluc and then expressed at a constant amount in the presence of increasing quantities of either receptors fused to the BRET acceptor YFP. Fusion of the tag to the C-tail of receptors modified neither their expression levels nor their functions (ie, internalization; data not shown), in keeping with our previous findings.[18,23,28] In the cells expressing Rluc-tagged CXCR4 (Figure 1A,C) or CXCR7 (Figure 1B,D), increasing the concentration of either of the 2 receptors tagged with YFP resulted in BRET signals that increased hyperbolically, thus reaching an asymptote when all Rluc-tagged receptors are associated with those fused to YFP ($BRET_{max}$). In contrast, the BRET signal varied linearly with the YFP/Rluc ratio in cells coexpressing the Rluc-tagged receptor either with YFP alone (Figure 1B) or with the $GABA_{B2}$-YFP receptor (data not shown), as expected for nonspecific random interactions. These data indicate that CXCR7 and CXCR4 form constitutive homodimers and heterodimers when they are coexpressed in the same cell. The $BRET_{50}$ values, which correspond to the acceptor/donor ratios yielding 50% of the $BRET_{max}$ and represent the propensities of receptors to interact with one another,[27,29] were found to be comparable for homodimer and heterodimer formation. This suggests that CXCR4/CXCR7 heterodimers form with the same efficiency as the receptor homodimers, thus implying that the expression levels of CXCR4 and CXCR7 contribute to the relative abundance of homodimers and heterodimers.

Modulation of CXCR7 expression levels has been reported in various normal[8] and malignant cells[30] and, in some cases, is correlated with changes in CXCL12 responses.[6,12,17,31] Although different mechanisms can be put forward to account for these changes,[14,16] this also raises the possibility that homodimers and heterodimers respond differentially to CXCL12. To address this possibility, we first used the BRET assay to monitor conformational changes in the receptor dimers upon CXCL12 stimulation, as we have shown for CXCR4 mutant receptor dimers.[20] Indeed, efficiency of energy transfer depends on the relative orientation



Dimers	$BRET_{50\ basal}$	$BRET_{50\ CXCL12}$
CXCR4-Rluc/CXCR4-YFP	0.16 ± 0.02	0.13 ± 0.02

Dimers	$BRET_{50\ basal}$	$BRET_{50\ CXCL12}$
CXCR7-Rluc/CXCR7-YFP	0.02 ± 0.003	0.02 ± 0.001
CXCR7-Rluc/YFP	ND	ND



Dimers	$BRET_{50\ basal}$	$BRET_{50\ CXCL12}$
CXCR4-Rluc/CXCR7-YFP	0.15 ± 0.03	0.14 ± 0.02

Dimers	$BRET_{50\ basal}$	$BRET_{50\ CXCL12}$
CXCR7-Rluc/CXCR4-YFP	0.05 ± 0.01	0.05 ± 0.01

Figure 1. CXCR4 and CXCR7 form homodimers and heterodimers in living HEK-293T cells. (A-D) BRET titration curves were generated in cells expressing a constant amount of either CXCR4-Rluc (A,C) or CXCR7-Rluc (B,D) and increasing quantities of YFP or YFP-tagged receptors, stimulated (open symbols) or not (closed symbols) with 1 μM CXCL12. $BRET_{50}$ values presented in the tables were deduced from data analysis using a nonlinear regression equation applied to a single binding site model and are representative of 3 to 5 independent experiments. $BRET_{max}$ signals from cells containing CXCR4 or CXCR7 homodimers or heterodimers were significantly increased by CXCL12 compared with basal conditions ($P < .05$).

and the distance between the BRET tags, 2 parameters that can be modulated upon the agonist-induced conformational rearrangements leading to receptor activation and interaction with signaling molecules. We found that CXCL12 increased the $BRET_{max}$ signal in a dose-dependent manner (Figure S1) in cells containing CXCR4 homodimers (Figure 1A), CXCR7 homodimers (Figure 1B), and CXCR4/CXCR7 heterodimers (Figure 1C,D), but not the unspecific signal measured in cells coexpressing CXCR7 and the negative control YFP (Figure 1B). Comparable $BRET_{50}$ values indicate that CXCL12 modifies the conformation of preformed dimers at the cell surface rather than the number of dimers. Our results thus support the view that CXCL12 does not behave as a neutral ligand once bound to CXCR7 homodimers and heterodimers. Rather, CXCL12 engagement to CXCR7 stabilizes receptor conformational states that may be associated with signal transduction processes.

CXCR7 does not trigger calcium responses but interferes with that of CXCR4

To assess homodimer and heterodimer functional properties, mobilization of intracellular calcium in response to CXCL12 was investigated in cells expressing CXCR4, CXCR7, or both receptors. CXCL12 was found to induce a rapid and transient rise of calcium level in CXCR4-expressing HEK-293T cells but not in cells expressing CXCR7 alone (Figure 2A). These results

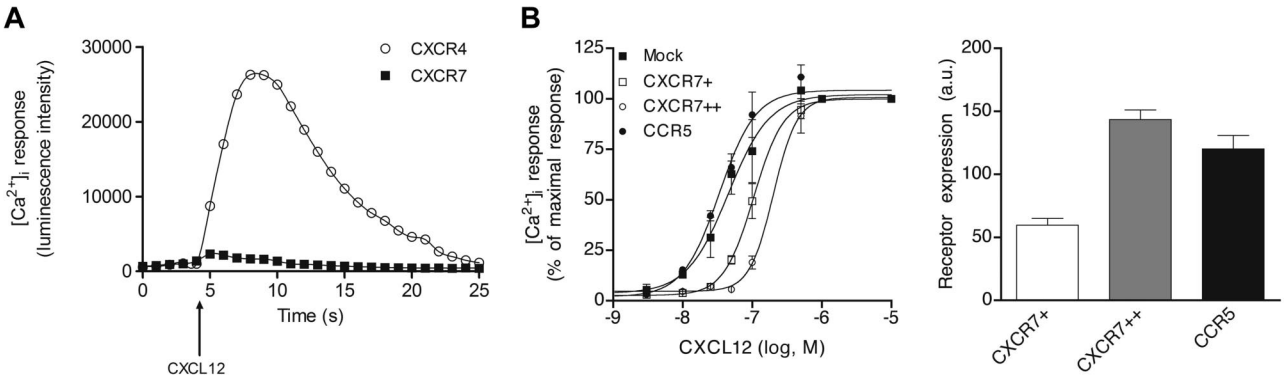


Figure 2. CXCR7 does not trigger calcium mobilization in response to CXCL12 and modulates that of CXCR4. (A) Representative Ca^{2+} responses triggered by 1 μM CXCL12 in HEK-293T cells transfected with a $G_{\alpha 16}$-apoaequorin encoding vector and either CXCR4 or CXCR7. (B) Calcium mobilization assay in CHO-K1-CXCR4 cells expressing or not either CXCR7-GFP or CCR5-GFP (left panel). Cells were loaded with Coelenterazine h, and $[Ca^{2+}]_i$ levels were measured after exposure to the indicated CXCL12 concentrations. Values (mean ± SEM, n = 3) are expressed as percentage of the maximal response. CXCR7 and CCR5 cell surface expression levels were determined by fluorescence intensity measurements (right panel). EC_{50} values for CXCL12 were significantly different in the presence of CXCR7 (CXCR4/CXCR7^{+} or CXCR4/CXCR7^{++} cells) compared with cells expressing CXCR4 alone ($P < .05$).

are in accordance with previous reports showing that CXCL12 binding to CXCR7 does not mediate typical G protein-dependent signaling.[11,12]

We next investigated the functional consequences of CXCR4/CXCR7 interactions upon expression of increasing amounts of GFP-tagged CXCR7 in CHO-K1 cells stably expressing CXCR4. Dose-response curves (Figure 2B left panel) indicate that CXCL12 potency decreases in a dose-dependent manner with increasing amounts of CXCR7, with EC_{50} values for the chemokine varying from 45 plus or minus 7 nM in the absence of CXCR7 up to 200 plus or minus 18 nM at the highest CXCR7 expression level. The maximal efficacy of CXCL12 remained the same in the presence of CXCR7 (Figure 3; and data not shown). In control experiments, we expressed the chemokine receptor CCR5-GFP, which also forms heterodimers with CXCR4[32] (and data not shown), at levels similar to those of CXCR7-GFP, as determined by fluorescence intensity (Figure 2B right panel). As shown in Figure 2B, expression of CCR5 did not alter CXCL12-induced calcium responses, thus demonstrating selectivity of the CXCR7 effect. Collectively, our data show that CXCR7, which does not govern per se calcium responses upon CXCL12 engagement, displays the capacity to alter CXCR4 signaling presumably through formation of CXCR4/CXCR7 complexes. We also noted that the hill slope values, which describe the steepness of sigmoidal dose-response curves, were consistently increased in the presence of CXCR7 (HiPP number $n_H = 1.4$ vs 2.5; see also Figure 3), thus suggesting

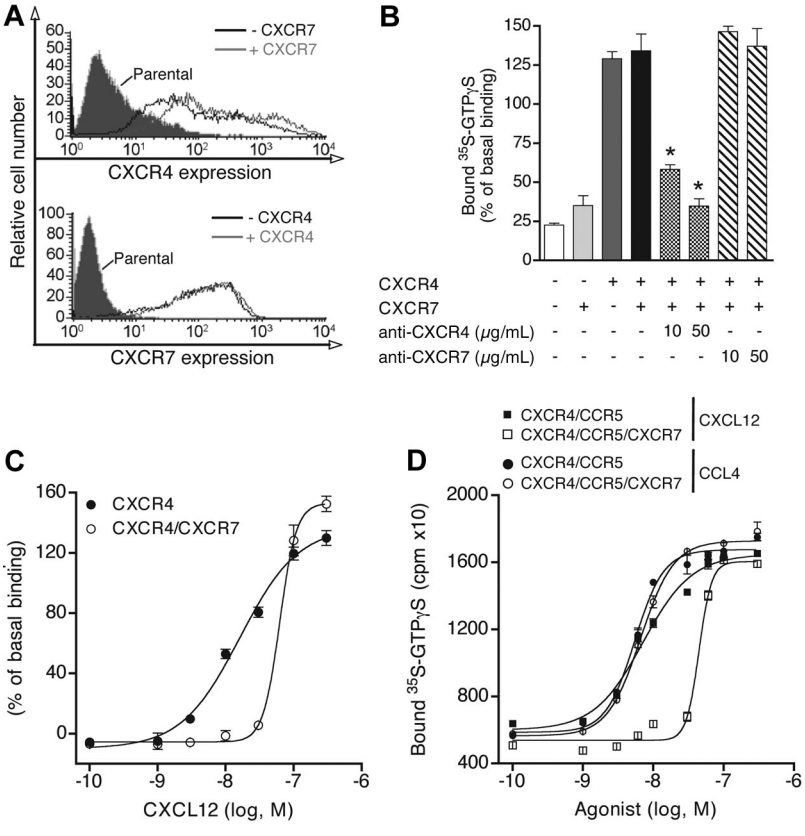


Figure 3. CXCR7 selectively interferes with CXCR4-mediated G protein activation. (A) CXCR7 expression was achieved in parental HEK-293T cells or CXCR4-expressing cells using a lentiviral-based strategy. Cell surface expression levels of CXCR4 (top panel) and CXCR7 (bottom panel) in parental (filled histograms) or cells expressing either (black histograms) or both (light gray histograms) receptors were determined by flow cytometry. (B) One hundred nanomoles of CXCL12-induced ^{35}S-GTP-γS binding to membranes from parental cells or cells expressing CXCR4, CXCR7, or both receptors, in the absence or presence of anti-CXCR4 12G5 or anti-CXCR7 9C4 mAbs at the indicated concentrations. Results are expressed as percentage of the basal binding measured in the absence of ligand. *$P < .05$, compared with CXCL12-induced binding to CXCR4- and CXCR7-expressing membranes without the anti-CXCR4 12G5 mAb. (C) ^{35}S-GTP-γS binding to membranes from cells expressing either CXCR4 alone or with CXCR7 in the presence of increasing CXCL12 concentrations. Results (mean ± SEM) are representative from 5 independent experiments performed in duplicate. EC_{50} values were significantly different in the presence of CXCR7 compared with membranes from cells expressing CXCR4 alone ($P < .01$). (D) CXCR7 expression was achieved in HEK-293T cells coexpressing CD4, CXCR4, and CCR5 using a lentiviral-based strategy. ^{35}S-GTP-γS binding to membranes was measured in response to CXCL12 or the CCR5 agonist CCL4/MIP-1β at the indicated concentrations. Representative results of 3 independent experiments are shown. EC_{50} values were significantly different in the presence of CXCR7 compared with control conditions with CXCR4 and CCR5 alone ($P < .05$).

that these complexes exhibit unique functional properties compared with homodimers.

CXCR7 interferes with CXCR4-mediated G protein activation

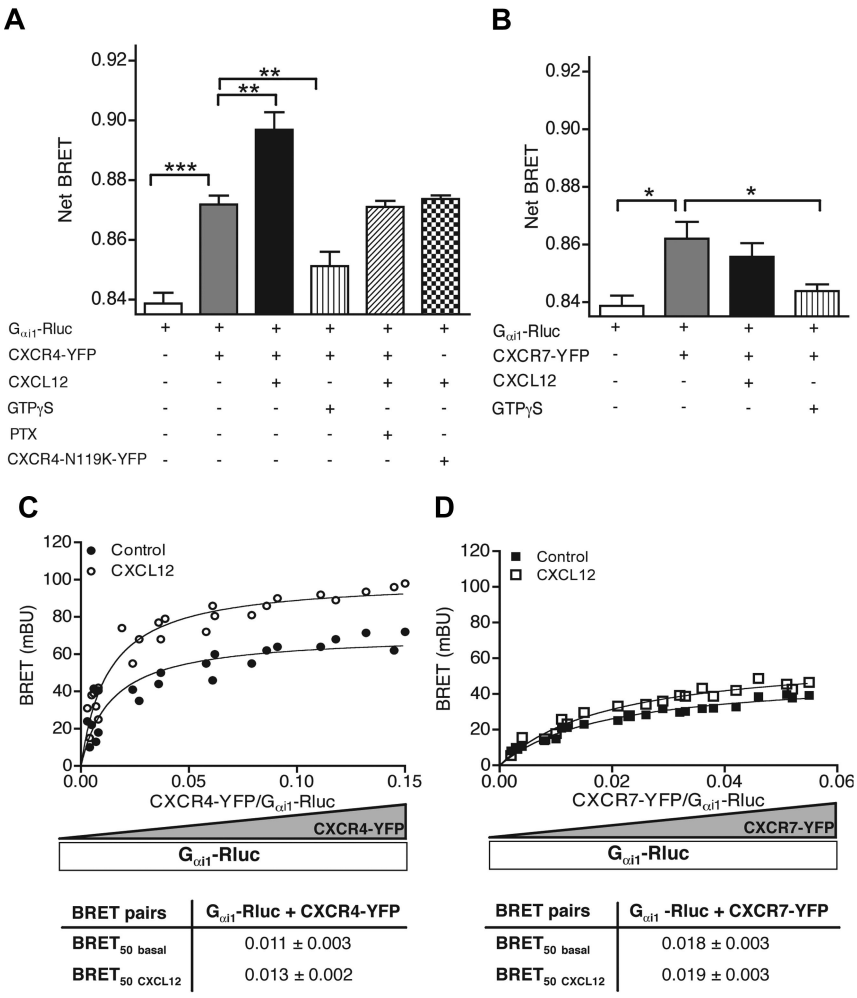
Alteration of CXCL12-mediated calcium responses in the presence of CXCR7 opened the possibility that CXCR4/CXCR7 heterodimers display an impaired ability to activate G proteins. We thus measured G protein activation upon CXCL12 stimulation using a ^{35}S-GTP-γS binding assay carried out on crude membranes from parental HEK-293T cells or these cells stably expressing CXCR4, CXCR7, or both receptors (Figure 3). Expression of CXCR7 had a marginal effect on the CXCR4 expression levels at the cell surface and conversely (Figure 3A). Stimulation of membranes from parental cells with CXCL12 at a saturating concentration led to a 25% increase in GTP-γS binding (Figure 3B), which relies on G protein activation by endogenous CXCR4. Expressing CXCR7 did not further enhance the GTP-γS binding, thus indicating that the receptor is unable to activate G proteins in response to CXCL12. The magnitude of G protein activation was 5-fold increased in membranes from cells stably expressing CXCR4 and was not changed by the subsequent expression of CXCR7. Therefore, these results indicate that the maximal efficacy of CXCL12 does not vary when CXCR4 associates with CXCR7. We also found that GTP-γS binding to membranes from CXCR4/CXCR7-expressing cells was inhibited in a dose-dependent manner by the neutralizing anti-CXCR4 mAb 12G5,[33,34] but not by the anti-CXCR7 mAb 9C4 (Figure 3B), thus supporting the conclusion that G protein activation downstream of CXCR4/CXCR7 heterodimers requires binding of CXCL12 to CXCR4. This is reminiscent of data with the leukotriene B4 receptor indicating that G protein signaling from a dimer occurs through the protomer where the agonist binds.[35] When dose-response curves to CXCL12 were performed (Figure 3C), data resembled those from the calcium mobilization assays with steeper and more right-shifted curves of the chemokine-promoted ^{35}S-GTP-γS binding in the presence of CXCR7 (EC_{50} = 16 ± 2 nM and n_H = 1 ± 0.1 nM vs EC_{50} = 63 ± 8 nM and n_H = 3.5 ± 0.5 nM in the absence or the presence of CXCR7, respectively). Agonist binding to receptors can be linked to cross-conformational changes of other receptors in heterodimers, thereby accounting for differential signaling pathways activated downstream of heterodimers and homodimers.[35,36] However, CXCR7-mediated conformational changes of CXCR4 may not be dependent on CXCL12 engagement to CXCR7, as revealed by the anti-CXCR7 mAb 9C4, which prevents CXCL12 from binding to CXCR7[10] but does not alter the effects this receptor has on dose-response curves of G protein activation by CXCL12 (Figure S2). Receptors can alter G protein–dependent signaling mediated by other receptors through sequestration of G proteins.[37] CXCR4 interacts with the $G_{\alpha i}$ subtype of G proteins, but we dismissed the possibility that CXCR7 sequesters these proteins. Indeed, CXCR7 did not affect G protein activation mediated by CCR5, another $G_{\alpha i}$-coupled receptor (Figure 3D; EC_{50} = 5.5 ± 0.4 nM and n_H = 2.1 ± 0.3 nM vs EC_{50} = 6.6 ± 0.3 nM and n_H = 1.8 ± 0.15 nM in the absence or the presence of CXCR7, respectively). Finally, we also observed that CXCR7 expression did not alter infection of CD4-containing HEK-293T cells by the X4-tropic HIV strain NL4-3 (Figure S3). Overall, the results are consistent with the view that heterodimerization with CXCR7 selectively impairs CXCR4 in its ability to activate G proteins after CXCL12 binding, whereas other functions of CXCR4 may be preserved.

Heterodimerization with CXCR7 changes CXCR4 in its ability to interact with G proteins

We next turned to BRET to examine in living cells whether CXCR7 could alter CXCR4/$G_{\alpha i}$ protein interaction. We used the $G_{\alpha i1}$-Rluc fusion protein previously described,[21] which was coexpressed with cognate β and γ subunits for proper targeting of functional heterotrimeric G protein complexes to the cell membrane.[38] Expressing CXCR4-YFP (Figure 4A) together with $G_{\alpha i1}$-Rluc resulted in a significant BRET signal indicating that the receptor constitutively interacts with $G_{\alpha i1}$ proteins. Surprisingly, we found that $G_{\alpha i1}$-Rluc also constitutively interacts with CXCR7-YFP (Figure 4B). BRET-titration experiments indicated that $G_{\alpha i1}$-Rluc associates as efficiently with CXCR4 as with CXCR7, as revealed by similar $BRET_{50}$ values from cells expressing CXCR4-YFP ($BRET_{50\ basal}$ = 0.011 ± 0.003) or CXCR7-YFP ($BRET_{50\ basal}$ = 0.018 ± 0.003; Figure 4C,D). Basal BRET signals decreased on addition of GTP-γS (Figure 4A,B), a nonhydrolyzable GTP analog that precludes inactivation of G proteins and thus limits the amount of GDP-linked α subunits available for interaction with the receptor. These data indicate that both CXCR4 and CXCR7 are associated basally with inactive $G_{\alpha i}$ proteins, as reported for other G protein–coupled receptors (GPCRs).[21,22,38-40] Stimulation of CXCR4-YFP with CXCL12 increased the $BRET_{max}$ signals between the receptor and $G_{\alpha i1}$-Rluc (Figure 4A,C) without affecting the $BRET_{50}$ values (Figure 4C). This indicates that the chemokine induces conformational rearrangements within preassembled CXCR4-YFP/$G_{\alpha i1}$-Rluc complexes, rather than a recruitment of G proteins to the receptor. These conformational changes have been proposed to be set in motion during the G protein activation process.[21,22,38,40] In line with this, $G_{\alpha i1}$ inactivation by a PTX treatment or the use of the CXCR4-N119K mutant receptor, which cannot activate G proteins,[20] suppressed the rising of BRET in response to CXCL12 (Figure 4A). In addition, CXCL12 increased BRET between CXCR4 and $G_{\alpha i1}$ in a dose-dependent manner (Figure S4), with an EC_{50} value (19.5 ± 2.2 nM) in the same range as those measured in ^{35}S-GTP-γS binding (EC_{50} = 16 ± 2 nM) and calcium (EC_{50} = 45 ± 7 nM) assays (Figures 2B, 3C). These observations further support a direct link between agonist-induced conformational reorganization of CXCR4-$G_{\alpha i1}$ protein complexes and G protein activation.

The chemokine failed to enhance BRET in CXCR7-expressing cells (Figure 4B,D), and BRET kinetic analysis in real-time ruled out the possibility that CXCL12-mediated conformational changes in the CXCR7-$G_{\alpha i}$ protein complex could occur at slower kinetic rates (Figure S5). The use of other $G_{\alpha i1}$ chimeric proteins gave similar results (Figure S5). Overall, these data indicate that CXCR7 can interact with $G_{\alpha i}$ proteins but is unable to activate them in agreement with our functional assays (Figures 2,3).

We then investigated whether CXCR4-$G_{\alpha i1}$ protein interactions are modulated in the presence of CXCR7. Expression of CXCR7 decreased the $BRET_{max}$ signal between CXCR4-YFP and $G_{\alpha i1}$-Rluc but did not modify the $BRET_{50}$ values. This indicates that CXCR7 modifies the relative orientation and/or distance between CXCR4 and $G_{\alpha i1}$ but does not induce $G_{\alpha i}$ protein sequestration (Figure 5). Finally, CXCL12-induced increases of BRET values are preserved in heterodimer-expressing cells, which is in keeping with the fact that heterodimers maintain functional responses to the chemokine (Figures 2, 3). Collectively, these data are consistent with the view that CXCR7 induces conformational rearrangements within



BRET pairs	$G_{\alpha i1}$-Rluc + CXCR4-YFP
$BRET_{50\ basal}$	0.011 ± 0.003
$BRET_{50\ CXCL12}$	0.013 ± 0.002

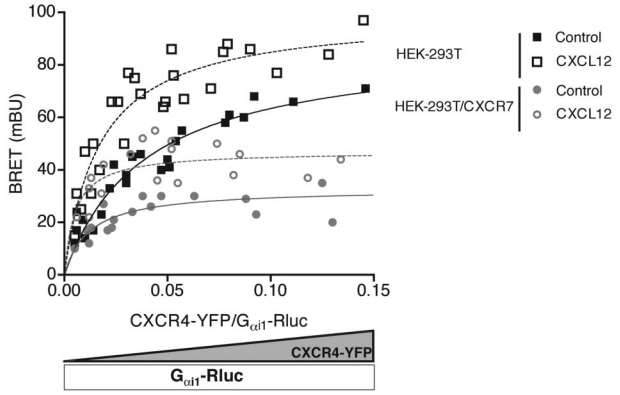
BRET pairs	$G_{\alpha i1}$ -Rluc + CXCR7-YFP
$BRET_{50\ basal}$	0.018 ± 0.003
$BRET_{50\ CXCL12}$	0.019 ± 0.003

Figure 4. CXCR7 constitutively interacts with, but does not activate, $G_{\alpha i1}$ proteins. (A,B) BRET signal between $G_{\alpha i1}$-Rluc and either CXCR4-YFP (A), CXCR4-N119K-YFP (A), or CXCR7-YFP (B) was measured 48 hours after transfection of HEK-293T cells, after preincubation or not with GTP-γS (200 μM) for 90 minutes at 25°C or PTX (100 ng/mL) overnight at 37°C and stimulation or not by 1 μM CXCL12. (C,D) BRET saturation assays were performed on cells transfected with a constant amount of the $G_{\alpha i1}$-Rluc fusion protein and increasing amounts of CXCR4-YFP (C) or CXCR7-YFP (D). BRET signals were determined in the absence (control) or presence of 1 μM CXCL12. Data represent 3 independent experiments. (C) $BRET_{max}$ signals were significantly increased by CXCL12 compared with basal conditions ($P < .05$). ***$P < .001$; **$P < .01$; *$P < .05$.

preassembled CXCR4/G protein complexes, which ultimately may account for the altered CXCL12-promoted calcium mobilization response.

CXCR7 regulates CXCL12-promoted chemotaxis of T lymphocytes

Chemotaxis mediated by CXCR4 depends on the release of Gβγ subunits from activated $G_{\alpha i}$ proteins and is abrogated by PTX,[18,41] so that we hypothesized that impairment of CXCR4 coupling to G proteins in the presence of CXCR7 might have consequences on cell migration to CXCL12. T lymphocytes express both receptors, thus making these primary cells as a suitable model for investigating the consequences of CXCR4/CXCR7 heterodimer formation on CXCL12 responses.[8,17] Indeed, CXCR7 is proposed to be transiently targeted to the plasma membrane of T cells, although a substantial pool of the receptor localizes intracellularly.[17] Of note, our previous work already mentioned that mAb-mediated blockade of CXCR7 engagement by CXCL12 resulted in the partial inhibition of T lymphocyte migration to CXCL12, thus further emphasizing that CXCR7 plays functional roles at the plasma membrane. Here, we used *CXCR7*-siRNA duplexes, which allowed us to reduce the levels of *CXCR7* transcripts by 40%, as determined by real-time polymerase chain reaction, and did not affect CXCR4 expression (data not shown). The decreased expression of *CXCR7* mRNAs led to an increased chemotaxis at a low CXCL12 concentration (ie, 0.3 nM) but had no effect at higher concentrations of the chemokine (Figure 6A). Thus, these results are reminiscent of the effects of CXCR7 in diminishing sensitivity of CXCR4 to low concentrations of CXCL12 for inducing G protein activation and calcium mobilization (Figures 2, 3). Somewhat different results were obtained when we used the anti-CXCR7 mAb 9C4 as a blocker of CXCL12-induced chemotaxis of T cells (Figure 6B). Indeed, in contrast to siRNA against *CXCR7*, 9C4 mAb does not affect the receptor ability to regulate CXCR4 signaling into heterodimers (Figure S2). As shown in Figure 6B, we reproduced our results that 10 μg/mL of the 9C4 mAb reduces chemotaxis of T cells toward 30 nM CXCL12,[10] but we also observed that cell migration was not affected (3 nM) or even significantly increased at a lower concentration of the chemokine (0.3 nM). The results provide an explanation for why anti-CXCR7 mAbs can differentially affect chemotaxis of T cells depending on the CXCL12 concentration used.[17] They also make it unlikely that the 9C4-induced decrease of chemotaxis at 30 nM CXCL12 results from inhibition of CXCR7 in its ability to mediate per se migration of T lymphocytes. Rather, the data suggest that preventing CXCL12/CXCR7 interaction mainly acts by shifting the chemotactic curve to the left and as this curve is known to be bell-shaped, this results in an increased migration at low CXCL12 concentrations and the reverse at high concentrations. These results are consistent with a recently proposed model where CXCR7 modulates CXCR4 signaling by scavenging CXCL12 and thus reducing the availability of the chemokine in the extracellular environment.[14] Overall, this suggests that CXCR7 can modulate CXCL12-mediated chemotaxis



BRET pairs	$G_{\alpha i1}$ -Rluc + CXCR4-YFP	
	HEK-293T	HEK-293T/CXCR7
$BRET_{50\ basal}$	0.017 ± 0.003	0.014 ± 0.004
$BRET_{50\ CXCL12}$	0.018 ± 0.001	0.012 ± 0.002

Figure 5. Heterodimerization with CXCR7 changes the ability of CXCR4 to interact with $G_{\alpha i1}$ proteins. BRET saturation assays were performed in parental (HEK-293T) or in CXCR7-expressing (HEK-293T/CXCR7) cells by transfecting a constant amount of the $G_{\alpha i1}$-Rluc fusion protein and increasing amounts of CXCR4-YFP, in the presence or absence of 1 μM CXCL12. The curves obtained were fitted and BRET signals were determined. Data represent 3 independent experiments. In parental or in CXCR7-expressing cells, $BRET_{max}$ signals were significantly increased by CXCL12 compared with basal conditions ($P < .05$). $BRET_{max}$ signals were significantly different in the presence of CXCR7 compared with parental cells in basal conditions and in the presence of CXCL12 ($P < .01$).

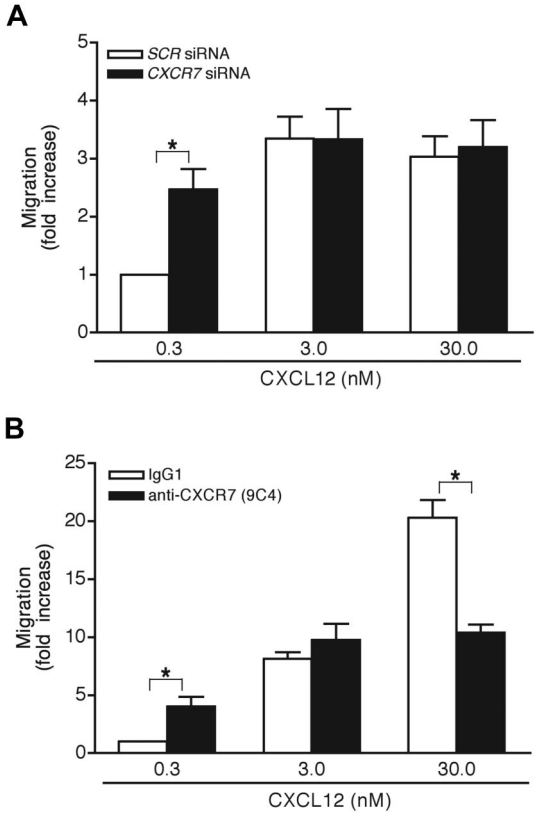


Figure 6. CXCR7 regulates CXCL12-promoted chemotaxis of T lymphocytes. (A) T lymphocytes were nucleoporated with 5 μg *SCR* or *CXCR7* siRNAs and tested for their ability to migrate in response to CXCL12 at the indicated concentrations. Data represent 3 independent experiments performed in duplicate. (B) T lymphocytes were tested for their ability to migrate in response to CXCL12 at the indicated concentrations in the presence of either mouse isotype control (IgG1) or the anti-CXCR7 mAb (9C4) at 10 μg/mL. The amount of input $CD4^+$-gated T cells that migrated to the lower chamber was compared with that of *SCR* siRNA- (A) or IgG1-treated cells (B) that migrated toward 0.3 nM CXCL12 (arbitrarily set at 1, and accounting for, on average, 3% of input cells). Data represent 3 independent experiments. *$P < .05$, compared with lymphocytes nucleoporated with *SCR* siRNA or incubated with IgG1.

of T lymphocytes both by sequestering CXCL12 and regulating CXCR4-promoted activation of G proteins.

Discussion

CXCR4 occupied by CXCL12 activates heterotrimeric Gαβγ proteins, which then trigger numerous downstream responses, such as calcium mobilization, actin polymerization, integrin-mediated adhesion, gene transcription, and proliferation. Signal transduction was initially thought to originate from sequential events, including recruitment of inactive G proteins to activated receptors, exchange of GDP for GTP on the G_α subunit, and then dissociation of G protein subunits from the receptor. In the present study, we found out that part of CXCR4 receptors constitutively interact with inactive, GDP-bound G_α subunits so that G protein activation arises from conformational changes within preassembled receptor—G protein complexes (Figure 4). Moreover, our kinetic analysis (Figure S5) revealed that these complexes do not disassemble after activation and persist over time. Similar observations have been reported for other receptor systems and more probably reflect general properties of GPCR functioning.[21,22,38,40] CXCR7 also constitutively interacts with inactive G proteins but dramatically fails to activate them and to mobilize intracellular calcium once engaged by CXCL12. Similarly, loss-of-function phenotypes have been reported for some mutant GPCRs that maintain coupling to G proteins, including GPR54 with a disease-causing single mutation in the second intracellular loop that alters the receptor conformational changes required for G protein activation.[37,42] This indicates that different structural determinants on GPCRs may be required for interaction with G proteins and G protein activation. Determinants in the sequence of CXCR7, which have been shown to be necessary for G protein–dependent signaling in other receptor systems,[43] might be involved in the $CXCR7/G_{\alpha i}$ protein interactions reported here. Although our present results are in keeping with others that also fail to demonstrate G protein signaling downstream of CXCR7,[8,11,12] CXCL12 engagement to CXCR7, however, can transmit a range of cellular responses, such as activation of ERK and AKT pathways,[30] receptor internalization,[10,14] cell survival,[6,9,11,30] proliferation,[44] adhesion,[6,17,30] and finally chemotaxis of CXCR4-negative cells.[10,31,44,45] Indeed, these observations are in line with our current data and others[46] showing that CXCL12 does not behave as a neutral ligand for CXCR7 but causes conformational changes of the receptor. It is also growingly appreciated that GPCRs can signal through mechanisms that function independently of G proteins, as is the case of CXCR4 for activation of the JAK2/STAT3 pathway and signaling through β-arrestins.[41] Such processes may also contribute to the aforementioned CXCL12/CXCR7-mediated responses that have been reported in different cellular contexts.[14]

G protein–independent chemotaxis mediated by some GPCRs[47] has been reported, and we cannot rule out that CXCR7 contributes in such a way to migration of T lymphocytes in response to CXCL12. From the data presented here and elsewhere,[14] we propose that CXCR7 expression in T lymphocytes regulates chemotaxis at 2 additional levels. The first one may involve the ability of CXCR7 to sequester CXCL12, thereby modifying the chemokine concentration in the extracellular environment, a process that has

been proposed to participate in the migration of primordial germ cells[14] and the lateral-line primordium.[15] The second level may not be dependent on CXCL12/CXCR7 interactions (Figure S2) and would involve the ability of CXCR7 to specifically alter CXCR4-mediated activation of G proteins through receptor heterodimerization. In line with this, distinct receptors can communicate with each other in heterodimers, so that one protomer can modify the function of the other one through trans-conformational changes.[36,48-50] Similarly, one can hypothesize that CXCR7 regulates CXCR4 functions in heterodimers through an allosteric mechanism, as reported for other chemokine receptors that can undergo functional modulations of allosteric nature affecting ligand binding and/or signaling once engaged in heterodimers.[51] Here, conformational changes within CXCR4/$G_{\alpha i}$ protein complexes that occurred in the presence of CXCR7 (Figure 5) may represent the molecular mechanism underlying alteration of G protein–dependent signaling downstream of CXCR4/CXCR7 heterodimers. This process could also account for the observation by Sierro et al that coexpression of CXCR7 with CXCR4 diminished early ERK activation on CXCL12 exposure.[12] Indeed, we recently stressed that CXCR4 stimulates ERK by 2 pathways: one occurring transiently and shortly after ligand stimulation and depending on G proteins, and the other being more prolonged and dependent on β-arrestin recruitment to the receptor.[18] Sierro et al showed that late ERK activation was not modified in the presence of CXCR7,[12] which may indicate that G protein–independent functions are maintained downstream of CXCR4/CXCR7 heterodimers.

In conclusion, we propose that CXCR7 can contribute differentially to CXCL12 responses either by signaling on its own through uncommon pathways or by regulating CXCR4 activity through heterodimer formation. In addition, heterodimerization might affect not only CXCR4 but also the pharmacologic properties of CXCR7. We and others often inferred the biologic activities of CXCR7 by the use of antagonists or mAbs, but our data that blocking CXCL12/CXCR7 interactions did not inhibit heterodimer functioning stress that these tools may not be sufficient to delineate all the activities of CXCR7. Finally, we showed here that CXCR4/CXCR7 heterodimers form as efficiently as the receptor homodimers. This implies that regulation of CXCR7 expression, which has been correlated to changes in response to CXCL12 during processes, such as organogenesis, B-cell lymphopoiesis, and tumorigenesis, might also arise from heterodimer formation.

Acknowledgments

The authors thank Drs M. Bouvier, S. Marullo, J.P. Pin, M. Parmentier, and M. Thelen for providing cDNAs and cells as detailed in the text; Drs R. Jockers and M.A. Ayoub (Institut de génomique fonctionnelle, Montpellier, France) for helpful discussions; and Dr K.Y. Chow (Unité de Pathogénie Virale, Institut Pasteur, Paris) for providing cDNAs and lentiviruses.

This work was supported by Ensemble Contre le SIDA (Paris, France), the Inserm, GIS-Network for Rare Diseases (Paris, France), the Ligue contre le Cancer (Paris, France), and the Agence Nationale de Recherches sur le SIDA (Paris, France). A.L. was supported by a grant from Ensemble Contre le SIDA.

Authorship

Contribution: A.L. designed and performed research, analyzed data, and wrote the manuscript; K.B. contributed to experiments, analyzed data, and edited the manuscript; F. Baleux performed chemokine synthesis; F. Bachelerie designed research, analyzed data, wrote the manuscript and provided funding for the study; and B.L. designed and performed research, analyzed data, wrote the manuscript, and provided funding for the study.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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