CXC Chemokine Receptor 3 Alternative Splice Variants Selectively Activate Different Signaling Pathways

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ABSTRACT

The G protein-coupled receptor (GPCR) C-X-C chemokine receptor 3 (CXCR3) is a potential drug target that mediates signaling involved in cancer metastasis and inflammatory diseases. The CXCR3 primary transcript has three potential alternative splice variants and cell-type specific expression results in receptor variants that are believed to have different functional characteristics. However, the molecular pharmacology of ligand binding to CXCR3 alternative splice variants and their downstream signaling pathways remain poorly explored. To better understand the role of the functional consequences of alternative splicing of CXCR3, we measured signaling in response to four different chemokine ligands (CXCL4, CXCL9, CXCL10, and CXCL11) with agonist activity at CXCR3. Both CXCL10 and CXCL11 activated splice variant CXCR3A. Whereas CXCL10 displayed full agonistic activity for Gαi activation and extracellular signal regulated kinase (ERK) 1/2 phosphorylation and partial agonist activity for β-arrestin recruitment, CXCL9 triggered only modest ERK1/2 phosphorylation. CXCL11 induced CXCR3β-mediated β-arrestin recruitment and little ERK phosphorylation. CXCR3Alt signaling was limited to modest ligand-induced receptor internalization and ERK1/2 phosphorylation in response to chemokines CXCL11, CXCL10, and CXCL9. These results show that CXCR3 splice variants activate different signaling pathways and that CXCR3 variant function is not redundant, suggesting a mechanism for tissue specific biased agonism. Our data show an additional layer of complexity for chemokine receptor signaling that might be exploited to target specific CXCR3 splice variants.

Introduction

Chemokine receptors are members of the G protein-coupled receptor (GPCR) family that bind peptidic chemotactic cytokines also known as chemokines. One hallmark of chemokine receptors is that they have multiple endogenous agonist ligands. Conversely, some chemokines can activate multiple separate chemokine receptor subtypes. Accumulating in vitro and in vivo evidence suggests that this promiscuity results in a diversity of specific chemokine receptor signaling, rather than mere functional redundancy (Schall and Proudfoot, 2011). Furthermore, chemokine receptor signaling is also controlled by chemokine binding to proteoglycans (Brady and Limbird, 2002; Proudfoot et al., 2003; Groom and Luster, 2011a,b; Zweemer et al., 2014), posttranslational modifications, and chemokine dimerization (Ludeman and Stone, 2014). Another potential mechanism controlling receptor function is the expression of alternative splice variants, which remains poorly explored (Wise, 2012). Because many chemokine receptors and chemokines are coexpressed by the same cell types or are present in the same cellular environment during inflammation, it is challenging to assess differences in the function of chemokine receptors and their alternative splice variants in response to different ligands in in vivo settings.

Inflammatory chemokine receptor CXCR3 is of increasing clinical interest because it controls leukocyte chemotaxis and is involved in inflammatory disorders such as atherosclerosis as well as in cancer metastasis (Hancock et al., 2000; Murphy et al., 2000; Groom and Luster, 2011a,b; Li et al., 2015; Lleo et al., 2015; Zhu et al., 2015). CXCR3 is expressed at the surface of a plethora of cells, including monocytes, lymphocytes, natural killer, and endothelial cells (García-López et al., 2001). CXCR3 displays classic receptor-ligand promiscuity and binds four chemokine ligands: CXCL4, CXCL9, CXCL10, and CXCL11 (Loetscher et al., 1996; Cole et al., 1998; Mueller et al., 2008). CXCR3 couples to Gαi/o heterotrimeric G proteins, which are pertussis toxin (PTX)-sensitive, and can also activate signaling pathways including MAPK, intracellular calcium flux, actin polymerization, and chemotaxis in response to chemokines (Loetscher et al., 1996; Smit et al., 2003; Kouroumalis et al.,

ABBREVIATIONS: BRET, bioluminescence resonance energy transfer; BSA, bovine serum albumin; CXCL, CXC chemokine ligand; CXCR, CXC chemokine receptor; ERK, extracellular signal regulated kinase; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; HEK, human embryonic kidney; PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline; PTX, pertussis toxin; RLuc, Renilla Reniformis luciferase; RT, room temperature; TM, transmembrane.
2005; Thompson et al., 2007). The CXCR3 receptor displays three alternative splice variants. After the identification of the canonical receptor CXCR3A (Loetscher et al., 1996), two naturally occurring alternative splice variants, CXCR3B and CXCR3Alt, were also identified (Lasagni et al., 2003; Ehler et al., 2004). Compared with CXCR3A, CXCR3B has an additional 51 amino acids at its N-terminal tail, whereas CXCR3Alt has a major truncation of intracellular loop three and transmembrane (TM) helices six and seven, which results in a mature protein with possibly only five TM helices and a short cytoplasmic C-terminal tail (Supplemental Figs. 1 and 2).

The expression patterns and function of CXCR3 splice variants in normal and disease states remain largely unexplored. Tissue-specific expression of CXCR3 alternative variants has been mainly quantified using reverse-transcription polymerase chain reaction, and variant-specific antibodies that might allow measurement of expression levels remain unavailable. Nevertheless, in prostate cancer specimens CXCR3A mRNA levels are upregulated, whereas CXCR3B mRNA levels are downregulated (Wu et al., 2012). These differences variant expression levels are reported to alter the migration and invasion capabilities of prostate cancer cells (Wu et al., 2012). In addition, mRNA levels of CXCR3A are decreased, whereas CXCR3Alt mRNA levels are increased in CD3+ peripheral blood lymphocytes in patients suffering from Crohn’s disease, suggesting a variant specific role of CXCR3Alt with Crohn’s disease (Manousou et al., 2008).

We set out to determine whether CXCR3 alternative splice variants activated different signaling pathways in response to their chemokine ligands. We dissected signaling profiles across multiple pharmacological assays when CXCR3 variants were individually expressed in an human embryonic kidney (HEK) 293T expression system. We carefully quantitated receptor expression levels and measured chemokine-induced Goi activity, β-arrestin recruitment, receptor internalization, and ERK1/2 phosphorylation to demonstrate that the nature of the activated signaling pathways depends on both the alternative receptor variant and the chemokine tested. For example, nearly all ligands induced CXCR3 internalization, whereas selected ligands induced β-arrestin recruitment in a PTX-sensitive manner, supporting a possible Goi-independent mechanism of β-arrestin recruitment to CXCR3A and CXCR3B. Moreover, our results strongly suggest that β-arrestin2 is recruited to both CXCR3A and CXCR3B in a ligand-independent manner. In summary, we show that CXCR3 splice variants have different signaling profiles in response to different ligands, which could lead to different pathophysiologic roles. Our data support the concept of biased agonism in CXCR3 variant-mediated signaling and suggest that individual CXCR3 variants might prove to be viable drug targets.

Materials and Methods

Materials. Recombinant chemokines were from PeproTech, Inc. (Rocky Hill, NJ). Coelenterazine 400A for BRET2 experiments was from Biotium (Hayward, CA). Forskolin, pertussis toxin (PTX), and poly-L-lysine were from Sigma (St. Louis, MO), and the anti-CXCR3 mAb (clone 1C6) directly coupled to phycoerythrin was from R&D Systems (Minneapolis, MN). Dulbecco’s modified Eagle’s medium Glutamax, 1% penicillin-streptomycin, and Lipofectamine 2000 were from Life Technologies (Carlsbad, CA). bovine serum albumin (BSA) fraction V, fatty acid free was from EMD Millipore (Bedford, MA), and 96-well white microplates with clear bottom and 384-well black microplates with clear bottom plates were from Corning (Woburn, MA).

Plasmids. The CXCR3A (Uniprot identifier P49682-2) plasmid was purchased from the Missouri &T cDNA Resource Center (Rolla, MO; www.cdna.org). The cDNA of splice variants CXCR3B (Uniprot identifier P49682-2) and CXCR3Alt (Uniprot identifier P49682-3) were synthesized by Bio Basic (Markham, Canada) and subcloned into pCDNA3.1+ using with BstEII and Xba restriction sites. The cAMP EPA2 sensor was a gift from Michel Bouvier (Université de Montréal, Canada). CXCR3A-GFP10, CXCR3B-GFP10, and CXCR3Alt-GFP10 were constructed by ligating the coding sequence of CXCR3 isoforms to GFP-GFP, amplified by PCR from the cAMP EPAC reporter including the linker region, using HindIII and XbaI unique restriction sites (Leduc et al., 2009). All sequences were verified by direct sequencing.

Cell Culture and Transfection. HEK293T cells (passage number 5 to 15, ATCC, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium Glutamax, 1% penicillin-streptomycin, and 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA). Transient transfection was performed in six-well plates using the polyethylenimine method as described previously (Percherancier et al., 2005). Transient high-throughput in-plate transfections were performed in 384-well plates using Lipofectamine 2000 according to manufacturer’s instructions with some modifications. Briefly, cells were trypsinized, counted, and mixed with the DNA-Lipofectamine 2000 complex then directly plated at 0.1% poly-L-lysine coated 384-well plates at a density of 20,000 cells/well. The total amount of transfected DNA was kept constant at 2 μg/well for six-well plates and 30 ng/well for 384-well plates by adding empty vector pCDNA3.1+. Transfected DNA amounts were adjusted to obtain comparable cell surface expression among the unfused and GFP10– fused CXCR3 splice variants in all experiments.

Flow Cytometry. HEK293T cells transfected in six-well plates with CXCR3 alternative variants were detached in ice-cold phosphate-buffered saline. Human peripheral blood mononuclear cells (PBMC) obtained by leukapheresis (Gift from Prof. Michel Nussenzweig, The Rockefeller University) and purified T cells (Gift from Dr. Helen Su, National Institute of Health/National Institute of Allergy and Infectious Diseases, NIH/NIAID) were obtained using a Pan T cell isolation kit (Miltenyi Biotec, San Diego, CA) were stimulated with 10 μg/ml phytohemagglutinin and cultured for 10 days in RPMI containing 10% fetal bovine serum and 50 IU/ml interleukin-2. Cells were labeled with monoclonal 1C6 phycoerythrin-conjugated antibody (BD Biosciences, San Jose, CA) that recognizes all three CXCR3 alternative variants for 30 minutes at 4°C in BRETT buffer [phosphate buffered saline (PBS) containing 0.5 mM MgCl2 and 0.1% BSA]. Cells were then washed three times in ice-cold PBS. Cell surface expression was quantified by flow cytometry using the Accuri C6 flow cytometer (BD Biosciences). We also determined receptor surface expression of transfected CXCR3 variants in HEK293T and the total CXCR3 quantities in both activated PBMC and T cells using the Quanti-BRITE standardization beads (BD Biosciences). Receptor quantities typically reached around 1.3 to 2.5 × 104 antibody-binding sites per cell for both HEK293T transfected CXCR3 alternative variants and endogenous CXCR3 expressed in activated PBMCs and purified T cells.

BRET Measurements. HEK293T cells transfected in six-well plates were seeded in 0.01% poly-L-lysine coated 96-well, white microplates with clear bottom 24 hours after transfection at a density of 90,000 cells/well. Forty-eight hours posttransfection, media was replaced with BRET buffer. Coelenterazine 400A was added at a final
into poly-D-lysine coated 96-well plates. We first determined the opti-
mum concentration of 5 μM followed by a 5 minutes incubation at room
temperature (RT). Luminescence and fluorescence readings were
collected using the Synergy NEO plate reader from Biotek (Winooski,
VT) and Gen5 software. BRETr readings between Rlu3c and GFP10
were collected by sequential integration of the signals detected in the
365 to 435 nm (Rlu3c) and 505 to 525 nm (GFP10) windows. BRET2
ratios were calculated as described previously (Leduc et al., 2009;
Berchiche et al., 2011). All BRETr experiments were performed while
cells remained attached to the 96-well plates.

Adenylyl Cyclase Activity. cAMP was determined by using the
Rlu3c-EPAC-GFP10, a BRETr cAMP sensor, as described previously
(Leduc et al., 2009). Briefly, cells cotransfected with 1.0 μg CXCR3A
or 1.5 μg CXCR3B or 1.0 μg CXCR3Alt and 0.03 μg Rlu3c-EPAC-
GFP10 reporter were seeded into poly-D-lysine coated 96-well plates
24 hours after transfection. Coelenterazine 400A was added to the cells
followed by a 5-minute incubation at RT. Cells were then stimulated
with ligand in the presence of 5 μM of forskolin at RT for 3 minutes.
For experiments involving PTX, 100 ng/ml of PTX was added to the media
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concentration of 30 minutes on ice following manufacturer
incubation was followed by two washes with ice-cold PBS. Cell surface
receptor expression was quantified by flow cytometry using the Accuri
C6 flow cytometer (BD Biosciences). Surface expression of CXCR3 after
incubation was followed by two washes with ice-cold PBS. Cell surface
expression compared with a sample drawn before addition of the ligands
resulted in a bystander curve, most likely due to random collision
throughout the experiment. We measured post t test when appropriate. When indicated, differences of top or
bottom values were also determined using sigmoidal dose response
simultaneous curve fitting.

Results
Goi Activity of CXCR3 Is Limited to Specific Alternative Splice Variants. We evaluated the ability of CXCR3
alternative splice variants to activate Gαi signaling in response to chemokines (Fig. 1). Receptor cDNA quantities were
adjusted to obtain comparable cell surface expression for all
CXCR3 variants, which were kept constant across all assays
(Supplemental Fig. 3). HEK293T cells coexpressing the EPAC
MOsensors previously described (Leduc et al., 2009) with
CXCR3 alternative variants were stimulated with increasing
concentrations of chemokines in the presence of forskolin in
live cells and the inhibition of forskolin induced cAMP
production as a consequence of Goi activation was measured.

Chemokine receptor CXCR3A inhibited cAMP production
when stimulated with CXCL10 and CXCL11, and both chemokines
had similar efficacies and potencies (Fig. 1A, Table 1). These results are in line with previous reports (Scholten et al.,
2012). Yet, chemokines CXCL9 and CXCL4 failed to trigger significant Gαi activity even at chemokine concentrations as
high as 100 nM. Furthermore, CXCR3B-mediated Gαi activation
was only detected after stimulation with 100 nM of
CXCL11 but not at lower concentrations or in the presence
of the other chemokine ligands (Fig. 1B). In addition, we
assessed the ability of chemokines to trigger Gαi activation
via CXCR3B using the EPAC biosensor in the absence of
forskolin (Leduc et al., 2009). As opposed to previously re-
ported Gαi activity in human microvascular endothelial cell
line-1 (HMEC-1) stably expressing CXCR3B (Lasagni et al.,
2003), we found that in our assays it failed to activate Gαi
upon treatment with its chemokine ligands (data not shown).
Chemokine receptor variant CXCR3Alt failed to induce significant Gαi activation in response to all chemokines tested
(Fig. 1C), although the truncated receptor was expressed
at the cell surface as measured by flow cytometry. Results
obtained with CXCR3Alt were similar to our results obtained
when cells were cotransfected with the EPAC biosensor and
empty vector pcDNA3.1+ (Fig. 1D).

CXCR3 Splice Variants Differentially Recruit β-Arrestins. We measured β-arrestin recruitment to CXCR3
in live cells using a BRETr proximity assay, which was extensively
used to study receptor interaction with arrestins, ligand-
induced receptor/arrestin conformational changes, as well as
receptor dimerization (Hamdan et al., 2005; Kalatskaya et al.,
2009; Berchiche et al., 2011). We performed acceptor/donor
titration experiments by coexpressing increasing quantities of
CXCR3-GFP10 acceptors with fixed quantities of β-arrestin1-
Rlu3c or β-arrestin2-Rlu3c donors (Fig. 2). Surprisingly, we
measured a significant and saturating basal BRETr signal in the
absence of ligands for CXCR3A with both β-arrestins and
CXCR3B with β-arrestin2, which further increased after stimu-
lation with 100 nM CXCL11 (Fig. 2, A–C). As for CXCR3B/
β-arrestin1, acceptor/donor titration in the absence of ligand
resulted in a bystander curve, most likely due to random collision
of coexpressed BRETr pairs (Bonneterre et al., 2016). However,
stimulation with CXCL11 resulted in a saturating curve sup-
porting ligand-induced β-arrestin1 recruitment to CXCR3B
(Fig. 2D). This suggests a preference of CXCR3B for β-arrestin2

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in the absence of ligand. Curve fitting of the data obtained from the titration experiments allowed us to determine both BRET_max and BRET_50 values in the absence and presence of 100 nM CXCL11 (Table 2). BRET_max corresponds to the best acceptor and donor concentrations determined at saturation with optimal sensitivity, which we used to assess ligand-induced CXCR3/β-arrestin interaction. Furthermore, BRET_50 corresponds to the acceptor/donor ratio giving 50% of the maximal

Fig. 1. CXCR3 alternative splice variants display different Goi activities in response to their chemokines. Receptor splice variants CXCR3A (A), CXCR3B (B), CXCR3Alt (C), or empty vector pcDNA3.1+ (D) were cotransfected with the cAMP Rluc3-EPAC-GFP10 sensor in HEK293T cells. Cells were exposed to 5 μM forskolin with increasing concentrations of the indicated ligands at room temperature for 5 minutes before readings. Results are expressed as the % of inhibition of forskolin-induced cAMP production. Data are reported as mean values of 4–6 independent experiments performed in triplicate ± S.E.M.

TABLE 1
Summary of fitted curve parameters for results shown in Figs. 1 (Goi activity), 5 (β-arrestin recruitment), and 8 (ERK1/2 phosphorylation).
pIC50 values are given and maximal responses are shown in absolute (β-arrestin recruitment) and relative (ERK1/2 phosphorylation and Goi activity) units. Errors are presented as ± S.E.M.

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<th>CXCR3A</th>
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<td>IC50 (nM)</td>
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<td>pIC50 ± S.E.M.</td>
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<td>E_max ± S.E.M.</td>
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<td>70 ± 7</td>
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<td>pEC50 ± S.E.M.</td>
<td>−8.2 ± 0.1</td>
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<td>BRET^2_max ± S.E.M.</td>
<td>0.11 ± 0.01</td>
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<td>β-arrestin1</td>
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<td>BRET^2_max ± S.E.M.</td>
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<td>E_max ± S.E.M.</td>
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<td>293 ± 32</td>
<td>N.D.</td>
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*Theoretical value, experimental curves did not reach saturation.
N.D., not detectable.
signal and reflects the propensity of BRET partners to interact.

Stimulation of CXCR3A/β-arrestin1, CXCR3A/β-arrestin2, as well as CXCR3B/β-arrestin2 pairs with 100 nM CXCL11 resulted in an increased BRETmax (Fig. 2, A–C, and Table 2) compared with basal conditions. This increase is a direct consequence of changes in the distance and/or orientation between the -Rluc donor and -GFP10 acceptor fusions. This strongly suggests that CXCL11 induces conformational changes in these receptor/β-arrestin preformed complexes. In addition, CXCL11 stimulation also decreased BRET50 values, shifting the saturation curve to the left for these receptor/arrestin pairs, which indicates a higher propensity of CXCR3A and B variants to interact with β-arrestins in the presence than in the absence of CXCL11 (Table 2).

In contrast, titration of the CXCR3Alt-GFP10 acceptor with both β-arrestins donors showed no ligand-dependent or -independent association, indicating that CXCR3Alt does not interact with β-arrestins (Fig. 2, E and F).

Moreover, we assessed the specificity of the interactions we measured to ascertain whether select CXCR3 variants interact with β-arrestins in the absence of ligand. We cotransfected cells expressing the BRET2 β-arrestin donor and CXCR3 acceptor fusions with the corresponding FLAG-β-arrestin and measured its impact on the basal BRET signal. We observed a transfected FLAG-β-arrestin quantity-dependent decrease of the basal BRET signal for CXCR3A and both β-arrestins as well as CXCR3B and β-arrestin2, whereas CXCR3B and β-arrestin1 remained unaffected (Fig. 3). A decrease in a dose-dependent manner of the basal BRET signal indicates a competition between the donor fused β-arrestin and the corresponding FLAG-tagged β-arrestin, which in turn supports a specific ligand-independent interaction.

![Fig. 2](https://example.com/image2.png)

**Fig. 2.** CXCR3 variants constitutively interact with select β-arrestins. Acceptor/donor titration curves of CXCR3 splice variants fused with GFP10 and β-arrestin-Rluc3 were obtained after transfection of HEK293T cells with increasing quantities of CXCR3-GFP10 fusion with 0.05 µg β-arrestin-Rluc3 fusions as indicated: A, CXCR3A/β-arrestin2; B, CXCR3A/β-arrestin1; C, CXCR3B/β-arrestin2; D, CXCR3B/β-arrestin1; E, CXCR3Alt/β-arrestin2; F, CXCR3Alt/β-arrestin1. Measurements were carried out at room temperature in the absence or presence of 100 nM CXCL11. Data were plotted and fitted to a one-site binding hyperbola equation. BRET50 and BRETmax values were calculated for each BRET2 couple. Results obtained from at least three independent experiments, carried out in triplicate ± S.E.M are shown.
interaction between select CXCR3 alternative variants and β-arrestin.

Next, we quantified ligand-mediated β-arrestin recruitment to CXCR3 at BRETmax by performing kinetic experiments. These were carried out at RT to fully capture the details of recruitment kinetics after chemokine stimulation (100 nM). CXCR3A treatment with CXCL11 or CXCL10 resulted in a rapid β-arrestin2 recruitment reaching a plateau in 5 to 7 minutes (Fig. 4A). Similar results were obtained with β-arrestin1 (Supplemental Fig. 4). A small but statistically significant elevation in β-arrestin2 recruitment to CXCR3A after stimulation with CXCL9 or CXCL4 was also observed (Fig. 4A, Supplemental Fig. 5). In contrast, CXCL9 or CXCL4 failed to induce β-arrestin1 recruitment to CXCR3A (Supplemental Fig. 4A). Our data show that CXCR3A and CXCR3B acceptor fusions are functional and are able to recruit both β-arrestins in response to ligands. In addition, these results indicate a receptor preference for specific β-arrestins, which is selectively influenced by chemokines.

Also, CXCL11 increased β-arrestin2 recruitment to CXCR3B with the signal reaching a plateau within the first 5 minutes of stimulation (Fig. 4B). CXCL10, CXCL9, and CXCL4, a chemokine suggested to be a selective high affinity ligand of CXCR3B (Lasagni et al., 2003), failed to trigger β-arrestin2 recruitment to CXCR3B. Similar results were obtained with β-arrestin1 (Supplemental Fig. 4B). As for CXCR3Alt, all chemokines failed to induce both β-arrestin1 and β-arrestin2 recruitment to this C-terminally truncated alternative variant (Fig. 4C, Supplemental Fig. 4C).

To catalog the pharmacology of CXCR3 variants in response to their chemokines, we measured the effect of increasing concentrations of ligands on β-arrestin recruitment to CXCR3A and CXCR3B (Table 1). Our results obtained with CXCL11 and CXCL10 are consistent with previously reported

![Fig. 3. Constitutive β-arrestin interaction is receptor variant specific. HEK293T cells transfected with the indicated receptor variant/β-arrestin at BRETmax in the absence (control) or presence of increasing levels of C-terminally FLAG-tagged β-arrestin. A, CXCR3A/β-arrestin2; B, CXCR3A/β-arrestin1; C, CXCR3B/β-arrestin2; D, CXCR3B/β-arrestin1. The fold increase shown on the x-axis represents the ratio of micrograms of β-arrestin-Rluc3 to β-arrestin-FLAG transfected in cells coexpressing CXCR3 variant GFP10 fusion. Statistical significance of the differences between the control, in the absence of FLAG-tagged β-arrestin and increasing levels of coexpressed C-terminally FLAG-tagged β-arrestin: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 [one-way analysis of variance (ANOVA), Bonferroni’s multiple comparison test]. Results obtained for three independent experiments carried out in triplicate ± S.E.M are shown.](molpharm.aspetjournals.org)
observations obtained with a BRET1 arrestin proximity assay (Scholten et al., 2012). Indeed, CXCL11 acts as a full agonist with a potency of 8 nM, whereas CXCL10 acts as a partial agonist with a potency of 70 nM (Fig. 5A) on the β-arrestin2 recruitment to CXCR3A. A similar rank order of potencies was observed with β-arrestin1 (Table 1, Supplemental Fig. 6A). As for CXCR3B, CXCL11 caused arrestin recruitment with potencies of 61 and 33 nM for β-arrestin2 and β-arrestin1, respectively (Fig. 5B, Table 1, Supplemental Fig. 6B). These results suggest that the longer N terminus of CXCR3B is sufficient to change the receptor’s ability to respond to chemokines and recruit β-arrestins. Moreover, these data suggest that although the N-terminal tail of CXCR3Alt is identical to that of CXCR3A, not surprisingly differences in the C terminus of CXCR3Alt are sufficient to perturb its ability to recruit β-arrestins.

**Chemokine Triggered β-Arrestin Recruitment to CXCR3 is PTX Insensitive.** To determine whether ligand-induced β-arrestin recruitment to CXCR3A and CXCR3B are linked to Gαi signaling, we tested their sensitivity to Gαi/o-inactivating PTX treatment (Fig. 6). Ligand-mediated β-arrestin2 (Fig. 6, A-C) and β-arrestin1 (Supplemental Fig. 7) recruitment to CXCR3A and CXCR3B were resistant to PTX treatment. As expected, PTX treatment inhibited ligand-induced Gαi activity mediated by CXCR3A (Fig. 6A). This result suggests that β-arrestin recruitment to CXCR3A occurs independently from Gαi signaling in response to CXCL11 and CXCL10. PTX treatment also impaired CXCL11 triggered
CXCR3B Gαi activity, even if it was only detected with 100 nM of chemokine (Fig. 6C) comparable to CXCR3A.

Chemokines Induce Internalization of All CXCR3 Splice Variants. We stimulated HEK293T cells expressing CXCR3 variants with chemokine ligands (100 nM) for various time periods at 37°C to assess ligand-induced receptor internalization. Excess chemokine was removed by acid wash and remaining surface receptors were quantified by flow cytometry. Stimulation with chemokines induced internalization of all receptor variants (Fig. 7). Chemokine CXCL11 induced CXCR3A internalization was modest in comparison with the other chemokines tested for this receptor variant, yet our results with CXCL11 remain comparable to previously reported observations (Scholten et al., 2012). Moreover, nearly 40% of CXCR3A receptors internalized within the first 10 minutes of stimulation with CXCL10 and CXCL4, compared with CXCL9, which required 30 minutes of stimulation to reach 40% internalization. Similarly, nearly 50% of CXCR3B receptors internalized during the first 10 minutes after incubation with CXCL10, CXCL9, and CXCL4.

Yet CXCL11 only induced moderate receptor internalization in the same fashion as CXCL11-induced CXCR3A

Fig. 5. CXCR3A and CXCR3B recruit β-arrestin2 in the presence of increasing concentrations of chemokine. HEK293T cells transiently coexpressing A, CXCR3A-GFP10 or B, CXCR3B-GFP10 with β-arrestin2-Rluc3 at BRET_max were incubated with increasing concentrations of the indicated ligands for 5 minutes at 37°C. BRET was measured 5 minutes after ligand addition. Data are reported as the mean values of three independent experiments performed in triplicate ± S.E.M (see Table 1 for curve fitting parameters).

Fig. 6. PTX treatment only affects chemokine induced Gαi activity of CXCR3A and CXCR3B. HEK293T cells transfected with CXCR3 and the EPAC cAMP biosensor or CXCR3-GFP10 fusion with β-arrestin2-Rluc3 were incubated with 100 ng/ml PTX for 16 hours at 37°C. Gαi activity of CXCR3A (A) and CXCR3B (C) are expressed as the % of forskolin-induced cAMP production in the presence of 100 nM of the indicated ligands in the absence or presence of PTX. The forskolin induced cAMP production measured in the absence of chemokine and PTX was set to 100% (white bars). β-Arrestin2 recruitment to CXCR3A (B) and CXCR3B (D) induced with 100 nM of the indicated ligands in the absence or presence of PTX. Results represent data obtained from three to five independent experiments performed in triplicate ± S.E.M.
internalization. Interestingly, CXCL11, CXCL9, and CXCL4 induced a robust and rapid CXCR3Alt internalization, whereas CXCL10 had little to no effect. Surprisingly, in our assay CXCL4 (100 nM), a CXCR3B ligand, induced robust receptor internalization of all three CXCR3 variants. CXCL4 chemokine is reported to bind to CXCR3A and trigger chemotaxis at high chemokine concentrations of 500 to 750 nM (Mueller et al., 2008; Korniejewska et al., 2011). Our results indicate that all ligands (100 nM) are able to bind CXCR3 variants expressed at the cell surface and thus trigger their function.

**CXCR3 Splice Variants Display Different ERK1/2 Phosphorylation Profiles.** We quantitatively measured ligand-induced ERK1/2 phosphorylation in HEK293T cells expressing CXCR3 variants. Transfected cells were stimulated with chemokines (100 nM) at 37°C for various time periods (Fig. 8, A, C, and D). ERK1/2 phosphorylation was quantified using an InCell Western approach. This method was previously used to measure ERK1/2 phosphorylation of cannabinoid receptor CB1, a member of the GPCR family coupled to Gi/o heterotrimeric G proteins (Daigle et al., 2008). Three out of the four chemokines tested induced CXCR3A-mediated ERK1/2 phosphorylation (Fig. 8A). In cells expressing CXCR3A, CXCL11 induced the longest and most significant response starting as early as 2 minutes after ligand addition (294 ± 37% of basal) and lasting 10 minutes (247 ± 21% of basal), with a peak phosphorylation measured at 5 minutes (402 ± 37% of basal). In comparison with CXCL11, CXCL10-mediated ERK1/2 phosphorylation through CXCR3A was shorter and more modest. Its signal was detected between 2 (250 ± 60% of basal) and 5 minutes (199 ± 25% of basal) after stimulation. Chemokine CXCL9 also triggered a moderate elevation of ERK1/2 phosphorylation starting at 5 minutes (225 ± 24% of basal), which declined after 10 minutes (217 ± 26% of basal). In contrast, CXCR3B activation by chemokines showed a different ERK1/2 phosphorylation profile than for CXCR3A (Fig. 8B). Indeed, CXCL11 triggered a modest ERK1/2 phosphorylation starting at 5 minutes (203 ± 18% of basal) and lasted 10 minutes (170 ± 13% of basal). Moreover, CXCL9 also induced a short ERK1/2 phosphorylation, peaking at 10 minutes (192 ± 22% of basal) and a short and weak, yet statistically significant, signal at 5 minutes (159 ± 22% of basal) after stimulation with CXCL4. Surprisingly, cells expressing CXCR3Alt variant also responded to ligands and we measured ERK1/2 phosphorylation after stimulation with CXCL11, CXCL10, and CXCL9 (Fig. 8D). Treatment with CXCL11 increased ERK1/2 phosphorylation between 5 to 10 minutes, with a peak

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**Fig. 7.** Chemokines induce CXCR3 splice variant’s internalization. HEK293T cells expressing CXCR3A; CXCRB, and CXCR3Alt were incubated with 100 nM of each chemokine at 37°C. Aliquots were removed on ice at the indicated times after ligand addition. Surface-bound ligand was removed by acid washing, and remaining surface CXCR3 was measured by flow cytometry. Data are mean of three to four independent experiments ± S.E.M.
phosphorylation detected after 7 minutes (195 ± 16% of basal). We also quantified modest ERK1/2 phosphorylation from 5 to 15 minutes after treatment with CXCL10. In contrast, CXCL9 triggered ERK1/2 phosphorylation at a single time point of 10 minutes (155 ± 14% of basal).

Compared with CXCR3A, chemokines induced weaker ERK1/2 phosphorylation through CXCR3B and CXCR3Alt. However, these changes were statistically significant compared with cells transfected with the receptor and treated with assay buffer only. Furthermore, to ensure that the signals were indeed the result of CXCR3 alternative variants, all experiments were performed in parallel with cells transfected with empty vector pcDNA3.1. These cells failed to respond to chemokines (data not shown), indicating that the responses measured for CXCR3 were ligand induced and receptor-variant specific. We also collected pharmacological data for CXCR3A by taking advantage of favorable dynamic range and the quantitative nature of the ERK1/2 phosphorylation InCell Western assay. We measured ERK1/2 phosphorylation in cells transfected with CXCR3A in response to increasing concentrations of CXCL11 and CXCL10 (Fig. 8B). Our results indicate that both CXCL11 and CXCL10 induce ERK1/2 phosphorylation with similar efficacy and potency and are full agonists for this pathway (Table 1).

Discussion

The goal of this study was to explore the specific intracellular signaling pathways activated by CXCR3 splice variants in response to their chemokine ligands. We report that different CXCR3 splice variants induce quantitatively distinctive signaling responses after ligand stimulation and that receptor signaling efficacy depends on the splice variant/chemokine pair assessed. Receptor variant/chemokine pairs and the corresponding pathways activated are summarized in Table 3. The ability of ligands to activate different intracellular signaling pathways via the same receptor to different extents is a widely observed phenomenon and has been documented for many GPCRs, including chemokine receptors. This characteristic has been given several names including biased agonism, stimulus trafficking, and functional selectivity. Biased agonism of GPCRs supports the existence of multiple active receptor conformations that ligands may differentially stabilize, leading to the activation of specific signaling pathways (Kenakin, 2007, 2009). The nonredundant signaling responses we report for CXCR3 alternative splice variants could be explained if chemokines stabilized distinct receptor splice variant conformations leading to the activation of different intracellular signaling pathways.

Indeed, CXCR3A-mediated Gai activity was only detectable in response to CXCL11 and CXCL10, which are in line with the Gai activity previously reported for this canonical receptor (Scholten et al., 2012). Interestingly, CXCL11 also induced CXCR3B-mediated Gai activity, yet only at a saturating concentration of 100 nM. Sulfation of Tyr27 and Tyr29 in the N-terminal region of CXCR3A is essential for chemokine binding and thus receptor function (Colvin et al., 2006). The longer N terminus of CXCR3B contains two additional potential sulfation sites (Tyr6 and Tyr40). These Tyr residues could also be sulfated or influence the sulfation of the Tyr
residues common to both variants, and therefore cotranslational Tyr sulfation might explain the Gαi activity measured for CXCR3B.

As expected, Gαi activation of CXCR3A was inhibited by PTX treatment. Surprisingly, CXCR3B-induced Gαi activation was also inhibited by PTX treatment, suggesting that this Gαi activity is specific. Relatedly, stimulation of CXCR3A with CXCL11 or CXCL10 and of CXCR3B with CXCL11 further increased basal β-arrestin recruitment to the receptor and was PTX insensitive. Taken together, our results indicate that ligand-mediated Gαi signaling and β-arrestin recruitment occur independently from each other. Our findings also suggest that chemokine receptor variant/ligand pairs selectively determine the receptor/β-arrestin conformation as well as the efficacy of Gαi activity. Similar to the ligand-initiated β-arrestin recruitment and Gαi signaling, we report that the CXCR3-mediated ERK1/2 phosphorylation intensity and signal duration measured depend on the splice variant/chemokine pair assessed. For example, CXCL11-mediated ERK1/2 phosphorylation through CXCR3A was stronger and lasted longer than the one mediated through both CXCR3B and CXCR3Alt.

Ligand-independent β-arrestin recruitment was previously reported for chemokine receptor CCR1 (Gilliland et al., 2013), atypical chemokine receptor ACKR2/D6 (McCulloch et al., 2008), as well as a chimeric receptor composed of ACKR3/CXCR7 with its C-terminal tail swapped with CXCR4 (Gravel et al., 2010). C-terminal tails of both D6 and CCR1 show basal phosphorylation, which plays an important role in their ligand independent activity. Our data are the first to indicate that ligand-independent β-arrestin recruitment can also differentially occur for the different CXCR3 alternative splice variants. Such constitutive β-arrestin recruitment to CXCR3A and CXCR3B might also arise from low levels of basal receptor phosphorylation. Indeed, the C-terminal tail of CXCR3A and CXCR3B contains nine Ser residues and one Thr residue that might allow for some basal level of Ser/Thr phosphorylation, favoring the interaction with β-arrestins. An earlier report of a low level of phosphorylation measured for transfected CXCR3 further supports this hypothesis (Colvin et al., 2004).

Receptor internalization may occur in both β-arrestin-dependent and -independent fashion. Here, we demonstrate that the internalization profiles of all three CXCR3 alternative splice variants fail to correlate with ligand-induced β-arrestin recruitment, supporting a β-arrestin-independent internalization mechanism. Similarly, Rajagopal et al. (2013) recently showed that CXCL11-induced β-arrestin recruitment to the canonical CXCR3 receptor does not correlate with CXCL11-induced internalization. Likewise, CXCL11 and CXCL10 provoked receptor internalization in a β-arrestin-independent fashion when CXCR3A was expressed in L1.2 murine cells (Meiser et al., 2008). Furthermore, β-arrestins are well recognized for their role as scaffold proteins for downstream signaling phosphorylation cascades (DeWire et al., 2007). ERK1/2 phosphorylation induced by CXCR3 splice variants does not correlate either with ligand-induced β-arrestin recruitment. This indicates that β-arrestin may not be required for ERK1/2 phosphorylation provoked by specific receptor variant/ligand pairs such as CXCR3B/CXCL9 and CXCR3Alt/CXCL10. Nevertheless, β-arrestins may play a role in ERK1/2 phosphorylation provoked by the CXCR3A/CXCL11 pair. Similarly, only CXCR3A/CXCL11 and CXCR3A/CXCL10 pairs provoke Gαi activity that correlates with their ERK1/2 phosphorylation profile, supporting the possibility that ERK1/2 phosphorylation may be a consequence of Gαi activation.

Many GPCRs with highly truncated alternative splice variants lacking some TM helices are reported in the literature (Wise, 2012). Alternative splice variant CXCR3Alt is a predicted five TM domain receptor with a short C-terminal end (Ehlert et al., 2004). CXCR3Alt lacks the third intracellular loop important for G-protein interaction and subsequent activation of intracellular components (Thelen and Thelen, 2008). Stimulation of this splice variant resulted in weak but statistically significant ERK1/2 phosphorylation and chemokine-induced receptor internalization. Nevertheless, CXCR3Alt failed to induce Gαi activation and β-arrestin recruitment in the absence or presence of chemokines. Compared with CXCR3A and CXCR3B, the C terminus of CXCR3Alt lacks the Ser and Thr phosphorylation sites typically phosphorylated by GRKs and required for β-arrestin recruitment. This fundamental difference of CXCR3Alt and its limited signaling further supports the idea that CXCR3 splice variants play different roles in fine tuning chemokine-induced signaling responses.

It may be speculated that CXCR3Alt splice variant behaves as an atypical chemokine receptor. Its role could be to scavenge chemokines and contribute to the establishment of a chemokine gradient, similarly to chemokine receptor CXCR7, which does not activate Gαi heterotrimeric G protein, yet still recruits β-arrestin and induces ERK1/2 phosphorylation and internalization in response to CXCL12 (Boldajipour et al., 2008; Kalatskaya et al., 2009; Levoye et al., 2009). Another explanation for our results could be that the role of CXCR3Alt is to modulate the function of a chemokine receptor such as CXCR3A, because these variants can be coexpressed in cells (Aksoy et al., 2006) and because truncated GPCRs

### TABLE 3
Summary of CXCR3 variant activated signaling pathways following stimulation with chemokines

<table>
<thead>
<tr>
<th></th>
<th>CXCR3A</th>
<th>CXCR3B</th>
<th>CXCR3Alt</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Goαi activation</strong></td>
<td>CXCL11, CXCL10</td>
<td>CXCL11*</td>
<td>N.D.</td>
</tr>
<tr>
<td><strong>β-arrestin2 recruitment</strong></td>
<td>CXCL11, CXCL10</td>
<td>CXCL11</td>
<td>N.D.</td>
</tr>
<tr>
<td><strong>β-arrestin1 recruitment</strong></td>
<td>CXCL11, CXCL10</td>
<td>CXCL11</td>
<td>N.D.</td>
</tr>
<tr>
<td><strong>Internalization</strong></td>
<td>CXCL11, CXCL10, CXCL9, CXCL4</td>
<td>CXCL11, CXCL9, CXCL4</td>
<td>CXCL11, CXCL9, CXCL4</td>
</tr>
<tr>
<td><strong>ERK1/2 phosphorylation</strong></td>
<td>CXCL11, CXCL10, CXCL9*</td>
<td>CXCL11, CXCL9, CXCL4*</td>
<td>CXCL11, CXCL10* CXCL9*</td>
</tr>
</tbody>
</table>

*Only measureable at 100 nM chemokine.
*Weak but statistically significant response.
N.D., not detectable.
could act as dominant negative of CXCR3A (Wise, 2012). The later possibility is further supported by the atypical and predicted five TM arrangement of CXCR3Alt. An additional possibility could be that activation of CXCR3Alt leads to other functional consequences we did not assess. For example, coupling to Go12/13 (Kouroumalis et al., 2005) or Gqi proteins isoforms i2 and i3 (Thompson et al., 2007) are also reported for CXCR3.

We cannot rule out that our observations are cell-type specific, yet CXCR3 natural ligands behave as perfect biased ligands (Kenakin and Christopoulos, 2013), which activate only specific signaling pathways but not others. For example, CXCL4 induces internalization of all variants, modestly stimulates ERK1/2 phosphorylation via CXCR3B and yet has no other effects on any of the receptor variants in the pathways assessed. Lack of signaling in our assays of specific CXCR3 variant/chemokine pairs could also be explained by the need of a GPCR modifying protein such as CXCR3A splice variant to induce signaling, which in turn could contribute to further diversity CXCR3-mediated signaling. Another possibility is that specific CXCR3 alternative splice variants that do not signal in our assays play a role to internalize specific chemokines and help establish gradients in specific tissues. Nevertheless, the differences we report support that CXCR3 ligands stabilize different conformations of CXCR3 splice variants. As a consequence, each CXCR3 variant/chemokine pair is likely to fulfill different functions in vivo. Although other chemokines receptors, such as CCR2 and (Charo et al., 1994) CCR9 (Yu et al., 2000), possess alternative splice variants with some differences expression and signaling levels (Wong et al., 1997; Sanders et al., 2000), our work is the first to quantify the function of chemokine receptor CXCR3 alternative splice variants in response to its four natural ligands across multiple four signaling pathways.

In addition, our work provides insights into the differences in the signaling abilities of CXCR3 alternative splice variants that are a direct consequence of their differences in the N- and C-terminal regions and intracellular loops. Our finding that chemokine receptor CXCR3 splice variants are able to selectively stimulate specific signaling pathways in response to different chemokines supports the idea that the chemokine system is not functionally redundant. Instead, the different activation patterns of CXCR3 splice variants indicate that the chemokine system displays an additional layer of complexity fine-tuned by receptor splice variants. The differences in CXCR3 variant/chemokine pair signaling must be taken into account in the context of targeting CXCR3 with small molecules. Furthermore, these differences could form a basis to design small molecules that selectively modulate in a biased manner specific receptor alternative splice variants in disease settings.

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Authorship Contributions

Participated in research design: Berchiche and Sakmar. Conducted experiments: Berchiche. Performed data analysis: Berchiche. Wrote or contributed to the writing of the manuscript: Berchiche and Sakmar.

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