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CXCR7/CXCR4 Heterodimer Constitutively Recruits β-Arrestin to Enhance Cell Migration*[S]

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G protein-coupled receptor hetero-oligomerization is emerging as an important regulator of ligand-dependent transmembrane signaling, but precisely how receptor heteromers affect receptor pharmacology remains largely unknown. In this study, we have attempted to identify the functional significance of the heteromeric complex between CXCR4 and CXCR7 chemokine receptors. We demonstrate that co-expression of CXCR7 with CXCR4 results in constitutive recruitment of β-arrestin to the CXCR4·CXCR7 complex and simultaneous impairment of G_i-mediated signaling. CXCR7/CXCR4 co-expression also results in potentiation of CXCL12 (SDF-1)-mediated downstream β-arrestin-dependent cell signaling pathways, including ERK1/2, p38 MAPK, and SAPK as judged from the results of experiments using siRNA knockdown to deplete β-arrestin. Interestingly, CXCR7/CXCR4 co-expression enhances cell migration in response to CXCL12 stimulation. Again, inhibition of β-arrestin using either siRNA knockdown or a dominant negative mutant abrogates the enhanced CXCL12-dependent migration of CXCR4/CXCR7-expressing cells. These results show how CXCR7, which cannot signal directly through G protein-linked pathways, can nevertheless affect cellular signaling networks by forming a heteromeric complex with CXCR4. The CXCR4·CXCR7 heterodimer complex recruits β-arrestin, resulting in preferential activation of β-arrestin-linked signaling pathways over canonical G protein pathways. CXCL12-dependent signaling of CXCR4 and its role in cellular physiology, including cancer metastasis, should be evaluated in the context of potential functional hetero-oligomerization with CXCR7.

Chemokine receptors belong to the superfamily of heptahelical G protein-coupled receptors (GPCRs)[4] and are involved in a vast array of physiological events (1–3). Among the 20 or so known chemokine receptors, CXCR4 and its cognate ligand, stromal cell derived factor-1 (SDF-1) or CXCL12, have been studied extensively given their essential role in directing cell migration during numerous developmental processes, including organogenesis, hematopoiesis, vascularization, and immune responses (2). In the adult, the CXCR4/CXCL12 axis regulates bone marrow stem cell homing and retention of hematopoietic progenitor cells and leukocyte trafficking (2, 4). In addition, CXCR4 is involved in pathological processes. It serves as a secondary co-receptor for HIV-1 cellular entry and is involved in mediating the development and targeted metastasis of primary endothelial tumors, including breast, prostate, ovary, and lung (5–7). CXCR4 has been found to be expressed in many tumor types and has been linked to survival and proliferation of tumor cells. Its expression has been correlated with the degree of malignancy and with metastasis formation in several tumor types (7–10).

Recently, CXCL12 was shown to bind with high affinity to chemokine receptor CXCR7, which was until then classified as an orphan receptor (11–13). The CXCR7-SDF-1 complex did not, however, activate the canonical G_i-mediated signaling network leading to the conclusion that CXCR7 may be a nonsignaling chemokine receptor, possibly akin to the erythrocyte chemokine scavenger, Duffy (also called the Duffy antigen receptor for chemokines) (11, 12, 14, 15). But interestingly, CXCR7 is found to be expressed in many tumor types and on activated tumor-associated endothelial cells and has been shown to be essential for the survival and growth of tumor cells (12, 16–19). Although the exact molecular mechanisms responsible for the ability of CXCR7, a putative nonsignaling receptor, to promote tumor growth and survival remain unclear, recent studies have attempted to provide mechanistic insight. Heterodimerization between CXCR4 and CXCR7 has been postulated as one mechanism for modulating CXCR4 function (20–22). Using firefly luciferase complementation assay, Luker *et al.* (21) demonstrated that CXCR4 and CXCR7 can each form homo- and heterodimers. Furthermore, co-expression of CXCR7 with CXCR4 resulted in modulation of CXCR4-mediated G_i activation and signaling (20). Additionally, even though CXCR7 does not signal through the canonical G protein pathways, it may signal in a biased fashion through the alternative β-arrestin-mediated signaling pathways (23–25).

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[4] The abbreviations used are: GPCR, G protein-coupled receptor; ITAC, interferon-inducible T-cell α chemoattractant; SEAP, secreted alkaline phosphatase; FK, forskolin; SAPK, stress-activated protein kinase; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; βArr, β-arrestin; DN, dominant negative; IP, immunoprecipitation; eGFP, enhanced GFP.

Based on these observations, we decided to test whether CXCR7 heterodimerization with CXCR4 would serve to create a distinct signaling entity with unique properties and serve to alter chemokine receptor pharmacology. We report here that the association of CXCR4 and CXCR7 causes impaired CXCR4-promoted G_i activation and signaling and promotes activation of alternative downstream β-arrestin-dependent signal transduction pathways. We demonstrate that the CXCR4·CXCR7 complex constitutively recruits β-arrestin and potentiates cell proliferative kinase pathways, including p38 MAPK, SAPK, and ERK1/2 activation leading to increased cell migration of CXCR4-expressing breast cancer cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HEK293, Neuro2A, and MDA-MB-231 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. U87 stable cell lines expressing CD4 or CD4 and CXCR4 were grown in DMEM under G418 (Invitrogen) selection. For all transfections, Lipofectamine™2000 (Invitrogen) was used on 60–80% confluent cells in a 6-well plate according to the manufacturer's protocol. For expression in HEK293 and Neuro2A cells, 3 μg of FLAG-tagged CXCR7 receptor cDNA was co-transfected with 1 μg of C9-tagged CXCR4 and/or 1 μg of eGFP-tagged β-arrestin1 (βarr-GFP) with pcDNA3.1 used to keep the total amount of transfected DNA constant in all cases. Similarly, 1 μg of HA-tagged CXCR4 was co-transfected with 1 μg of C9-tagged CCR5 or 3 μg of FLAG-tagged CXCR7. The FLAG epitope was introduced by PCR on the C-terminal tail of CXCR7 using the forward oligonucleotide ATTGGATCCCCATGGATCTGCATCTCTTCGACTAC and the reverse oligonucleotide TAACTCGAGTTAGTCATCATCGTCCTTGTAGTCTTTGGTGCTCTGCTCCAAGG. The amplified fragment was cloned into pcDNA3.1 using the introduced BamHI and XhoI sites.

ELISA and Immunostaining—5×10^4 cells/well were plated on a 96-well plate 24 h after transfection. The next day, the wells were washed with phosphate-buffered saline (PBS) and incubated with either 12G5, 2D7, or 11G8 monoclonal antibodies (BD Biosciences) in PBS, 0.5% BSA on ice for 2 h. ELISA was performed as described (26). For immunostaining, cells were plated 24 h after transfection on glass coverslips coated with poly-D-lysine (Sigma). The next day, cells were fixed with methanol and incubated with polyclonal anti-FLAG and monoclonal 1D4 antibodies in PBS, 0.5% bovine serum albumin (BSA) at room temperature for 1 h. Cells were then incubated for 1 h at room temperature with secondary Alexa-594 anti-rabbit antibodies and Alexa-488 anti-mouse or Alexa-647 anti-mouse antibodies. Coverslips were mounted on Superfrost/Plus slides (ThermoFisher), and fluorescence was observed using a Zeiss LSM 510 confocal microscope.

Co-immunoprecipitation and Western Blotting—HEK293T cells were transfected as above. 48 h after transfection, cells were washed three times and lysed in a buffer containing 1% CHAPSO (ThermoFisher), 10% glycerol, 250 mM NaCl, 50 mM Tris-Cl (pH 8), 0.5 mM EDTA, and protease inhibitor mixture (Sigma) for 1 h. The supernatant fraction collected after 20 min of centrifugation was then incubated overnight at 4 °C with 10% (v/v) ImmunoPure immobilized protein A/G (Pierce) and 3–5 μg of anti-FLAG polyclonal or M2 monoclonal (Sigma) antibodies. For IP of cell surface receptors, cells were washed with PBS and incubated with 3–5 μg of 11G8 monoclonal antibodies for 2 h on ice before lysis step and supernatant fractions were incubated with the same amount of beads. The beads were washed three times in lysis buffer, and elution was performed in 100 μl of 1× Laemmli buffer at 37 °C for 2 h on shaker. Samples were separated using NuPAGE system (Invitrogen) and subjected to Western blotting using anti-FLAG, anti-GFP (Cell Signaling), anti-HA (Covance) polyclonal or 1D4 monoclonal antibodies. Detection was performed after incubation with peroxidase-labeled anti-rabbit or anti-mouse secondary antibodies (Kirkegaard & Perry Laboratories) on an AlphaImager system (Alpha Innotech Corp.). For protein kinases studies, 24 h after transfection, cells were plated in a 24-well plate coated with poly-D-lysine and serum-starved overnight. Cells were then treated with 100 nM SDF-1α (PeproTech) for indicated times and lysed with 100 μl of 1× Laemmli buffer prewarmed at 65 °C. Samples were sonicated, and 30 μl was separated as above and subjected to Western blotting using anti-phospho or anti-total p38, SAPK, or ERK1/2 antibodies (Cell Signaling).

SEAP Assay—In a 6-well plate, 0.8 μg of CXCR4 with or without 0.4 or 1.2 μg of CXCR7 or CCR5 cDNA were co-transfected with 4 μg of responsive element-secreted alkaline plasmid per well. 24 h after transfection, cells were plated onto a poly-D-lysine-coated 384-well plate and serum-starved overnight. The next day, cells were stimulated for 6 h in media without serum with 10 μM forskolin and prespecified concentrations of SDF-1α. For pretreatments, 500 nM ITAC (PeproTech) or 1 μM AMD3100 or 0.4 μg/well of 12G5 or 11G8 antibodies were incubated in media without serum for 1 h prior to SDF-1 stimulation. 1.5 μl of each well supernatant fraction was transferred into a second 384-well plate, and the levels of alkaline phosphatase activity were quantified as described using an EnVision plate reader (PerkinElmer Life Sciences) (26).

β-Arrestin2 siRNA—β-Arrestin2 expression was silenced using prevalidated ON-TARGET plus SMART pool siRNA duplexes targeting human β-arrestin2 (catalogue no. L-007292-00-0005, Thermo Scientific). ON-TARGET plus nontargeting pool (catalogue no. D-001810-10^{-05}, Thermo Scientific) was used as control. The day before transfection, cells were seeded in 6-well plates in regular growth medium without antibiotics and grown overnight. The day of transfection, Lipofectamine™2000 (Invitrogen)·plasmid·siRNA complexes were prepared according to the manufacturer's protocol. Cells were transfected with either 100 nM β-arrestin2 siRNA or control nontargeting siRNA complexes for 6 h followed by replacement with fresh growth medium, including antibiotics. Transfected cells were assayed 48 h post-transfection. Suppression of gene target was confirmed using quantitative PCR. Briefly, total RNA was generated from siRNA-transfected cells followed by first strand cDNA synthesis. cDNA template was used to determine gene expression levels using gene-specific primers against β-arrestin2 (forward primer CCAGGGTCTTCAAGAAGTC and reverse primer TTGCCCAAGTACACGGT and probe (6-FAM)CTAACTGCAAGCTCACCG(TAMRA-6-FAM)) and GAPDH (hsGAPDH-forward CGACATCAGCCGCATCT

and hsGAPDH-reverse CTAGCCTCCCGGGTTTCTCT, and hsGAPDH probe (6-FAM)TCGCCAGGTGAAGACGGGCG-(TAMRA-6-FAM)). Quantitative PCR was performed in 96-well plates on ABI 7900HT sequence detection system, and SDS 2.3 software was used for data analysis. β-Arrestin2 knockdown was calculated using $\Delta\Delta C_T$ method normalized to GAPDH reference gene and nontargeting siRNA control treated cells.

Cell Migration—Cell migration was assayed using transwells (8 μm pore; Falcon). The lower chamber was filled with 0.5 ml of cell migration medium (DMEM with 2 mg/ml bovine serum albumin) in the presence or absence of ligand (SDF-1α, ITAC). U87-CXCR4 and MDA-MB-231 cells transfected with varying concentrations of CXCR7 expression vector were trypsinized and resuspended to 5×10^5 cells/ml. For β-arrestin inhibition experiment, β-arrestin2 targeting siRNA or β-arrestin dominant negative (βArr DN) mutants were used. βArr DN (V53D) (27) was generated using site-directed mutagenesis, and the mutation was confirmed by DNA sequencing. Cell suspension (200 μl) was added to the top chamber, and cells were allowed to migrate at 37 °C, 5% CO_2, for 3–6 h. For U87-CXCR4 stable cell line (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health; U87.CD4.CXCR4 was from Dr. HongKui Deng and Dr. Dan R. Littman) (28), 12.5 nM SDF-1α in cell migration medium was added to the bottom chamber. For MDA-MB-231 cells, an SDF-1α dose-response curve was generated using 0–100 nM SDF-1α. The upper surface of the insert was wiped with a cotton tip to mechanically remove the nonmigratory cells. The migrated cells attached to the lower surface were imaged on an inverted Zeiss Axiovert followed by lysis in buffer containing CyQUANT® DNA-binding dye (Invitrogen). Each sample was measured in triplicate in a fluorescent plate reader (excitation 488 nm and emission 530 nm) 30–60 min after staining. Migration index was calculated as the fluorescent intensity measured from cells that migrated in response to chemokine divided by the fluorescent intensity measured from cells present in the lower chamber in the absence of chemokine. The data presented are an average of three different experiments. Each cell line was measured in triplicate wells in three different experiments.

RESULTS

CXCR7 and CXCR4 Form Heterodimers—Based on the overlap in tissue expression profile of CXCR4 and CXCR7 and their ability to bind to the same chemokine ligand CXCL12/SDF-1 (henceforth referred to as SDF-1), we investigated the potential of these two chemokine receptors to form heterodimers or higher order oligomeric complexes at the cell surface. Co-expression of differentially C-terminally tagged CXCR4-C9 and CXCR7-FLAG in HEK293 cells resulted in co-immunoprecipitation (co-IP) of CXCR4-C9 together with CXCR7-FLAG (Fig. 1*A*). Conversely, reciprocal co-IP of CXCR7 upon immunoprecipitation of CXCR4-C9 was also observed (data not shown). Similar results were obtained using HA-tagged CXCR4 (CXCR4-HA) (data not shown). Moreover, we found that immunoprecipitation of cell surface expressed CXCR7 from intact cells using 11G8, a conformation-specific CXCR7 antibody, led to the detection of co-immunoprecipitated CXCR4 (Fig. 1*B*). In contrast, co-expression of CXCR4 and CCR5 resulted in very little co-IP between CXCR4 and CCR5 (supplemental Fig. 1*A*). Immunofluorescence studies of CXCR4-C9 and CXCR7-FLAG performed in Neuro2A cells co-expressing both receptors revealed co-localization of the two receptors on the cell surface as well as distinct puncta within the cell (Fig. 1*C*). Taken together, these results strongly point toward the existence of a CXCR4·CXCR7 complex in cells expressing both receptors.

CXCR7 Regulates CXCR4-dependent G Protein Signaling— We next examined the functional effects of the CXCR4· CXCR7 complex on SDF-1-mediated signaling. Active CXCR4 stimulates heterotrimeric inhibitory G protein (G_i), which inhibits cellular adenylyl cyclase activity and decreases cAMP levels. We used a cAMP reporter gene assay, based on SEAP to study the effects of SDF-1 on G_i-mediated inhibition of cAMP production (26). SDF-1 stimulation leads to strong inhibition of forskolin (FK)-induced cAMP production in HEK293 cells expressing CXCR4 (Fig. 1*D*). In contrast, SDF-1 treatment of cells expressing CXCR7 failed to induce a similar effect (Fig. 1*D*). However, CXCR7 co-expression along with CXCR4 strongly attenuated SDF-1-induced inhibition of cellular cAMP production. The effect of CXCR7 on SDF-1-induced CXCR4 signaling was dose-dependent (Fig. 1*D*). The effect was also specific to CXCR7 because co-expression of the same amounts of CCR5, which is also G_i-coupled, did not induce a similar shift in EC_{50} (Fig. 1*E*). This shift is not due to altered cell surface expression of CXCR4 because we did not see any significant decrease in cell surface levels of CXCR4 in the presence of CXCR7 (supplemental Fig. 1*B*). These results indicate that CXCR7 plays a role in regulating the coupling of CXCR4 to G_i to affect G protein-mediated downstream signaling. Our findings are in general agreement with a recently published study wherein CXCR7 co-expression resulted in decreased coupling of G proteins and decreased mobilization of intracellular calcium levels, another signaling pathway activated by G_i downstream of CXCR4 stimulation (20).

We next investigated the effect of CXCR7-specific ligand interferon-inducible T-cell α chemoattractant/CXCL11 on the CXCR4·CXCR7 complex. Similar to SDF-1, ITAC binds to CXCR7 but does not induce G protein signaling via CXCR7 (15). Interestingly, preincubation of CXCR4/CXCR7 co-expressing cells with 500 nM ITAC relieves the negative effect of CXCR7 on SDF1-mediated CXCR4 signaling (Fig. 1*F*). ITAC restores SDF-1-dependent inhibition of cellular cAMP production downstream of CXCR4 activation (Fig. 1*F*).

CXCR4·CXCR7 Complex Constitutively Recruits β-Arrestin—β-Arrestin plays a prominent role in both signaling and internalization events downstream of GPCRs. Given that recent studies have demonstrated the ability of CXCR7 to recruit β-arrestin in a ligand-dependent manner (23–25), we decided to investigate β-arrestin recruitment by the CXCR4·CXCR7 receptor complex. CXCR7-FLAG and βArr-GFP were co-transfected with or without CXCR4-C9 in HEK293 cells. We observed low levels of basal and ligand-dependent interaction between CXCR7 and β-arrestin (Fig. 2, *A* and *B*). Surprisingly,

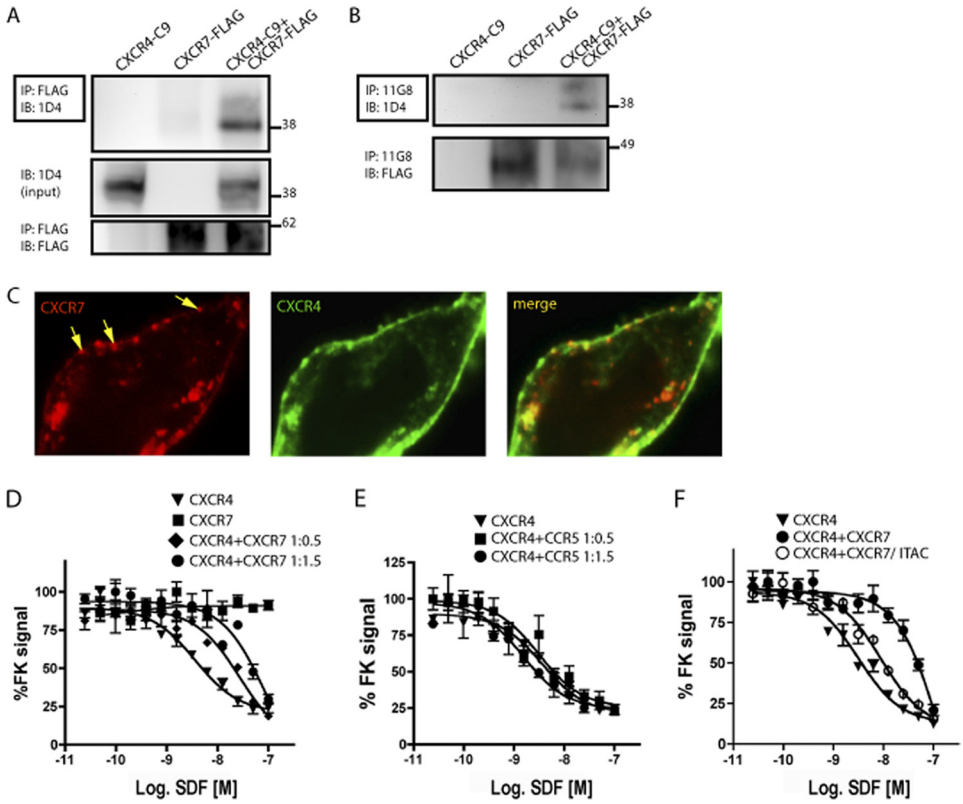

FIGURE 1. **CXCR7 alters SDF-1 signaling by forming a complex with CXCR4.** *A,* CXCR4 and CXCR7 co-immunoprecipitate when co-expressed in HEK293 cells. Detergent-soluble lysates from HEK293 cells transfected with CXCR4-C9 and CXCR7-FLAG, as indicated, were treated with anti-FLAG antibody to immunoprecipitate (*IP*) CXCR7-FLAG. The pulldown was subjected to SDS-PAGE followed by Western immunoblot (*IB*) analysis using 1D4 mAb (*upper panel*). 1D4 and anti-FLAG antibodies were used to monitor CXCR4-C9 (*middle panel*) and CXCR7-FLAG expression levels (*lower panel*), respectively. *B,* same experiment as in *A* except transfected cells were preincubated with 11G8, a CXCR7-specific antibody sensitive to receptor conformation, prior to lysis. Co-IP CXCR4-C9 was detected using 1D4 antibody (*upper panel*), and CXCR7 levels were monitored using ant-FLAG antibody (*lower panel*). *C,* immunofluorescent staining of CXCR4 and CXCR7 expressing Neuro2A cells demonstrates co-localization of CXCR4 and CXCR7 at the membrane. *D,* pCRE-SEAP assay showing inhibition of FK-induced cAMP production by CXCR4 and CXCR7. SDF-1 stimulation inhibits FK-induced cellular cAMP production through CXCR4 but not through CXCR7. Co-expression of CXCR7 along with CXCR4 results in a decrease in FK-induced cellular cAMP production through CXCR4 in a dose-dependent manner. CXCR4 alone, $EC_{50} = 3.4 \pm 0.8$; CXCR4 ± CXCR7 1:1.5, $EC_{50} = 60 \pm 11$. *E,* unlike CXCR7, co-expression of CCR5 with CXCR4 does not lead to alteration in SDF-1-stimulated signaling in the pCRE-SEAP assay. CXCR4 alone, $EC_{50} = 3.4 \pm 0.8$; CXCR4 ± CCR5 1:1.5, $EC_{50} = 3.1 \pm 0.5$. *F,* pCRE-SEAP assay shows recovery of SDF-1 signaling when CXCR4-CXCR7 co-expressing cells were preincubated with 500 nM of ITAC. Data are expressed as mean ± S.E. ($n = 3$). CXCR4 alone, $EC_{50} = 3.4 \pm 0.8$; CXCR4 ± CXCR7 1:1.5, $EC_{50} = 60 \pm 11$; CXCR4 + CXCR7/ITAC, $EC_{50} = 5.5 \pm 1.7$.

co-expression of CXCR4 and CXCR7 resulted in a dramatic increase in both basal and SDF-1-induced β-arrestin co-immunoprecipitation with CXCR7 (Fig. 2, *A* and *B*). The enhanced recruitment of β-arrestin is specific for the CXCR4·CXCR7 complex, because co-expression of CCR5 did not result in increased association of β-arrestin with CXCR7 (Fig. 2, *A* and *B*). Further studies are needed to delineate the structural and molecular determinants involved in β-arrestin recruitment that could presumably be located on either CXCR4 and CXCR7 or distributed on both receptors (see under "Discussion"). Interestingly, as opposed to the effect of treatment with SDF-1, treatment of CXCR4/CXCR7-co-expressing cells with ITAC did not result in a further increase in β-arrestin recruitment (Fig. 2*C*). In fact, there was a slight but reproducible decrease in β-arrestin recruitment to the CXCR4·CXCR7 complex upon treatment with ITAC (Fig. 2*C*).

In addition to co-IP, we also observed co-localization of CXCR4 and CXCR7 with β-arrestin in Neuro-2A cells co-expressing all three components (Fig. 2*D*). Notably, we did not observe any co-localization between CXCR7 and β-arrestin in the absence of CXCR4 (data not shown), suggesting that CXCR4 is required for the enhanced interaction between CXCR7 and β-arrestin.

Taken together, our data demonstrate that CXCR7 not only induces a shift in G protein coupling of CXCR4 but also constitutively interacts with β-arrestin in the presence of CXCR4. Additionally, CXCR7-specific ligand ITAC pretreatment restores SDF-1-mediated G_i signaling as well as leads to reduced association of β-arrestin to the CXCR4·CXCR7 complex. This suggests a potentially interesting role for ITAC in modulating the CXCR4·CXCR7 heteromeric complex and/or its signaling pathways.

Effect of CXCR7 on ERK1/2 Signaling Pathways—Both CXCR4 and CXCR7 have been shown independently to potentiate cell signaling pathways that are important for cell proliferation and tumor growth (9, 12, 17, 29). We examined the effects of CXCR4/CXCR7 co-expression on the SDF-1-mediated ERK1/2 signaling pathway. Interestingly, CXCR7 co-expression with CXCR4 leads to elevated and sustained ERK1/2 activation upon SDF-1 stimulation (Fig. 3*A*). This is in accordance with previous evidence showing that CXCR7 co-expression leads to sustained ERK1/2 activation (22), although in the

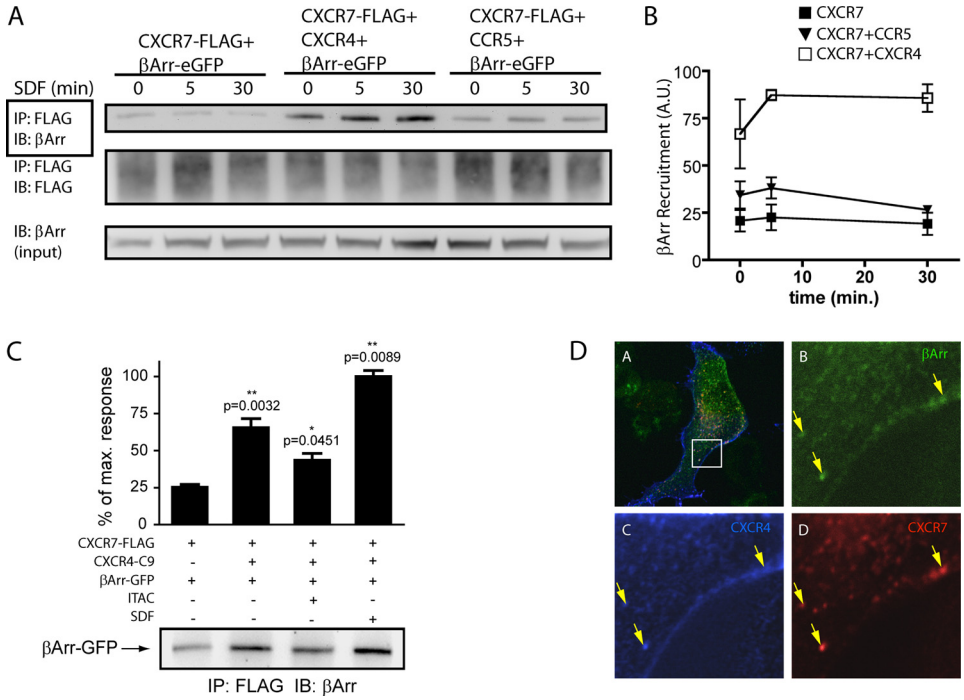

FIGURE 2. **CXCR4·CXCR7 complex constitutively recruits β-arrestin.** *A,* HEK293 cells were co-transfected with CXCR7-FLAG and β-arrestin-eGFP, along with either CXCR4 or CCR5, and treated with 100 nM SDF-1 for the indicated time. Lysates were immunoprecipitated (*IP*) with polyclonal anti-FLAG antibodies and subjected to Western immunoblot (*IB*) analysis using anti-GFP (*upper panel*) and anti-FLAG (*middle panel*) antibodies, respectively. Increased recruitment of β-arrestin to membranes containing CXCR7 is observed at base line in the presence of CXCR4 but not CCR5 (*upper panel*). The same trend is observed after 5 and 30 min of stimulation with SDF-1. The *lower panel* shows the input levels of β-arrestin-eGFP expression using anti-GFP antibody. *B,* quantification of β-arrestin recruitment kinetics in membranes containing CXCR7 alone or when CXCR7 is co-expressed with CXCR4 or CCR5. Data represent mean ± S.E. (n = 3). *A.U.,* arbitrary units. *C,* HEK293 cells were co-transfected with CXCR7-FLAG and β-arrestin-eGFP, along with either control vector or CXCR4-C9, and treated with 100 nM SDF-1 or ITAC for 30 min. Lysates were immunoprecipitated with polyclonal anti-FLAG antibody and subjected to Western immunoblot analysis using anti-GFP antibody (*bottom panel*). Quantification of β-arrestin-recruitment is depicted in the *top panel*. Data represent results from at least three independent experiments and are expressed as mean ± S.E. The maximum response from β-arrestin recruitment to CXCR4-CXCR7 membranes is taken as 100%. Stimulation with ITAC does not result in increased recruitment of β-arrestin to the CXCR4-CXCR7-expressing membranes. *D,* immunofluorescence image of Neuro2A cell co-expressing CXCR4, CXCR7, and βArr-eGFP. *A* shows the merged image of CXCR4/CXCR7/βArr-eGFP. *B–D* show a higher magnification of a small area (*white square, A*) demonstrating co-localization of all these partners.

reported study, the authors did not observe a further increase in ERK1/2 activation.

In addition to ERK1/2, we extended our analysis to p38 MAPK and SAPK, MAPK signaling pathways linked to cell proliferation, and cell survival. As shown in Fig. 3, CXCR4 expression alone leads to very little activation of p38 MAPK or SAPK. Co-expression of CXCR4 with CXCR7 leads to an increase in both basal and SDF-1 stimulated p38 MAPK and SAPK activation in a dose-dependent manner (Fig. 3, *A* and *B,* and supplemental Fig. 2). This effect is specific for CXCR7 because transfection with CCR5 does not result in an activation of p38 MAPK and SAPK in response to SDF-1 (supplemental Fig. 2). Both CXCR4 and CXCR7, when expressed alone, exhibited very low levels of p38 MAPK and SAPK activation (basal and SDF-1-stimulated) (Fig. 3, *A* and *B*). We hypothesize that the CXCR4·CXCR7 complex may preferentially couple to the p38 MAPK and SAPK pathways as compared with the signaling mediated by each receptor alone. Taken together, co-expression of CXCR7 with CXCR4 leads to a decrease in G protein-coupled inhibition of cellular cAMP production but an increase in β-arrestin recruitment and an increase in signaling pathways implicated in cell proliferation and survival.

CXCR7 Potentiation of SDF-1-induced Cell Signaling Pathways Is a β-Arrestin-mediated Process—Given that CXCR7 did not induce typical G protein-mediated response but did lead to increased β-arrestin recruitment, we next investigated whether the increased activation of various cell signaling pathways by the CXCR4·CXCR7 heteromer may be a β-arrestin-dependent process. We co-transfected CXCR4 and CXCR7 along with either β-arrestin2 targeting siRNA or with nontargeting control siRNA to deplete endogenous levels of β-arrestin2. siRNA targeting β-arrestin2 resulted in 87.7 ± 0.01% reduction in β-arrestin2 levels as quantified using RT-quantitative PCR and $\Delta\Delta C_T$ analysis (data not shown). As shown in Fig. 4, depletion of β-arrestin resulted in an overall decrease in ERK activation. There was also a marked decrease in both basal and SDF-1-stimulated p38 and SAPK activation (Fig. 4). We therefore conclude that β-arrestin is an important component of the CXCR4·CXCR7 heteromer-mediated signaling and that CXCR7-mediated potentiation of SDF-1-induced signaling may be a β-arrestin-dependent process.

CXCR7 Enhances SDF-1-induced Cell Migration in a β-Arrestin-dependent Manner—Given the increased activation of MAPK signaling pathways, we decided to investigate further the functional consequences of the CXCR4·CXCR7 heteromer complex on cell migration. We investigated the effect of CXCR7 expression on SDF-1-stimulated chemotaxis of MDA-MB-231 (231) breast cancer cells. These cells respond to SDF-1 in a dose-dependent manner and move forward in an increasing SDF-1 concentration gradient (Fig. 5*A*). The maximal chemo-

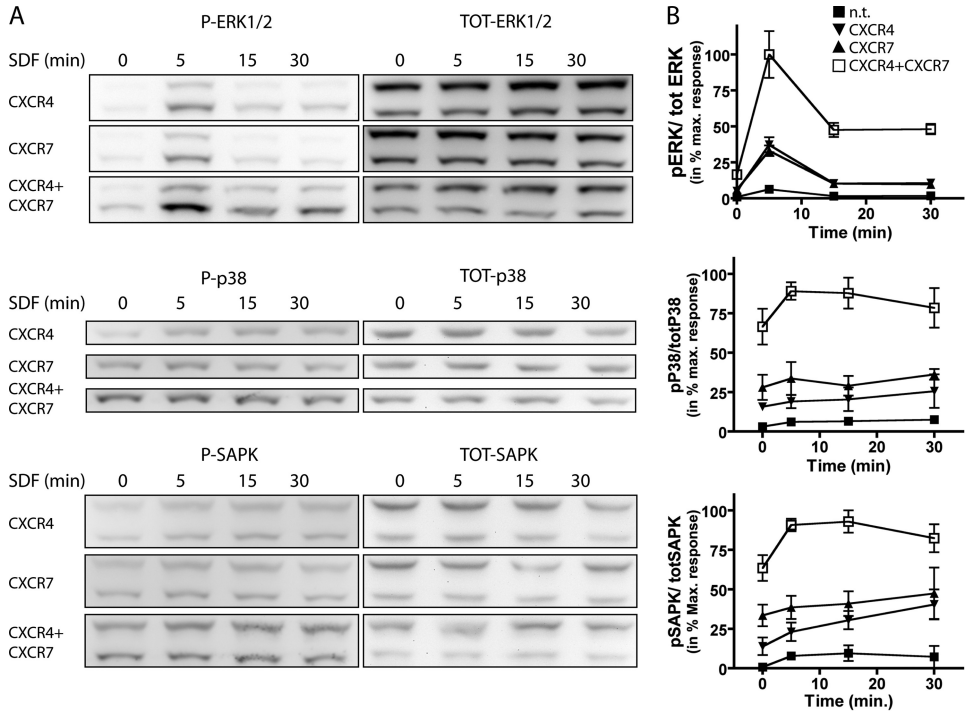

FIGURE 3. **CXCR7 potentiates SDF-1-induced cell signaling pathways.** *A,* kinetics of SDF-1-induced ERK, p38 MAPK, and SAPK/JNK activation in CXCR4-, CXCR7-, and CXCR4/CXCR7-expressing cells. HEK293 cells transfected with CXCR4 and CXCR7 alone, or in combination, were treated with 100 nM SDF-1 for 0, 5, 15, and 30 min. Cell lysates were subjected to Western immunoblot using phospho- and total ERK1/2, p38 MAPK, and SAPK/JNK antibodies. *B,* amount of ERK, p38 MAPK, and SAPK phosphorylation was quantified by densitometry and normalized by expressing the data as a ratio of the phosphorylated signal over the total signal. Results are expressed as a percentage of the maximum response at 5 min. Data represent mean ± S.E. ($n = 3$). Quantification of ERK1/2, p38 MAPK, and SAPK/JNK activation kinetics performed on samples from cells expressing CXCR4, CXCR7, or both. *n.t.*, nontargeting.

tactic response to SDF-1 was observed at 10 nM. Cell migration index as a function of SDF-1 concentration has been shown to be a bell-shaped curve, where higher levels of SDF-1 actually induce less chemotactic response (30). We found that the SDF-1-induced chemotactic response can be potentiated when 231 cells are transfected with increasing amounts of CXCR7 (Fig. 5*A*). As shown in Fig. 5*A*, the enhanced chemotaxis of 231 cells was maximal at 2 μg of CXCR7. In addition to an increase in the overall chemotactic index of 231 cells in the presence of over-expressed CXCR7, we also noticed a shift in potency of SDF-1. We observed increased ability of 231 cells transfected with CXCR7 to respond to lower concentrations of SDF-1 (Fig. 5*A*).

Next, we decided to use a stable cell line expressing CXCR4 as a model to study the effect of CXCR7 on SDF-1-induced cell migration. As shown in Fig. 5*B*, U87-CXCR4 cells co-transfected with CXCR7 showed increased SDF-1-stimulated migration in a dose-dependent manner. ITAC stimulation only led to a marginal increase in migration in the higher dose of CXCR7-co-expressing cells (Fig. 5*B*). Interestingly, pretreatment with ITAC markedly attenuated the increased SDF-1-stimulated migration of CXCR4/CXCR7-co-expressing cells suggesting that ITAC stimulation negatively regulates the CXCR4·CXCR7 complex. We also found that the enhanced migration induced by CXCR7 is dependent on the presence of functional CXCR4 because pretreatment of parental and CXCR7-expressing cells with AMD-3100, a CXCR4-specific small molecular inhibitor, resulted in abrogating all SDF-1-induced cell migrations (data not shown). Interestingly depleting functional β-arrestin2 levels using siRNA knockdown or using a previously described dominant negative mutant of β-arrestin (27, 31) also abrogated the migratory advantage provided by CXCR7 expression (Fig. 5*C*).

DISCUSSION

Recent studies have shown that CXCR7 is a novel alternative receptor for the chemokine SDF-1, a known agonist ligand for CXCR4 (11, 12). However, CXCR7 is an atypical chemokine receptor that apparently does not signal through canonical G protein-linked signaling pathways even in the presence of saturating SDF-1 concentrations (11, 12, 14, 15). Even though the signaling pathways activated by CXCR7 are still under investigation, there is increasing evidence that CXCR7 may function as a biased receptor for selective recruitment and activation of β-arrestin-mediated signaling (23–25, 32). In this study, we show that CXCR7 modulates CXCR4 function by self-assembling in cell membranes to form heterodimers or possibly higher order hetero-oligomers with CXCR4. Heterodimerization of CXCR4 and CXCR7 results in attenuation of classical SDF-1-mediated G_i-activated signaling as measured by classical G_i activation assays that monitor inhibition of adenylyl cyclase (Fig. 1). Surprisingly, cells co-expressing CXCR4 and CXCR7 display increased ligand-stimulated, as well as basal, plasma membrane recruitment of β-arrestin (Fig. 2). Plasma membrane-localized β-arrestin has been shown to bind active phosphorylated GPCRs to act as an obligate signaling scaffold for MAPK cascades, including ERK1/2, JNK, and p38 MAPK kinases (33, 34). Here, we show that co-expression of CXCR7 in the presence of CXCR4 causes increased and sustained activa-

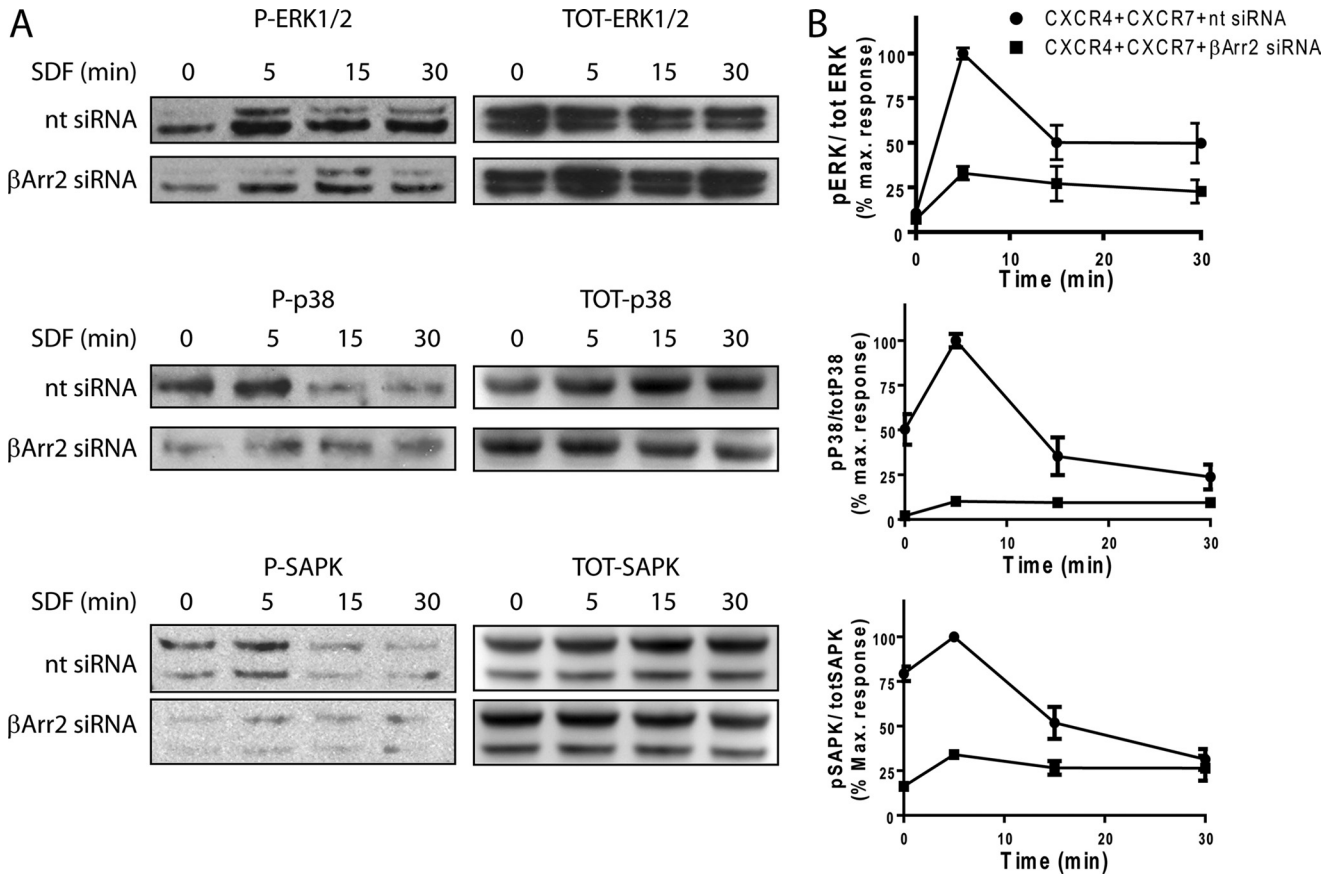

FIGURE 4. **β-Arrestin is required for SDF-1-induced cell signaling pathways activated by the CXCR4·CXCR7 complex.** *A,* kinetics of SDF-1-induced ERK, p38 MAPK, and SAPK/JNK activation in cells expressing CXCR4/CXCR7 along with either nontargeting siRNA (*nt siRNA*) or β-arrestin2 targeting siRNA (*βArr2 siRNA*). Transfected cells were treated with 100 nM SDF-1 for 0, 5, 15, and 30 min. Cell lysates were subjected to Western immunoblot using phospho- and total ERK1/2, p38 MAPK, and SAPK/JNK antibodies. *B,* amount of ERK, p38 MAPK, and SAPK phosphorylation was quantified by densitometry and normalized by expressing the data as a ratio of the phosphorylated signal over the total signal. Results are expressed as a percentage of the maximum response at 5 min. Data represent means ± S.E. ($n = 3$). *nt*, nontargeting.

tion of the ERK1/2 cascade as well as increased activation of p38 MAPK and SAPK (Fig. 3 and supplemental Fig. 2). This potentiation of SDF-1-induced signaling by CXCR7 seems to be a β-arrestin-dependent process because siRNA knockdown of β-arrestin resulted in significant attenuation of all signaling pathways investigated (Fig. 4). This switch from G protein-mediated signaling to β-arrestin-dependent signaling correlates with an apparent increased sensitivity of the CXCR4·CXCR7 heteromer to SDF-1-dependent cell migration of MDA-MB-231 breast cancer cells as well as motile cells heterologously expressing CXCR7 in the presence of endogenous CXCR4 (Fig. 5). Direct inhibition of β-arrestin using siRNA knockdown or dominant negative mutant of β-arrestin abrogates the increased migration mediated by the CXCR4·CXCR7 heteromer suggesting an important role of β-arrestin downstream of the receptor heterodimer complex (Fig. 5*C*). Our results support the model wherein CXCR4 monomers/homodimers would signal predominantly via G protein-dependent signaling pathways, whereas CXCR4·CXCR7 heteromers would predominantly engage β-arrestin-dependent pathways in a biased fashion (Fig. 5*D*).

Receptor dimerization/oligomerization has emerged as a key paradigm in GPCR biology and has been implicated in almost all aspects of GPCR function, including intracellular trafficking, receptor internalization, pharmacological inhibition, and signal transduction (35, 36). Although it has been challenging to ascertain functional consequences of receptor heterodimerization, it has been demonstrated that heterodimerization can change completely the signaling pathways activated as well as the trafficking of the receptors (37, 38). In the case of the CXCR4·CXCR7 heterodimer, we observed a switch in signaling pathways that are induced downstream of SDF-1 stimulation, a decrease in G protein-dependent signaling and an increase in β-arrestin recruitment and signaling. Indeed, there are examples wherein the heterodimer has been shown to possess unique pharmacological profiles. For example, it was shown that the μ-δ opioid receptor heterodimer selectively recruits β-arrestin2 resulting in biased signaling leading to sustained ERK activation (37). In the case of the V1a and V2 vasopressin receptor, heterodimerization regulates interaction with β-arrestin and the fate of the internalized receptors (38). Heterodimerization can also modulate ligand selectivity as seen in the case of the CCR5·CCR2 and CXCR4·CCR2 heterodimers wherein heterodimerization resulted in negative binding cooperativity with only one chemokine ligand binding with high affinity to the receptor dimer (39–41).

β-Arrestin can act independently of G proteins to activate signaling pathways (33, 34). This has been best described for the

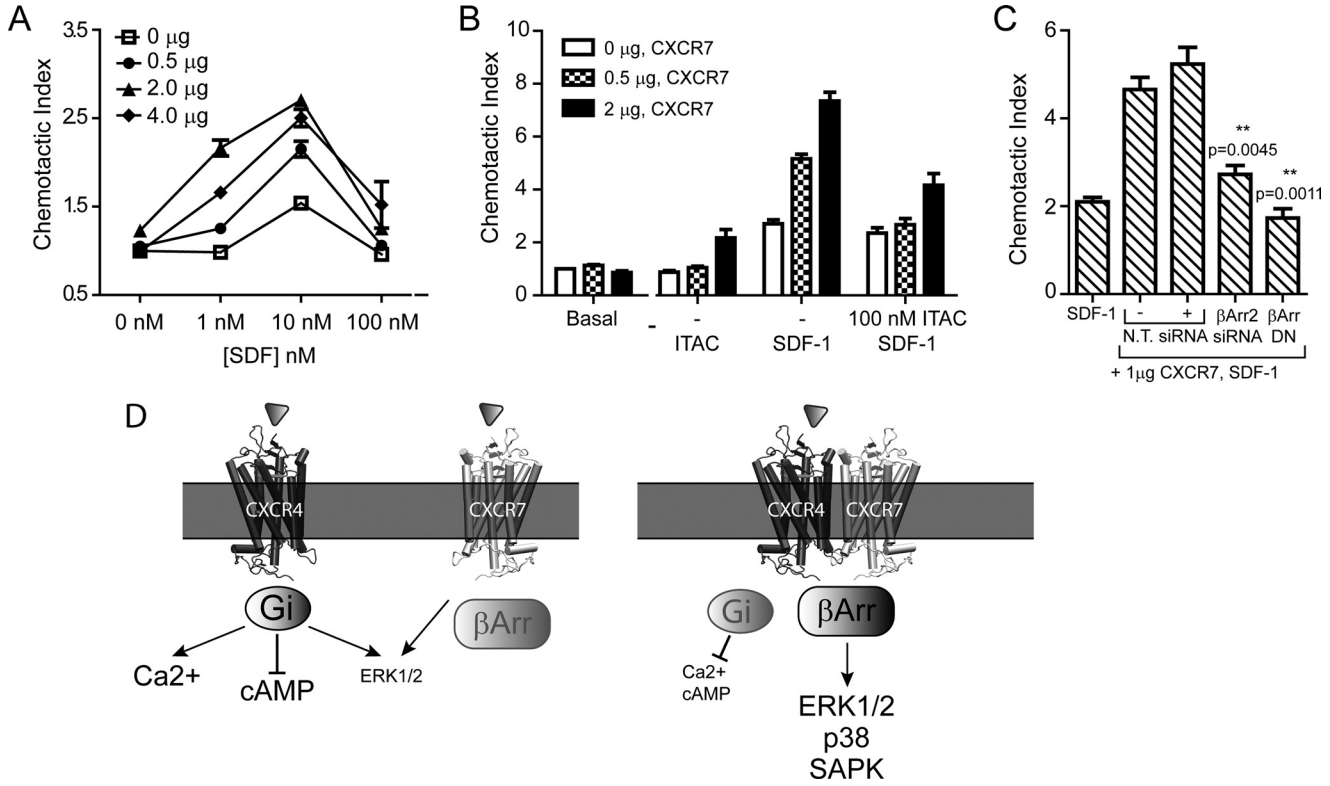

FIGURE 5. **CXCR7 expression enhances chemotaxis.** *A,* chemotaxis of MDA-MB-231 breast cancer cells induced by 0, 1, 10, and 100 nM of SDF-1 in the presence of increasing amounts of transfected CXCR7. Cells become more chemotactic to SDF-1 when increasing amounts of CXCR7 are expressed. *B,* chemotaxis of U87-CD4 cells stably expressing CXCR4 (U87-CD4-CXCR4) induced by 12.5 nM SDF-1 is enhanced in the presence of CXCR7 in a dose-dependent manner (0, 0.5, and 2 μg of CXCR7). Pretreatment of cells with 100 nM ITAC results in decreased SDF-1-stimulated migration of CXCR7 co-expressing cells. *C,* U87-CD4-CXCR4 cells were transfected with nontargeting (*N.T.*) siRNA, βArr2 siRNA, or βArr DN along with 1 μg of CXCR7. Potentiation of SDF-1-stimulated migration of U87-CD4-CXCR4 cells by CXCR7 is reverted in the presence of βArr2 siRNA and βArr DN. Data are expressed as means ± S.E. ($n = 3$). *D,* proposed model showing that in cells treated with SDF-1, CXCR4 triggers inhibition of intracellular cAMP production and Ca^{2+} mobilization, and both CXCR4 and CXCR7 stimulate ERK 1/2 activation (*left panel*). Co-expression of CXCR4 and CXCR7 leads to apparent heterodimerization of CXCR4 and CXCR7, and the heteromeric CXCR4·CXCR7 complex demonstrates a dramatically altered signaling profile. The CXCR4·CXCR7 heteromer is unable to trigger inhibition of cellular cAMP production. The constitutive recruitment of β-arrestin couples CXCR4 stimulation in the CXCR4·CXCR7 complex to proliferative pathways (ERK1/2, p38 MAPK, and SAPK).

angiotensin 1 receptor and confirmed for several additional GPCRs (42, 43). Both G protein-dependent and G protein-independent pathways for ERK activation were demonstrated downstream of activated angiotensin 1 receptors. Specifically, G protein-mediated signaling led to transient nuclear ERK activity, and β-arrestin-dependent signaling resulted in sustained cytosolic ERK activity (42). This distinct spatial and temporal activation of ERK was translated into different signaling cascades. These results and others suggest that biased ligands or conditions that lead to selective activation of one pathway over the other will result in distinct physiological effects, possibly by stabilizing different "active" receptor conformations (33, 34). There are indeed such examples of selective ligands; for example, CCL19 and CCL21 are two CCR7-specific endogenous chemokine ligands that are equally effective in their ability to activate G proteins, but only CCL19 can induce β-arrestin recruitment and β-arrestin-dependent ERK activation (44). Similarly, both β_2-adrenergic and parathyroid hormone receptors have been shown to have specific ligands that are biased toward either G protein-dependent or β-arrestin-dependent signaling (45, 46).

β-Arrestin-mediated ERK and p38 MAPK activation have been linked to stimulus-driven cell migration and cell survival downstream of CXCR4 (47–50). Moreover, lymphocytes derived from β-arrestin2 knock-out animals were found to be impaired in SDF-1-mediated chemotaxis (47). Interestingly, co-expression of β-arrestin with CXCR4 resulted in attenuation of chemokine-stimulated G protein activation and inhibition of cAMP production while increasing receptor internalization and ERK1/2 activation (51). In the same study, a dominant negative inhibitor of β-arrestin had no effect on G protein signaling, although strongly inhibiting receptor internalization and ERK1/2 activation. Taken together, β-arrestin-dependent signaling downstream of chemokine receptor activation provides a G protein-independent mechanism for linking GPCRs to several signaling pathways involved in cell proliferation, survival, and migration.

Our results, as well as other recently published data, show that CXCR7 co-expression shifts CXCR4 signaling away from the G protein-linked pathways (20). But to date, there has been no mechanistic insight into the signaling induced by the CXCR4·CXCR7 heteromeric complex. We show that the CXCR4·CXCR7 complex potently induces β-arrestin membrane recruitment and enhances activation of SDF-1-induced ERK1/2, p38, and SAPK pathways. The enhanced activity of the CXCR4-CXCR7 heterodimer in recruiting the β-arrestin scaffold complex provides mechanistic insight into the growth, survival, and migratory advantage provided by CXCR4 and

CXCR7 co-expression in cancer cells. An interesting but challenging follow up of this work will be to identify the determinants guiding the enhanced β-arrestin recruitment to the CXCR4·CXCR7 complex we observed. For example, a study conducted by Gravel *et al.* (32) demonstrated that a chimeric CXCR7 bearing the C-terminal tail of CXCR4 resulted in constitutive recruitment of β-arrestin. Such recent studies have demonstrated the usefulness of bioluminescence resonance energy transfer, luciferase complementation, and imaging techniques to investigate the β-arrestin-CXCR7 interaction (20, 21, 23, 24, 32). Although analogous bioluminescence resonance energy transfer/FRET techniques can be implemented to identify the specific partner of the CXCR4·CXCR7 dimer pair that plays a key role in β-arrestin recruitment, the localization and identification of amino acids involved in contacting β-arrestin may require more focused techniques such as unnatural amino acid mutagenesis, bio-orthogonal labeling, photochemical cross-linking, and reconstitution in membrane nanoparticles. We and others have successfully used these techniques to identify intra-receptor conformational changes (52) as well as receptor-G protein/arrestin binding and activation (53, 54). For example, both monomeric and dimeric receptors states have been reported as functional units for β-arrestin binding and activation (54, 55). In our system, the CXCR4·CXCR7 complex constitutively recruits β-arrestin and will therefore serve as a good model system to map the β-arrestin-interacting sites. Taken together, the techniques described above, along with known modulators of CXCR4 that have recently been shown to affect selectively the ability of CXCR7 to recruit β-arrestin (32, 56), will allow further delineation of the observed enhanced recruitment of β-arrestin to the CXCR4·CXCR7 complex.

Both CXCR4 and CXCR7 expression levels have been correlated with increased cell proliferation *in vitro* and enhanced tumor growth and metastatic potential of breast, lung, and prostate tumors *in vivo* in mouse cancer models (9, 29). We used the U87-CXCR4 cell line to study the role of CXCR7 in CXCR4-responsive cell proliferation. As reported earlier, we did observe a modest dose-dependent SDF-1-induced cell proliferation. However, in our model system, transient co-expression of CXCR7 seems to uncouple ligand-induced cell proliferation resulting in ligand-independent cell proliferation. These types of experiments in transfected cells have limited import, but relevant findings utilizing various transformed cell lines have been reported. For example, glioblastoma cells show SDF-1α dose-dependent proliferation, which is reduced by AMD3100 pretreatment (57–59). Interestingly, CXCR7 confers a growth advantage to the breast tumor cell line MDA MB435s, which was seen as an increase in the live cell count, but not necessarily the total cell count, an effect that can be reduced with the specific CXCR7 ligand CCX754 (12). Similar CXCR7-dependent proliferation was also seen in NIH 3T3 cells (60). Although SDF-1 did not significantly increase the proliferation of A764 glioma cells endogenously expressing CXCR7, ligand stimulation reduced camptothecin-induced apoptosis, which could be reversed using the CXCR7-specific ligand CCX733 (19). In an analogous study with U373 glioma cells, AMD3100 and CCX733 independently reduced SDF-1-induced proliferation, indicating the role of CXCR4 and CXCR7 in cell proliferative pathways (62). Calatozzolo *et al.* (62) also report statistically significant differences between untreated and CCX733-treated cells that had not been stimulated with SDF-1. CXCR4 and CXCR7 expression levels were also linked to early and metastatic recurrence in non-small cell lung cancer (63). High CXCR7 expression was inversely correlated with 5-year disease-free survival of patients with non-small cell lung cancer and renal cancer (63, 64). Interestingly, targeted overexpression of CXCL11/ITAC in tumor sites displayed antitumor activity in a murine cancer model, and ITAC also served as a potent antagonist of transendothelial migration of CXCR4- and CXCR7-positive human tumor cells (25, 65).

Our results provide a mechanistic insight into these previous observations. We show that treatment of the CXCR4·CXCR7 complex with ITAC modulates the signaling induced by the CXCR4/CXCR7 heterodimer. ITAC reverses the effect of CXCR7 on CXCR4-induced inhibition of cAMP production, bringing it back to levels normally observed with CXCR4 alone (Fig. 1). ITAC treatment reduces the levels of β-arrestin recruited to the CXCR4·CXCR7 complex and also reduces the migratory advantage provided by CXCR7 co-expression (Figs. 2 and 5). Hence, ITAC could be functioning as an allosteric modulator of the CXCR4·CXCR7 dimer complex. Conceivably, ITAC binding to CXCR7 may induce cross-conformational changes within CXCR4 restoring its coupling to G_i. Similar allosteric modulation has been observed in other chemokine receptor heterodimer complexes (61, 66). Further studies are warranted to evaluate the functional consequences of targeting CXCR7 using CXCR7-specific ligands or small molecules to modulate the CXCR4/CXCR7 heterodimer and its effect on tumor growth and metastasis. The central role of CXCR4 in numerous disease processes, including cancer, HIV infection, atherosclerosis, and rheumatoid arthritis, and the emerging role of CXCR7 in tumor progression and metastasis validate both receptors as promising therapeutic targets for possible pharmacological intervention.

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