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Imaging CXCR4 Signaling with Firefly Luciferase Complementation

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Abstract

Chemokines and their cognate receptors have key functions in cell growth, survival, and tissue-specific homing of cells. While these functions first were identified in normal immune cells, cancer cells may co-opt chemokine receptor signaling to promote primary tumor growth and metastasis. Our knowledge of signaling by chemokines and chemokine receptors in cancer is lacking, particularly as this signaling occurs in vivo. New insights into chemokine receptor signaling in cancer are needed to understand molecular regulation of primary and metastatic disease and develop targeted therapies to improve patient survival. To meet this need, we have developed a molecular imaging reporter to investigate activation of CXCR4, a chemokine receptor that regulates tumor growth and metastasis in a variety of common cancers. The reporter system uses a firefly luciferase-based protein fragment complementation assay to detect interactions between CXCR4 and β -arrestin molecules, a common early step in chemokine receptor signaling. In cell-based assays, incubation with the chemokine ligand CXCL12 (SDF-1) produced dose-dependent increases in bioluminescence with >7-fold induction above basal levels of association between these proteins. Reporter activation could be blocked with specific inhibitors of CXCR4 signaling. These reporters enabled in vivo imaging of CXCR4 activation and inhibition in living mice. Overall, this research establishes a new imaging reporter for probing CXCR4 signaling in cancer and other diseases regulated by this chemokine receptor.

Chemokines are a family of small, secreted proteins that bind to specific chemokine receptors, a subgroup of the family of seven transmembrane (G-protein-coupled) receptors, expressed on the cell membrane of target cells. Signaling through chemokine receptors produces cytoskeletal rearrangement, integrin-mediated adhesion to endothelium, and directional migration of cells.^{1,2} Chemokines originally were identified because of functions in development and regulation of immune cell trafficking,³ but chemokines also regulate angiogenesis,⁴ cell cycle progression,⁵ and resistance to apoptosis.⁶ Due to the central importance of chemokine receptor signaling in a wide array of cellular and molecular

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SUPPORTING INFORMATION AVAILABLE

PCR primers given in Table 1. This material is available free of charge via the Internet at <http://pubs.acs.org>.

functions, chemokine receptors are critical to pathophysiology of common diseases including cancer, atherosclerosis, infection, and autoimmunity. Therapeutic compounds targeted against chemokine receptors likely will have widespread applications in many different clinical settings, making chemokine receptors a focus of ongoing drug discovery and development.

Chemokine receptor CXCR4 and its cognate ligand CXCL12 have drawn particular attention as key regulators of normal development, physiology, and disease. CXCL12–CXCR4 signaling promotes proliferation, survival, and migration of normal and malignant cells. Loss of CXCR4 signaling in neuronal progenitor cells perturbs development of the central nervous system,^{7–9} and this receptor controls trafficking of hematopoietic stem and progenitor cells.¹⁰ CXCL12–CXCR4 signaling also is essential for normal vasculogenesis of the gastrointestinal tract.¹¹ Normal physiologic functions of CXCR4 may be co-opted in diseases such as cancer, where expression of the receptor increases metastatic disease and correlates with poor survival in malignancies including breast, prostate, and osteosarcoma.^{12–14} Disrupting CXCR4 signaling also may benefit cancer therapy by inhibiting tumor angiogenesis,⁴ while proangiogenic functions of CXCR4 may be exploited for cell-based therapies in ischemic vascular disease.¹⁵ In addition, CXCR4 acts as a coreceptor for HIV, and binding of the Nef protein from HIV to CXCR4 initiates an apoptotic signaling cascade in cells.¹⁶ Collectively, these studies highlight the diverse biologic effects of CXCL12–CXCR4 and the need to better understand how activation and inhibition of this signaling pathway regulates mechanisms of disease, especially as these processes occur in vivo.

Signaling by CXCR4 proceeds through a common pathway shared among all chemokine receptors and other seven transmembrane receptors. Binding of CXCL12 to CXCR4 causes ligand-dependent conformational changes in the receptor, resulting in phosphorylation of the intracellular C-terminus of CXCR4 by protein kinase C and G protein-coupled receptor kinases (GRK) such as GRK3.¹⁷ The phosphorylated receptor then recruits a member of the β -arrestin family of cytosolic scaffolding proteins, either β -arrestin-1 or β -arrestin-2. The interaction between activated chemokine receptor and β -arrestin causes internalization of the bound chemokine–receptor pair and regulates signaling through downstream effector molecules such as ERK1/2. In particular, assays for recruitment of β -arrestin to seven transmembrane receptors have been used to interrogate ligand-dependent and -independent activation of a specific receptor using either optical or enzymatic reporter systems.^{18–20} These assays have been used to monitor chemokine receptor signaling in cell-based assays, and one such system based on β -galactosidase has been used to image activation of nonchemokine seven transmembrane receptors in living mice.²¹ However, the β -galactosidase assay is linked indirectly to the imaging signal and produces modest signal induction (2–4-fold) in vivo, emphasizing the need for an improved assay to study seven transmembrane receptors in disease and quantify pharmacodynamics of therapeutic agents.

To image CXCL12–CXCR4 signaling in intact cells and living mice, we used protein fragment complementation assay (PCA) based on fragments of firefly luciferase to quantify interactions between CXCR4 and β -arrestin molecules. The firefly luciferase complementation pair used in this study originally was identified through a library screen to

select enzyme fragments with minimal background association and high reconstitution of bioluminescence when brought together by interacting proteins.²² We tested various orientations of fusion proteins and complementation with either β -arrestin-1 or β -arrestin-2 to optimize the reporter system for chemokine receptor signaling. Using the optimized reporter, we were able to quantify activation and inhibition of CXCR4 in cell-based assays and enhance reporter activation for in vivo imaging. By directly linking the PCA to a bioluminescent signal with higher induction for in vivo imaging, this research establishes an improved reporter assay for monitoring chemokine receptor signaling and screening for effective inhibitors in cells and living animals.

MATERIALS AND METHODS

DNA Constructs

Luciferase complementation plasmids in vector pEF for the N-terminal fragment of firefly luciferase (NLuc)-416, the C-terminal fragment of firefly luciferase (FL) (CLuc)-398, and c-fos-CLuc-398 were provided by Alnawaz Rehemtulla (University of Michigan). Human CXCR4 (gift of Bryan Cullen, Duke University) was amplified by PCR and inserted with *SaII/NotI* to fuse the carboxy terminus of CXCR4 to the amino terminus of NLuc-416 using a linker of amino acids AAAQISYASRGGGSSGGG. β -Arrestin 1 and β -arrestin 2-green fluorescent protein (GFP) plasmids (provided by John Tesmer, University of Michigan) were amplified by PCR and inserted with *SaII/NotI* or *NotI/XbaI* to generate N- or C-terminal fusions with CLuc 398. Amino acids in the linkers between the proteins were GGGSSGGGQISYASRGSGR and RARDPPVGGGSSGGG for N- or C-terminal fusions, respectively. NLuc-398 and C-Luc 394 were amplified by PCR using pGL3 basic (Promega) as a template and inserted with *NotI/XbaI* to replace NLuc-416 or CLuc-398 as fusion proteins with CXCR4 or β -arrestin 1 or β -arrestin 2, respectively. All PCR products were verified by DNA sequencing. Sequences of PCR primers are provided in Supporting Information, Table 1.

To coexpress pairs of complementation reporters from the same plasmid, NLuc and CLuc fragments were excised with *SaII* and *XbaI* and blunted with Klenow. CXCR4–NLuc416 was inserted into the blunted *BamHI* site of pBudCE4.1 (Invitrogen), while β -arrestin 2–CLuc398 and c-fos–CLuc398 were ligated into the blunted *KpnI* site. Lentiviral vectors for these reporter constructs were prepared in FUGW or pSico, which express GFP from a human ubiquitin promoter or CMV promoter, respectively.²⁴ A corresponding vector expressing fluorescent protein mPlum (gift of Roger Tsien, University of California San Diego) was prepared by digesting FUGW with *XbaI* and replacing GFP with mPlum at the blunted *XbaI* sites.²⁵ The resultant vector was designated FUPW. CXCR4–NLuc416, driven by a human EF-1 α promoter, was removed from pEF using *BglII* and *NotI* and inserted into the blunted *EcoRI* site of FUGW. The same strategy was used to insert β -arrestin 2–CLuc398, or c-fos–CLuc398 into FUPW.

Cells

Human embryonic kidney 293 cells stably expressing large T antigen from SV40 virus (293T) obtained from Open Biosystems were cultured in DMEM (Invitrogen), 10% fetal

bovine serum, 1% glutamine, and 0.1% penicillin/streptomycin/gentamicin. Cells were maintained in a 37 °C incubator with 5% CO₂.

To generate stable cell lines coexpressing CXCR4–NLuc416 and β -arrestin 2–CLuc398 or c-fos–CLuc398, we prepared recombinant lentiviruses expressing each of these proteins as described previously.^{23,26} The 293T cells first were transduced with supernatants containing CXCR4–NLuc416 in FUGW. Transduction efficiency was 100% as determined by fluorescence microscopy. These cells then were transduced with supernatants for β -arrestin 2–CLuc398 or c-fos–CLuc398 in FUPW. Again, all cells were transduced with CLuc constructs, as determined by fluorescence microscopy for mPlum.

Live Cell Imaging

Cells were transfected by calcium phosphate precipitation as described previously.²³ One day after transfection, cells were split into black-walled 96-well plates (Corning) at 1×10^4 cells/well, and experiments were performed 2 days after transfection. For experiments with stable cell lines, cells were plated into 96-well plates as described above using a Multidrop 384 dispensing system (Labsystems) 1 day prior to each assay. Quadruplicate samples were used for all experimental conditions. Cells were treated with various concentrations of CXCL12 (R & D Systems), AMD3100 (Sigma), or TF14013 (generous gift of N. Fujii) as described in figure captions. Incubation times for each agent also are listed in figure captions.

Bioluminescence imaging of live cells was performed on a cryogenically cooled camera system (IVIS 100 or 200, Caliper) as described previously,²² using 1–5-min acquisition times, high sensitivity, and field of view B.

Western Blots

Cells were harvested from 96-well plates or parallel cultures of transfected cells as described previously.²² In some experiments, cells were transfected with human CXCR4–GFP as a positive control. Total cell lysates were probed with 1:500 dilutions of antibodies to phosphorylated or total Erk1/2 (Santa Cruz), CXCR4 (Santa Cruz), or firefly luciferase (Promega). Primary antibodies were detected with appropriate species-specific secondary antibodies conjugated with horseradish peroxidase.

In Vivo Bioluminescence Imaging

All animal procedures were approved by the University of Michigan Committee for use and care of animals. The 293T cells were transfected with combinations of plasmids with N- and C-terminal fragments of luciferase as described in the figure caption. Cells were cotransfected with a plasmid expressing mPlum²⁵ to control for total number of cells injected into mice. Cells were harvested 3 days after transfection, and 5×10^6 cells in 200 μ L of sterile 0.9% NaCl were injected intraperitoneally (ip) into 10–12-week-old female SCID mice (Charles Rivers). To activate CXCR4 signaling, mice were treated with 2 μ g of CXCL12 in 100 μ L of 0.9% NaCl solution or vehicle control alone. In separate experiments, mice were pretreated ip with 60 μ g of AMD3100 in sterile phosphate-buffered saline or vehicle control 30 min before injecting CXCL12.

Bioluminescence imaging was performed on an IVIS Spectrum system (Caliper). Fluorescence imaging of mPlum was performed with excitation and emission filters of 620 and 680 nm, respectively. Images were acquired for 1 s using high sensitivity. Following fluorescence imaging, bioluminescence imaging was performed as described previously.²⁷ Sequential bioluminescence images (1-min acquisition, high sensitivity) were acquired for up to 30 min. Data for fluorescence and bioluminescence were quantified as efficiency and photons, respectively, using Living Image software (Caliper). Photon data for bioluminescence were normalized to fluorescence efficiency to account for differences in injected cells.

Statistics

Data are plotted as mean values with standard error of the mean (SEM). Pairs of data were analyzed by *t* test to determine statistically significant differences (GraphPad Prism). EC₅₀ values were calculated with BioDataFit 1.02 software.

RESULTS

Optimization of Firefly Luciferase PCA To Monitor CXCR4 Activation

PCA strategies measure interactions between proteins, such as two proteins in a signaling pathway, by fusing target proteins of interest to separate fragments of a reporter protein. Activity of the reporter protein is reconstituted when individual fragments are brought in proximity by interactions between the fused proteins of interest (reviewed in ref 28). We used the FL as the PCA reporter for CXCR4 signaling, based on optimized FL enzyme fragments (NLuc416 and CLuc398) we identified through a library screen.²² This PCA method builds on the established sensitivity of FL in imaging studies in intact cells and living animals, as well as the favorable pharmacology of the D-luciferin substrate for in vivo imaging.²⁹

CXCL12 binding to CXCR4 causes phosphorylation of the receptor and recruitment of a β -arrestin molecule to the third intracellular loop and cytoplasmic tail of CXCR4 as an early step in CXCR4 signaling. We used CXCR4 and β -arrestin as the interacting proteins of interest for the FL-based PCA imaging reporter. In this assay, binding of chemokine ligand CXCL12 to its receptor CXCR4 results in recruitment of a β -arrestin molecule to the activated receptor, bringing together fragments of FL and restoring activity of the reporter enzyme. Therefore, the magnitude and kinetics of CXCR4 signaling can be quantified by changes in bioluminescence (Figure 1A).

We fused the C-terminus of CXCR4 to NLuc416 (CXCR4–NLuc416), positioning the FL fragment in the intracellular space. FL enzyme fragments must interact in the same cellular compartment, so positioning NLuc416 in the extracellular space on the N-terminus of CXCR4 would not allow complementation with CLuc-398 on cytoplasmic β -arrestin 2. We fused CLuc398 to either the N- or C-terminus of β -arrestin 2 (CLuc398– β -arrestin 2 or β -arrestin 2–CLuc398, respectively) to test the extent to which complementation was affected by position of the CLuc fragment relative to β -arrestin 2. We cotransfected 293T cells with pairs of CXCR4–NLuc416 and CLuc398– β -arrestin 2 or β -arrestin 2–CLuc398,

respectively. We also transfected cells with single plasmids for CXCR4–NLuc416, CLuc398– β -arrestin 2, or β -arrestin 2–CLuc398 to measure bioluminescence from isolated FL fragments.

Forty-eight hours after transfection, cells cultured in medium containing 10% serum were treated with vehicle control or 100 ng/mL CXCL12, a concentration of chemokine known to produce robust activation of CXCR4 signaling pathways.³⁰ Bioluminescence produced by interaction of CXCR4 and β -arrestin 2 in living cells was measured on sequential images obtained for 30 min after adding chemokine ligand. In cells treated with vehicle only, imaging signal was significantly greater in cells transfected with CXCR4–NLuc416 and β -arrestin 2–CLuc398 relative to CXCR4–NLuc416 and CLuc398– β -arrestin 2 ($p < 0.01$). Bioluminescence in the absence of CXCL12 indicates that there is baseline association of CXCR4 and β -arrestin 2 without ligand, consistent with previous data obtained using purified proteins and coimmunoprecipitation of proteins from cultured cells.³⁰ Treatment with CXCL12 increased bioluminescence from the CXCR4–NLuc416 and β -arrestin 2–CLuc398 pair by ~6-fold, while the signal increased by only 1.5-fold in cells coexpressing CXCR4–NLuc416 and CLuc398– β -arrestin 2 ($p < 0.01$). For both pairs of reporters, peak luciferase activity occurred ~3–4 min after adding chemokine ligand. There was no detectable bioluminescence above background levels in cells expressing only single NLuc or CLuc fusion proteins, and incubation with CXCL11, a chemokine that does not bind to CXCR4, did not affect the imaging signal (data not shown). Western blotting for CXCR4 or the CLuc fragment of FL verified that all proteins were expressed at comparable levels (data not shown). Collectively, these data establish that FL complementation can detect interactions between CXCR4 and β -arrestin 2 and identify a preferred orientation for the β -arrestin 2–CLuc398 fusion protein.

Interaction of CXCR4–NLuc416 with β -arrestin 2–CLuc398 suggests that the CXCR4 fusion protein remains competent to activate downstream signaling pathways. To investigate signaling by CXCR4–NLuc416, we transiently transfected 293T cells with expression plasmids for CXCR4–NLuc416 or pGL4 luciferase (negative control). As a positive control, we transfected 293T cells with CXCR4 fused to GFP (CXCR4–GFP). Previous studies have established that CXCR4 remains functional when the intracellular C-terminus of the receptor is fused to GFP.³¹ Twenty-four hours after transfection, cells were switched to medium containing 0.5% serum for overnight and then treated with 100 ng/mL CXCL12 for 5 min. Total cell lysates were probed by Western blot to detect phosphorylation of ERK1/2, a well-established downstream effector molecule activated by CXCL12–CXCR4 signaling.³² Incubation with CXCL12 activated ERK1/2 in cells expressing either CXCR4–NLuc416 or CXCR4–GFP (Figure 1C). Cells expressing CXCR4–GFP had relatively increased levels of phosphorylated ERK1/2 in the absence of CXCL12, consistent with ligand-independent activation of downstream signaling pathways that may occur in cells overexpressing a seven transmembrane receptor.³³ By comparison, CXCL12 did not change phosphorylation of ERK1/2 in negative control cells transfected with pGL4 luciferase. These data establish that CXCR4–NLuc416 can initiate CXCL12-dependent signaling.

In addition to β -arrestin 2, epithelial cells express a second β -arrestin family member, β -arrestin 1, which also binds to chemokine and other seven transmembrane receptors. A

previous study showed that association of CXCR4 with β -arrestin 1 is lower than that for β -arrestin 2 in the absence of ligand, while CXCL12-dependent interactions between CXCR4 and β -arrestin 1 are comparable to those for CXCR4 and β -arrestin 2.³⁰ These data suggest that luciferase complementation between CXCR4 and β -arrestin 1 may have lower basal signal and greater induction in response to stimulation, thereby enhancing signal-to-background ratios for imaging. To test this hypothesis, we cotransfected cells with CXCR4–NLuc416 and either β -arrestin 2–CLuc398 or β -arrestin 1–CLuc398. As a negative control, we also cotransfected cells with CXCR4–NLuc416 and c-fos fused to CLuc398. The c-fos protein has no known interactions with CXCR4, allowing us to quantify bioluminescence produced by nonspecific association of NLuc and CLuc within cells. In the absence of CXCL12, the pair of CXCR4–NLuc416 and β -arrestin 2–CLuc398 produced bioluminescence comparable to CXCR4–NLuc416 and β -arrestin 1–CLuc398 (Figure 2A). The response to 1 ng/mL CXCL12 was comparable for CXCR4–NLuc416 and β -arrestin 1–CLuc398, but peak bioluminescence at 100 ng/mL CXCL12 was significantly less for β -arrestin 1 relative to β -arrestin 2 ($p < 0.01$). For CXCR4–NLuc416 and β -arrestin 2–CLuc398, CXCL12 produced a dose-dependent increase in bioluminescence that reached ~8-fold above baseline at 100 ng/mL CXCL12. There was significantly less baseline bioluminescence from CXCR4–NLuc416 and c-fos–CLuc398 ($p < 0.001$), and CXCL12 had no effect on the low levels of light produced by this pair. Data with CXCR4–NLuc416 and c-fos–CLuc398 exclude the possibility that bioluminescence is produced by nonspecific interactions between CXCR4 and a different cytoplasmic protein. Western blotting was performed on quadruplicate samples of each PCA pair 48 h after transfection and without treatment with CXCL12 (Figure 2B). There were small variations in amounts of various reporter proteins due to differences in cell numbers, but these do not account for the greater signal from CXCR4–NLuc416 and β -arrestin 2–CLuc398. Collectively, these data established that bioluminescence was due to specific interaction between CXCR4 and β -arrestin and demonstrate that the complementation pair of CXCR4–NLuc416 and β -arrestin 2–CLuc398 provides significantly greater dynamic range for imaging activation of CXCR4.

Recently, Paulmurugan et al. reported an alternative FL complementation pair, NLuc398 and CLuc394, with improved background signal and induction for monitoring interactions between cytosolic proteins.³⁴ We constructed CXCR4–NLuc398 and β -arrestin 2–CLuc394 fusion proteins to allow direct comparison with the complementation pair used for the preceding experiments. We cotransfected cells with CXCR4–NLuc416 and β -arrestin 2–CLuc398 or the CXCR4–NLuc398 and β -arrestin 2–CLuc394 pair and then quantified baseline and CXCL12-dependent bioluminescence in living cells. Association between CXCR4–NLuc416 and β -arrestin 2–CLuc398 produced the expected baseline bioluminescence and dose-dependent increase in light following treatment with CXCL12. However, no bioluminescence above background could be detected in cells coexpressing the CXCR4–NLuc398 and β -arrestin 2–CLuc394 pair under baseline conditions or in response to CXCL12 (data not shown). Collectively, data from these experiments support the use of CXCR4–NLuc416 and β -arrestin 2–CLuc398 for imaging CXCR4 activation.

Cell-Based Assays for CXCR4 Activation and Inhibition

Having established an optimized FL complementation strategy for monitoring CXCR4 activation, we then used lentiviral transduction to generate stable populations of 293T cells expressing either the CXCR4 reporter (CXCR4–NLuc416/ β -arrestin 2–CLuc398) or negative control reporter (CXCR4–NLuc416/c-fos–CLuc398). We used these cells for more detailed analyses of the CXCR4 reporter in cell-based assays under various experimental conditions. Cells stably expressing the negative control reporter were undetectable above background bioluminescence without or with CXCL12 treatment, so these cells were not analyzed further in experiments described below.

CXCR4 reporter cells were incubated with increasing concentrations of CXCL12 for various periods of time up to 45 min. For each time point, we quantified bioluminescence from recruitment of β -arrestin 2 to CXCR4 in intact cells using an IVIS system. At times 5 and 10 min after adding CXCL12 to intact cells, there were dose-dependent increases in bioluminescence that peaked at ~7-fold induction at 10 min (Figure 3A). The EC_{50} for CXCL12-dependent induction of the CXCR4 reporter was 30 ng/mL ($r^2 = 0.96$ for a 4-parameter model), which is slightly lower than that previously reported for CXCR4-dependent chemotaxis in response to CXCL12 ($EC_{50} \approx 60$ ng/mL).³⁵ Bioluminescence subsequently declined to ~2-fold induction after 45 min of treatment with 10 ng/mL or greater concentrations of CXCL12. Kinetics of reporter activity is consistent with known events in CXCL12–CXCR4 signaling: (1) chemokine-receptor binding; (2) recruitment of β -arrestin 2; (3) internalization of the receptor into endosomes; and (4) dissociation of β -arrestin 2 from CXCR4.

Treatment with CXCL12 causes internalization of CXCR4 from the cell surface, effectively desensitizing the receptor for further activation and signaling. To investigate dose-dependent effects of CXCL12 on this process, we treated reporter cells with vehicle control or increasing concentrations of CXCL12 for 1 h. After this pretreatment period, parallel cultures of cells then were restimulated with 100 ng/mL CXCL12 immediately before quantifying bioluminescence (Figure 3B). One-hour incubation with 10 ng/mL or higher CXCL12 blunted reporter activity in response to restimulation with chemokine. Interestingly, pretreatment with very low concentrations of CXCL12 (0.1–1 ng/mL) potentiated association of CXCR4 with β -arrestin 2 as compared with cells pretreated with vehicle only before stimulation with CXCL12. These data suggest that low doses of CXCL12 prime the pathway for further activation, potentially by recruiting additional receptors to the cell surface.

To establish that this reporter can quantify inhibition of CXCR4 signaling, we tested AMD3100 and TF14013, two known inhibitors of the CXCL12–CXCR4 pathway.^{36,37} CXCR4 reporter cells were pretreated for 30 min with increasing concentrations of each compound and then treated with a maximal stimulatory dose (100 ng/mL) of CXCL12. Both agents blocked CXCL12-dependent activation of bioluminescence with EC_{50} values of ~0.8 and ~0.3 nM for AMD3100 and TF14013, respectively ($r^2 = 0.99$ and 0.94, respectively for a sigmoidal model) (Figure 4). These values are comparable to EC_{50} data previously reported for these compounds to block CXCL12 binding to CXCR4.^{38,39} Low concentrations of TF14013 (0.001 and 0.01 nM), but not AMD3100, enhanced reporter

activity above incubation with CXCL12 alone, similar to pretreatment with low concentrations of CXCL12. These data suggest bimodal potentiation and inhibition and CXCR4 signaling with TF14013. Collectively, these data validate the CXCR4 reporter system as a quantitative assay for CXCR4 activation and inhibition in cell-based assays.

In Vivo Imaging of CXCR4 Activation and Inhibition

There currently is no method to detect and quantify CXCR4 signaling in living animals, so we tested the hypothesis that the CXCR4–NLuc-416 and β -arrestin 2–CLuc-398 PCA would enable in vivo imaging of CXCR4 activation. We cotransfected cells with CXCR4–NLuc-416 and β -arrestin 2–CLuc-398 or the negative control pair of CXCR4–NLuc-416 and c-fos–CLuc-398. All cells also were transfected with fluorescent protein mPlum as an internal control for variations in numbers of cells introduced into mice. Transiently transfected cells were used for these experiments to produce high levels of mPlum in cells, enhancing sensitivity for fluorescence imaging to detecting small differences in injected cells in each animal and normalize the bioluminescence reporter signal.

Recent studies show that CXCL12–CXCR4 signaling promotes intraperitoneal metastases from a variety of cancers, including gastric and ovarian. CXCL12 is present in intraperitoneal ascitic fluid associated with malignant cells, providing the chemokine ligand for CXCR4 on cancer cells.^{40–43} Clinical trials in patients with these cancers have established that direct intraperitoneal administration of chemotherapeutic agents improves survival. Therefore, intraperitoneal injection of reporter cells serves as a model of CXCR4 signaling and response to therapy in these common cancers.

Transfected reporter cells were injected intraperitoneally into female SCID mice and then imaged sequentially for mPlum fluorescence and bioluminescence. Under baseline conditions, low levels of bioluminescence were produced by the association of CXCR4–NLuc-416 and β -arrestin 2–CLuc-398 (Figure 5A, B). Similar to experiments in cell culture, basal bioluminescence from CXCR4–NLuc-416 and β -arrestin 2–CLuc-398 was greater than that produced by CXCR4 and c-fos. Mice then were injected with 2 μ g of CXCL12 to model elevated levels of this chemokine found with intraperitoneal metastases, and bioluminescence from the CXCR4 imaging reporter was monitored with sequential images over a period of 30 min. Treatment of mice with CXCL12 increased bioluminescence from the CXCR4 reporter by 8-fold above levels of light produced under baseline conditions ($p < 0.001$), showing robust in vivo activation of CXCR4 signaling. By comparison, CXCL12 had no effect on the minimal bioluminescence from CXCR4–NLuc-416 and c-fos–CLuc-398.

To further validate the CXCR4–NLuc-416 and β -arrestin 2–CLuc-398 complementation pair for in vivo studies of CXCR4 activation, we quantified inhibition of CXCR4 signaling in living animals. Mice were implanted with cells cotransfected with CXCR4–NLuc-416, β -arrestin 2–CLuc-398, and mPlum as described above. After obtaining baseline images, mice were treated for 30 min with 60 μ g of AMD3100 or vehicle alone injected intraperitoneally to model treatment of patients with intraperitoneal metastases. Repeat imaging showed that AMD3100 produced a modest increase in bioluminescence, consistent with data showing this compound may function as a partial agonist of CXCR4 (Figure 5C).⁴⁴ Reporter activity

was unaffected by injection of vehicle control. Animals then were injected with CXCL12 and imaged over a 30-min period for activation of CXCR4. Pretreatment with AMD3100 completely blocked the CXCL12-dependent activation of CXCR4 observed in mice treated with vehicle control ($p < 0.01$). Overall, these data establish the high inducibility of FL complementation for imaging CXCR4 activation and the ability to use this reporter to assay inhibition of this receptor in vivo.

DISCUSSION

Chemokine receptors are essential for normal development and physiology, and members of this receptor family are emerging as central regulators of multiple disease processes. In particular, CXCL12 signaling through CXCR4 controls physiologic and pathophysiologic trafficking of cells including stem and progenitor cell types, differentiated immune cells, and malignant cells from a wide variety of cancers. CXCR4 is a coreceptor for HIV infection, and the receptor also functions in normal and pathologic angiogenesis. As a result, modulating CXCR4 function in vivo is proposed as a new treatment strategy for a variety of diseases. Stimulating CXCL12–CXCR4 signaling may enhance therapeutic trafficking of stem cells to injured or ischemic tissues, while blocking this pathway may be effective against cancer, lung fibrosis, HIV, and autoimmune diseases. Understanding activation and inhibition of this receptor in cell-based assays is critical to defining functions of this molecule and developing new therapeutic agents targeted against it. Real-time, repetitive quantification of chemokine receptor signaling in mouse models of disease also is needed to reveal spatial and temporal patterns of activation during disease initiation and progression and to monitor pharmacodynamics of therapeutic agents.

We have developed a FL PCA strategy for monitoring CXCR4 activation in intact cells and living mice. The reporter was optimized for several different parameters, including orientation of fusion proteins, β -arrestin molecule, and FL enzyme fragments. The CXCR4–NLuc416 fusion protein activated downstream signaling pathways in response to CXCL12, establishing that the imaging reporter construct reproduces functions of CXCR4. The resultant CXCR4 imaging reporter detects activation of the receptor based on recruitment of β -arrestin-2, producing bioluminescence as a quantitative measure of signaling. Using this reporter system in cell-based assays, we were able to detect dose-dependent activation of CXCR4 by its cognate chemokine ligand, CXCL12, and inhibition of this process with two different inhibitors. EC₅₀ values for activation and inhibition were comparable to those measured with other biologic assays. The imaging reporter allowed CXCR4 activation and inhibition to be quantitatively imaged in living mice, emphasizing the utility of the assay for translating between cell culture assays and preclinical mouse models. Collectively, these studies validated the CXCR4 FL complementation reporter for studying biologic functions of CXCL12–CXCR4 in cell culture assays and in vivo models of normal physiology and disease.

In cell-based assays, the CXCR4 reporter showed unexpected effects of low concentrations of CXCL12 or the inhibitor TF14013 on subsequent activation of the chemokine receptor. Incubation of cells with 0.1 or 1 ng/mL CXCL12 had minimal effects on reporter bioluminescence, but these concentrations enhanced recruitment of β -arrestin-2 in response

to a stimulatory dose of CXCL12 relative to cells not pretreated with this chemokine. Similarly, pretreatment with low concentrations of TF14013 (1 or 10 pM) potentiated reporter activation in response to CXCL12 as compared with cells treated only with CXCL12. However, pretreatment with AMD3100, a structurally unrelated inhibitor, did not produce this effect. These results suggest that these pretreatment conditions with CXCL12 or TF14013 increase levels of CXCR4 on the cell surface to respond to ligand or facilitate CXCL12-dependent interactions between CXCR4 and β -arrestin-2. Further experiments will be required to define mechanisms of action and implications for activating downstream effector pathways and biologic functions.

Recently, von Degenfeld et al. described a related complementation system for assaying activation of seven transmembrane receptors including the β_2 -adrenergic receptor.²¹ This technique used complementation of β -galactosidase to detect recruitment of β arrestin-2 to the target receptor. These authors were able to measure ligand-dependent activation of nonchemokine seven transmembrane receptors in cultured cells and mice, although imaging signals increased by only 2–4-fold in vivo. An important limitation of β -galactosidase complementation for in vivo bioluminescence imaging is that the assay relies upon β -galactosidase activity to convert a precursor substrate to an active substrate for firefly luciferase, which then produces bioluminescence. This two-step process requires that cells express FL in addition to the complementation reporters, increasing the complexity of the system. The protein interaction and enzyme complementation event also may be in a cellular compartment separate from FL, which may dissociate the protein interaction of interest from the imaging readout. Furthermore, the pharmacology and biodistribution of the luminescent β -galactosidase substrate are not well-defined, so there may be unexpected challenges with using this reporter for in vivo imaging studies. Using FL PCA to quantify CXCR4 activation takes advantage of the well-characterized parameters for administering D-luciferin substrate and imaging FL activity and allows bioluminescence to be produced directly from the reaction of FL with its substrate. Both of these factors likely contribute to enhanced signal-to-background in vivo and make the FL PCA an improved strategy for imaging studies in mice.

In conclusion, the validated CXCR4 imaging reporter is a reproducible, quantitative assay for CXCR4 signaling in intact cells and living mice. Unlike many commonly used assays for CXCR4 signaling, the FL PCA reporter does not require cells to be starved prior to adding CXCL12. The bioluminescence output is compatible with high-throughput screening technology, which will facilitate identification of new compounds that block CXCR4 signaling. This imaging reporter will allow CXCR4 signaling to be quantitatively analyzed in mouse models of diseases including cancer, allowing activation and inhibition of this receptor to be monitored during disease progression and therapy. FL complementation also should be generalizable to other chemokine and seven transmembrane receptors, providing an improved method for studying this important family of receptors in intact cells and living animals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Campbell J, Butcher E. *Curr. Opin. Immunol.* 2000; 12:336–341. [PubMed: 10781407]
2. Rossi D, Zlotnik A. *Annu. Rev. Immunol.* 2000;18.
3. Luster A. N. *Engl. J. Med.* 1998; 338:436–445. [PubMed: 9459648]
4. Guleng B, Tateishi K, Ohta M, Kanai F, Jazag A, Ijichi H, Tanaka Y, Washida M, Morikane K, Fukushima Y, Yamori T, Tsuruo T, Kawabe T, Miyagishi M, Taira K, Sata M, Omata M. *Cancer Res.* 2005; 65:5864–5871. [PubMed: 15994964]
5. Khan M, Brandimarti R, Musser B, Resue D, Fatatis A, Meucci O. *J. Neurovirol.* 2003; 9:300–314. [PubMed: 12775414]
6. Broxmeyer H, Kohli L, Kim C, Lee Y, Mantel C, Cooper S, Hangoc G, Shaheen M, Li X, Clapp D. *J. Leukocyte Biol.* 2003; 73:630–638. [PubMed: 12714578]
7. Zou Y, Kottman A, Kuroda M, Taniuchi I, Littman D. *Nature.* 1998; 393:595–599. [PubMed: 9634238]
8. Lu M, Grove E, Miller R. *Proc. Natl. Acad. Sci. U. S. A.* 2002; 99:7090–7095. [PubMed: 11983855]
9. Luo Y, Cai J, Xue H, Miura T, Rao M. *J. Neurochem.* 2005; 93:452–462. [PubMed: 15816868]
10. Peled A, Petit I, Kollett O, Magid M, Ponomaryov T, Byk T, Nagler A, Ben-Hur H, Many A, Shultz L, Lider O, Alon R, Zipori D, Lapidot T. *Science.* 1999; 283:845–848. [PubMed: 9933168]
11. Tachibana K, Hirota S, Iizasa H, Yoshida H, Kawabata K, Kataoka Y, Kitamura Y, Matsushima K, Yoshida N, Nishikawa S, Kishimoto T, Nagasawa T. *Nature.* 1998; 393:591–594. [PubMed: 9634237]
12. Li Y, Pan Y, Wei Y, Cheng X, Zhou B, Tan M, Zhou X, Xia W, Hortobagyi G, Yu D, Hung M. *Cancer Cell.* 2004; 6:459–469. [PubMed: 15542430]
13. Laverdiere C, Hoang B, Yang R, Sowers R, Qin J, Meyers P, Huvos A, Healey J, Gorlick R. *Clin. Cancer Res.* 2005; 11:2561–2567. [PubMed: 15814634]
14. Akashi T, Koizumi K, Tsuneyama K, Saiki I, Takano Y, Fuse H. *Cancer Sci.* 2008; 99:539–542. [PubMed: 18201276]
15. Zemani F, Silvestre J, Fauvel-Lafeve F, Bruel A, Vilar J, Bieche I, Laurendeau I, Galy-Faroux I, Fischer A, Boisson-Vidal C. Ex vivo priming of endothelial progenitor cells with SDF-1 before transplantation could increase their proangiogenic potential. *Arterioscler. Throm. Vasc. Biol.* In press.
16. James C, Huang M, Khan M, Garcia-Barrio M, Powell M, Bond V. *J. Virol.* 2004; 78:3099–3109. [PubMed: 14990729]
17. Balabanian K, Levoe A, Klemm L, Lagane B, Hermine O, Harriague J, Baleux F, Arenzana-Seisdedos F, Bachelier F. Leukocyte analysis from WHIM syndrome patients reveals a pivotal role for GRK3 in CXCR4 signaling. *J. Clin. Invest.* In press.
18. Yan Y, Boldt-Houle D, Tillotson B, Gee M, D'Eon B, Chang X, Olesen C, Palmer M. *J. Biomol. Screening.* 2002;7.
19. Oakley R, Hudson C, Cruickshank R, Meyers D, Payne R Jr, Rhem S, Loomis C. *Assay Drug Dev. Technol.* 2002; 1:21–30. [PubMed: 15090153]
20. Eglen R. *Comb. Chem. High Throughput Screening.* 2005; 8:311–318.
21. von Degenfeld G, Wehrman T, Hammer M, Blau H. *FASEB J.* 2007; 21:3819–3826. [PubMed: 17942828]
22. Luker K, Smith M, Luker G, Gammon S, Piwnica-Worms H, Piwnica-Worms D. *Proc. Natl. Acad. Sci. U. S. A.* 2004; 101:12288–12293. [PubMed: 15284440]
23. Lois C, Hong E, Pease S, Brown E, Baltimore D. *Science.* 2002; 295:868–872. [PubMed: 11786607]

24. Ventura A, Meissner A, Dillon C, McManus M, Sharp P, Van Parijs L, Jaenisch R, Jacks T. *Proc. Natl. Acad. Sci. U. S. A.* 2004; 101:10380–10385. [PubMed: 15240889]
25. Wang L, Jackson W, Steinbach P, Tsien R. *Proc. Natl. Acad. Sci. U. S. A.* 2004; 101:16745–16749. [PubMed: 15556995]
26. Smith M, Luker K, Garbow J, Prior J, Jackson E, Piwnica-Worms D, Luker G. *Cancer Res.* 2004; 64:8604–8612. [PubMed: 15574767]
27. Luker G, Bardill J, Prior J, Pica C, Piwnica-Worms D, Leib D. *J. Virol.* 2002; 76:12149–12161. [PubMed: 12414955]
28. Michnick S, Ear P, Manderson E, Remy I, Stefan E. *Nat. Rev. Drug Discovery.* 2007; 6:569–582. [PubMed: 17599086]
29. Paroo Z, Bollinger R, Braasch D, Richer E, Corey D, Antich P, Mason R. *Mol. Imaging.* 2004; 3:117–124. [PubMed: 15296676]
30. Cheng Z, Zhao J, Sun Y, Hu W, Wu Y, Cen B, Wu G, Pei G. *J. Biol. Chem.* 2000; 275:2479–2485. [PubMed: 10644702]
31. van Buul J, Voermans C, van Gelderen J, Anthony E, van der Schoot C, Hordijk P. *J. Biol. Chem.* 2003; 278:30302–30310. [PubMed: 12766157]
32. Ganju R, Brubaker S, Meyer J, Dutt P, Yang Y, Qin S, Newman W, Groopman J. *J. Biol. Chem.* 1998; 273:2316–23175.
33. Milano C, Allen L, Rockman H, Dolber P, McMinn T, Chien K, Johnson T, Bond R, Lefkowitz R. *Science.* 1994; 264:582–586. [PubMed: 8160017]
34. Paulmurugan R, Gambhir S. *Anal. Chem.* 2007; 79:2346–2353. [PubMed: 17295448]
35. Murphy J, Cho Y, Sachpatzidis A, Fan C, Hodsdon M, Lolis E. *J. Biol. Chem.* 2007; 282:10018–10027. [PubMed: 17264079]
36. De Clercq E. *Nat. Rev. Drug Discovery.* 2003; 2:581–587. [PubMed: 12815382]
37. Tamamura H, Hiramatsu K, Mizumoto M, Ueda S, Kusano S, Terakubo S, Akamatsu M, Yamamoto N, Trent J, Wang Z, Peiper S, Nakashima H, Otaka A, Fujii N. *Org. Biomol. Chem.* 2003; 1:3663–3669. [PubMed: 14649897]
38. Este J, Cabrera C, Schols D, Cherepanov P, Gutierrez A, Witvrouw M, Pannecouque C, Debyser Z, Rando R, Bonaventura C, Desmyter J, De Clercq E. *Mol. Pharm.* 1998; 53:340–345.
39. Tamamura H, Hori A, Kanzaki N, Hiramatsu K, Mizumoto M, Nakashima H, Yamamoto N, Otaka A, Fujii N. *FEBS Lett.* 2003; 550:79–83. [PubMed: 12935890]
40. Yasumoto K, Koizumi K, Kawashima A, Saitoh Y, Arita Y, Shinohara K, Minami T, Nakayama T, Sakurai H, Takahashi Y, Yoshie O, Saiki I. *Cancer Res.* 2006; 66:2181–2187. [PubMed: 16489019]
41. Armstrong D, Bundy B, Wenzel L, Huang H, Barergen R, Lele S, Copeland L, Walker J, Burger R. Group, f. t. G. O. N. *Engl. J. Med.* 2006; 354:34–43. [PubMed: 16394300]
42. Yan T, Black D, Sugarbaker P, Zhu J, Yonemura Y, Petrou G, Morris D. *Ann. Surg. Oncol.* 2007; 14:2702–2713. [PubMed: 17653801]
43. Kajiyama H, Shibata K, Terauchi M, Ino K, Nawa A, Kikkawa F. *Int. J. Cancer.* 2008; 122:91–99. [PubMed: 17893878]
44. Zhang W, Navenot J, Haribabu B, Tamamura H, Hiramatsu K, Omagari A, Pei G, Manfredi J, Fujii N, Broach J, Peiper S. *J. Biol. Chem.* 2002; 277:24515–24521. [PubMed: 11923301]

Figure 1.

Firefly luciferase complementation reporter for CXCR4 activation. (A) Diagram of complementation strategy showing fusion of the N- and CLuc fragments of FL to the membrane protein CXCR4 and the cytoplasmic adapter molecule β -arrestin, respectively. CXCL12 binding to CXCR4 recruits β -arrestin to intracellular domains in the receptor, bringing NLuc and CLuc enzyme fragments into proximity and reconstituting FL activity. Bioluminescence provides a quantitative measure of protein interactions between CXCR4 and β -arrestin, a well-established early step in CXCR4 signaling. (B) 293T cells were

transfected with indicated complementation plasmids and assayed 48 h later under baseline conditions or after treatment with 100 ng/mL CXCL12. Bioluminescence in living cells was quantified with a CCD camera device (IVIS) ($n = 4$, representative of 2 independent experiments). Error bars denote SEM. (C) Western blot showing CXCR4–NLuc416-dependent activation of downstream signaling pathways. 293T cells were transiently transfected with CXCR4–NLuc416, pGL4 negative control plasmid, or CXCR4–GFP positive control plasmid. Twenty-four hours after transfection, cells were switched to medium containing 0.5% serum overnight. Cells then were treated with 100 ng/mL CXCL12 or bovine serum albumin (BSA) for 5 min or left untreated (NT). Total cell lysates were analyzed by Western blot for phosphorylation of ERK1/2 (P-ERK). Blots then were stripped and reprobed for total ERK1/2 (total ERK).

Figure 2.

Enhanced reporter signal from association of CXCR4 with β -arrestin-2. (A) 293T cells were transiently transfected with complementation reporters shown in the figure caption. Forty-eight hours after transfection, cells were treated with increasing concentrations of CXCL12. Bioluminescence produced by living cells was quantified using an IVIS ($n = 4$). Error bars represent SEM. (B) Western blot for β -arrestin-1 or -2-CLuc, c-fos-CLuc, and CXCR4-NLuc in quadruplicate wells of transfected, untreated cells from (A). Immunoblotting was performed with antibodies to luciferase or CXCR4, respectively.

Figure 3.

CXCL12-dependent activation of CXCR4 reporter. (A) Stably transduced CXCR4 reporter cells were incubated with increasing concentrations of CXCL12 for various periods of time prior to adding D-luciferin and quantifying bioluminescence in intact cells ($n = 4$, representative of 2 independent experiments). Data are reported as the fold increase in photons relative to control. Error bars denote SEM. The inset figures show a bioluminescent photo of the reporter cells and a dose-response curve with additional data points from the 10-min time point. (B) Cells were treated with vehicle control (0) or increasing

concentrations of CXCL12 for 1 h, and then all cells were stimulated with an additional 100 ng/mL CXCL12. Bioluminescence was quantified 5–10 min after adding CXCL12 ($n = 4$, representative of 2 independent experiments).

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Figure 4.

Inhibition of CXCR4 signaling reporter. Stable CXCR4 reporter cells were treated with vehicle control or increasing concentrations of CXCR4 inhibitors AMD3100 (AMD) or TF14013 for 30 min. Cells treated with vehicle control only then were treated with 100 ng/mL CXCL12 (CXCL12 only) or BSA (NT), while cells pretreated with inhibitors were incubated with 100 ng/mL CXCL12. Bioluminescence was quantified 5–10 min after adding CXCL12 ($n = 4$ per condition, representative of 2 independent experiments). Error bars are SEM.

Figure 5.

In vivo imaging of CXCR4 activation and inhibition. (A) Mice were injected ip with 5×10^6 cells expressing the activation reporter (CXCR4–NLuc-416 and β -arrestin 2–CLuc-398) or the negative control pair (CXCR4–NLuc-416 and c-fos–CLuc-398). Bioluminescence imaging was performed before and immediately after ip injection of 2 μ g of CXCL12. Fluorescence from mPlum was used to control for numbers of injected cells. Representative bioluminescence and fluorescence images of mice are shown. The fluorescence images correspond to mice in the left bioluminescence images. (B) Quantified data from (A),

expressed as bioluminescence photons divided by mPlum fluorescence efficiency ($n = 3$ per group). (C) Cells expressing CXCR4–NLuc416 and β -arrestin 2–CLuc398 were injected ip into mice as in (A). Bioluminescence imaging was performed immediately after injection, following 30-min treatment with AMD3100 or vehicle, and after ip injection of CXCL12. Data are expressed as bioluminescent photons divided by fluorescence efficiency ($n = 3$ per group).