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The Chemokine Receptor CCR1 Is Constitutively Active, Which Leads to G Protein-independent, β-Arrestin-mediated Internalization*

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Background: CCR1 is a chemokine receptor of significant importance in human health, yet little is known about its ligand-independent behavior.
Results: CCR1 exhibits constitutive activity leading to basal signaling and β-arrestin-mediated receptor internalization.
Conclusion: Constitutive activity may enable CCR1 to engage in dual functions of canonical signaling and non-canonical chemokine scavenging.
Significance: This may suggest a new function for CCR1 and avenue for drug development.

Activation of G protein-coupled receptors by their associated ligands has been extensively studied, and increasing structural information about the molecular mechanisms underlying ligand-dependent receptor activation is beginning to emerge with the recent expansion in GPCR crystal structures. However, some GPCRs are also able to adopt active conformations in the absence of agonist binding that result in the initiation of signal transduction and receptor down-modulation. In this report, we show that the CC-type chemokine receptor 1 (CCR1) exhibits significant constitutive activity leading to a variety of cellular responses. CCR1 expression is sufficient to induce inhibition of cAMP formation, increased F-actin content, and basal migration of human and murine leukocytes. The constitutive activity leads to basal phosphorylation of the receptor, recruitment of β-arrestin-2, and subsequent receptor internalization. CCR1 concurrently engages $G\alpha_i$ and β-arrestin-2 in a multiprotein complex, which may be accommodated by homo-oligomerization or receptor clustering. The data suggest the presence of two functional states for CCR1; whereas receptor coupled to $G\alpha_i$ functions as a canonical GPCR, albeit with high constitutive activity, the CCR1·β-arrestin-2 complex is required for G protein-independent constitutive receptor internalization. The pertussis toxin-insensitive uptake of chemokine by the receptor suggests that the CCR1·β-arrestin-2 complex may be related to a potential scavenging function of the receptor, which may be important for maintenance of chemokine gradients and receptor responsiveness in complex fields of chemokines during inflammation.

G protein-coupled receptors (GPCRs)[4] constitute the largest family of cell surface molecules involved in signal transduction and mediate physiological responses to extraordinarily diverse extracellular stimuli, including light, odorants, neurotransmitters, chemoattractants, and peptides (1). They are one of the most therapeutically important family of receptors in the human genome and constitute the targets of roughly half of all drugs in clinical use (2). The historical paradigm of GPCR signaling suggests that receptors activate G proteins only upon agonist binding (3). However, this paradigm has significantly shifted since the seminal 1989 discovery by Costa and Herz (4) of negative intrinsic efficacy of δ-opioid receptor inhibitors. In the roughly two decades since, increasing evidence has shown that many GPCRs exhibit some level of ligand-independent signaling and that constitutive activity can play an important role in both normal and diseased tissues and cells (5). Spontaneous receptor isomerization from an inactive to an active state, resulting in elevated basal signaling to effector proteins and consequent cellular responses, is a hallmark of receptor constitutive activity (6). Dozens of studies have demonstrated mutations capable of inducing basal signaling by GPCRs (7); however, constitutive activity occurs among many wild-type endogenous receptors as well. For example, the ghrelin receptor (8), melanocortin 4 receptor (9), histamine H4 receptor (10), and multiple orphan receptors (11, 12) exhibit variable levels of ligand-independent G protein coupling or effector signaling. A review of constitutive GPCR activity documented more than 60 naturally occurring GPCRs from multiple receptor families that displayed this behavior (5). Although constitutive activity may be an intrinsic feature of many GPCRs, it can be modulated by receptor expression, cell type, and microenvironment as well as endogenous ligands that shift the equilibrium between the active and inactive conformational states. Its functional impli-

[4] The abbreviations used are: GPCR, G protein-coupled receptor; β₂AR, β₂-adrenergic receptor; PT, pertussis toxin; BRET, bioluminescence resonance energy transfer; Rluc, *R. reniformis* luciferase; PE, phycoerythrin; MEF, mouse embryonic fibroblast; GTPγS, guanosine 5′-*O*-(thiotriphosphate); RIPA, radioimmunoprecipitation assay.

cations are, however, poorly understood, as are the molecular interactions that promote or regulate such behavior. Nevertheless, the physiological significance of this phenomenon is underscored by the key role that loss of GPCR constitutive activity can play in human disease. Examples include mutations that reduce ligand-independent activity in the ghrelin receptor and melanocortin 4 receptor, resulting in familial short stature syndrome and obesity, respectively (13).

Chemokine receptors belong to the class A rhodopsin-like family of GPCRs. As mediators of directional migration and localization of leukocytes, chemokine receptors are essential to the development, maintenance, and proper functioning of the immune system (14). This subfamily of GPCRs has proven difficult for drug development, with only two Food and Drug Administration-approved compounds on the market (Selzentry® targeting CCR5 in HIV/AIDS treatment and Mozobil® targeting CXCR4 (CXC-type chemokine receptor 4) for hematopoietic stem cell mobilization) despite significant pharmaceutical industry investment (15). Among the chemokine family of GPCRs, CCR1 is one of the most prevalent targets for drug development according to the distribution of patents for small molecule inhibitors of chemokine receptors (16). CCR1 was originally cloned in 1993 and was shown to be expressed by neutrophils, T cells, B lymphocytes, natural killer cells, monocytes, and $CD34^+$ bone marrow cells. It has 10 known human ligands and, like most chemokine receptors, is a $G\alpha_i$-coupled receptor (17). Gene deletion of CCR1 in mice is not lethal; however, knockout of the receptor revealed both beneficial and detrimental effects dependent on the cellular context. In some studies of immune system challenge, mice lacking CCR1 exhibited an increased rate of pathogen clearance (18), attenuation of an excessive inflammatory response (19), and suppression of tissue allograft rejection (20). In non-challenged mice, CCR1 has been shown to play an important role in osteoclastogenesis (21) and in mobilization of bone marrow progenitor cells to the spleen (22). CCR1 has been demonstrated to play a key role in diseases associated with inappropriate leukocyte infiltration and activation, such as multiple sclerosis (23, 24), rheumatoid arthritis (25, 26), progressive kidney disease (27–29), and transplant rejection (20, 30, 31). Many attempts have been made to develop small molecule drugs that effectively inhibit receptor signaling, but thus far all have failed during clinical trials primarily due to lack of efficacy (32–34). Despite its biomedical relevance, relatively little has been reported on the molecular pharmacology of the receptor in its apo (*i.e.* basal, non-ligand bound) state.

With the clinical relevance of CCR1 clearly established, our laboratory set out to study the behavior of the receptor in its apo state, following initial observations that cells expressing CCR1 showed significant basal migration compared with cells expressing other chemokine receptors. The purpose of this study was to validate and explore the extent of CCR1 constitutive activity and to investigate the effect of agonist-independent signaling on the interactions between the receptor and intracellular proteins as well as its functional consequences. Previous studies have demonstrated constitutive activity among both wild type (35–39) and mutant (40–43) CC- and CXC-type chemokine receptors but not for CCR1 to the best of our knowl-

edge. This report demonstrates for the first time that CCR1 expression is sufficient to induce ligand-independent inhibition of cAMP (consistent with $G\alpha_i$ stimulation) and migration of both murine and human leukocytes. Constitutive phosphorylation, β-arrestin association, and pertussis toxin (PT)-independent internalization were also demonstrated as well as the ability of CCR1 to oligomerize and assemble a preformed CCR1·G protein·β-arrestin-2 complex. Preliminary data suggest that the β-arrestin-mediated constitutive internalization may be related to a chemokine scavenging activity, which in turn may be important for the responsiveness of CCR1-expressing cells to chemokine gradients.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—HEK293, HeLa, and COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with Glutamax (Invitrogen) supplemented with 10% fetal bovine serum (FBS). The THP-1 human acute monocytic leukemia cell line (ATCC) was maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS and the L1.2 murine pre-B lymphoma cell line (kind gift of Brian Zabel, Palo Alto Institute for Research and Education, Palo Alto, CA) was maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, 1% non-essential amino acids (Invitrogen), 1% sodium pyruvate, and 0.1% β-mercaptoethanol. Last, wild-type and β-arrestin-2$^{-/-}$ mouse embryonic fibroblasts (MEFs; kind gifts of Robert Lefkowitz, Duke University, Durham, NC) were cultured in DMEM with Glutamax supplemented with 10% FBS. All cells were maintained at 37 °C and 5% CO_2. Transfection of HEK293 cells was carried out in 6-well plates at 50–60% confluence using TransIT-LT1 reagent (Mirus Bio) as per the manufacturer's protocol. HeLa, COS-7, and MEF cells were transfected on glass coverslips at 80–90% confluence using Lipofectamine^{TM}2000 (Invitrogen) as per the manufacturer's protocol. Stable L1.2 lines were generated by electroporation of 10 μg of CCR1, CCR2, CCR5, or CCR10 in pcDNA3.1 into 1×10^7 cells, followed by G418 selection and identification of high expressers through limiting dilution. Stable CCR1 expression in the inducible pACMV-TetO/HEK293 vector/cell system was generated as described previously (44, 45).

In Vitro Migration and Actin Polymerization—Migration assays were performed using 24-well transwell plates with 5-μm pore size filter inserts (Corning). Prior to migration assays, L1.2 or THP-1 cells were pretreated with either 100 μM BX-471 (kind gift of Richard Horuk, University of California, Davis), 0.2 μg/ml pertussis toxin (List Biological Laboratories), or DMSO as a control for 1 h at 37 °C. Cells were resuspended at a concentration of 2×10^6 cells/ml in RPMI 1640 + 10% FBS, and 100 μl of cells were distributed in the upper part of each well with 600 μl of RPMI 1640 + 10% FBS in the bottom well. Cells were allowed to migrate for 2 h at 37 °C and 5% CO_2, after which time cells that migrated to the bottom chamber were counted on a guava® easyCyte^{TM} flow cytometer (Millipore). Wells with cells only (no filter) were used to quantify maximal migration. Migration was plotted as the percentage of cells migrated in relation to the total number of cells ± S.D. For the actin polymerization assay, L1.2 cells were resuspended in assay buffer (1× HBSS, 0.5% BSA, 10 mM HEPES, pH 7.4) at a con-

centration of 6×10^6 cells/ml and fixed with 4% paraformaldehyde (PFA). Cells were then permeabilized with 0.1% Triton X-100 in assay buffer and stained with 2.5 units/ml Alexa Fluor® 488 phalloidin (Invitrogen). The extent of F-actin staining was measured using the guava® easyCyte™ flow cytometer (Millipore).

GloSensor™ cAMP Assay—The GloSensor™ cAMP assay (Promega) uses a genetically encoded biosensor with cAMP binding domains fused to a mutant form of *Photinus pyralis* luciferase. Upon binding to cAMP, conformational changes occur that yield large increases in luminescence. HEK293 cells stably expressing the pGloSensor™-22F plasmid were seeded in 6-well plates and transiently transfected with 1 μg of HA-CCR1, HA-M3, or empty pcDNA3.1 vector for 24 h. Cells were then seeded at 2×10^5 cells/well into 96-well white assay plates (BD Biosciences) in Opti-MEM™ medium (Invitrogen) and incubated with 4% (v/v) GloSensor™ substrate for 1 h at 37 °C. Upon maintaining equilibrium at room temperature, the luminescent signal following stimulation with 10 μM forskolin was measured using a VictorX Light multilabel plate reader (PerkinElmer Life Sciences).

Flow Cytometry and Receptor Internalization—For receptor internalization assays, HEK293 cells stably expressing FLAG-CCR1 were cultured in 6-well plates. Cell surface receptor was labeled with mouse anti-CCR1 antibody (clone 53504, R&D Systems) for 30 min on ice in wash buffer (DMEM, 0.5% BSA, 10 mM HEPES, pH 7.4), unbound antibody was washed away with cold wash buffer, and then the medium was replaced with prewarmed wash buffer for specified periods of time. Cells were washed with phosphate-buffered saline (PBS) + 0.5% BSA, and the remaining cell surface receptor was labeled with anti-mouse antibody conjugated to phycoerythrin (PE) (R&D Systems). The relative amount of receptor remaining on the surface at each time point was quantified using a guava® easyCyte™ flow cytometer (Millipore) and analyzed using FlowJo software (Tree Star). A similar approach was taken to measure transfected or endogenous CCR1 expression and subsequent internalization from the surface of L1.2 and THP-1 cells, respectively, grown in suspension.

Chemokine Scavenging—For chemokine scavenging experiments, L1.2 cells stably expressing CCR1 were cultured at 2×10^6 cells/ml in the absence or presence of 0.2 μg/ml pertussis toxin or 100 μM BX-471 for 1 h at 37 °C in serum-free RPMI 1640 supplemented with 0.5% BSA and 10 mM HEPES, pH 7.4. Wild-type and CCR5-expressing L1.2 cells were included as controls. Cells were then incubated with 100 nM CCL7 (chemokine (C-C motif) ligand 7) conjugated to Cy3B maleimide[5] for up to 30 min. Non-internalized CCL7-Cy3B was removed by repeated washing with cold PBS supplemented with 0.5% BSA. The relative amount of internalized CCL7-Cy3B was quantified using a guava® easyCyte™ flow cytometer and analyzed using FlowJo software.

CCR1 Phosphorylation—HEK293t cells were transiently transfected in 6-well plates as described above with vector containing FLAG-CCR1. Forty-eight hours post-transfection, cells

were labeled with 250 μCi of [^{32}P]orthophosphate (PerkinElmer Life Sciences) in phosphate-free DMEM for 2 h at 37 °C. Cells were incubated with or without 1 μM CCL14 for up to 6 min and then lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 7.4, 300 mM NaCl, 1% IGEPAL/Nonidet P-40, 0.25% deoxycholate, 0.1% SDS) supplemented with protease inhibitor mixture (Sigma) and phosphatase inhibitor mixture II and III (Sigma) for 1 h at 4 °C. Cell lysates were immunoprecipitated with anti-FLAG M2 affinity gel (Sigma), and immunoprecipitates were resolved by SDS-PAGE and transferred to PVDF membrane. Membranes were analyzed by autoradiography and then probed with anti-FLAG (Sigma) antibody to detect FLAG-CCR1 expression by Western blotting.

Bioluminescence Resonance Energy Transfer (BRET) Assay—The BRET assay protocol has been described extensively elsewhere (46–48). In brief, the chemokine receptors CCR1, CCR2, CCR5, and the β_2-adrenergic receptor (β_2AR) were genetically fused to *Aequoria victoria* YFP on the receptor C terminus followed by a short linker in pcDNA3.1 (receptor-pYFP3.1). The *Renilla reniformis* luciferase (Rluc) genetic sequence was fused to the C terminus of β-arrestin-2 in the phRluc-N2 vector. YFP (energy acceptor) and Rluc (energy donor) constructs were generous gifts from M. Bouvier (University of Montréal). HEK293t cells were transiently transfected in defined stoichiometric ratios of each vector, usually 1.5–2 μg of a receptor-YFP construct and 0.05–0.1 μg of β-arrestin-2-Rluc. Forty-eight hours post-transfection, cells were washed; suspended in PBS supplemented with 0.5% (w/v) glucose; aliquoted at 1×10^5 cells/well in triplicate into a white, clear bottom 96-well plate (BD Biosciences); and incubated for 1 h at 37 °C prior to BRET measurement. YFP-tagged receptor expression was quantified by measuring fluorescence of the wells at 485-nm excitation and 538-nm emission wavelengths on a SpectraMax fluorescence spectrometer (Molecular Devices). The luciferase substrate coelenterazine-*h* (Biotium) was added to a final concentration of 50 μM in each well 10 min prior to the beginning of the BRET assay. Luminescence and fluorescence measurements were collected at room temperature with 1-s exposure times using a VictorX Light multilabel plate reader (PerkinElmer Life Sciences) at repeating time intervals. The BRET signal was calculated as the ratio of YFP emission (550 ± 40 nm) to Rluc emission (470 ± 30 nm). The BRET$_{net}$ signal is calculated by subtracting the background BRET ratio of cells expressing only the Rluc fusion from the BRET ratio of cells expressing both the YFP- and Rluc-fused proteins. CCL14 was prepared as described previously (49) and diluted in PBS and added following incubation with coelenterazine-*h* but prior to BRET measurement. BRET saturation curve experiments were carried out in which the levels of β-arrestin-2-Rluc were kept constant while increasing amounts of CCR1-YFP were cotransfected. The BRET signal was then plotted against the acceptor/donor ratio; a hyperbolic curve is indicative of a specific interaction as opposed to random collisions within the cell that would yield a quasilinear relationship (46).

Co-immunoprecipitation and Western Blotting—HEK293t cells were transfected as above in 6-well plates with FLAG-CCR1 and β-arrestin-2-HA constructs in pcDNA3.1 vectors. Forty-eight hours after transfection, cell medium was replaced

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[5] T. Kawamura, B. Stephens, L. Qin, X. Yin, M. M. Fuster, C. L. Salanga, and T. M. Handel.
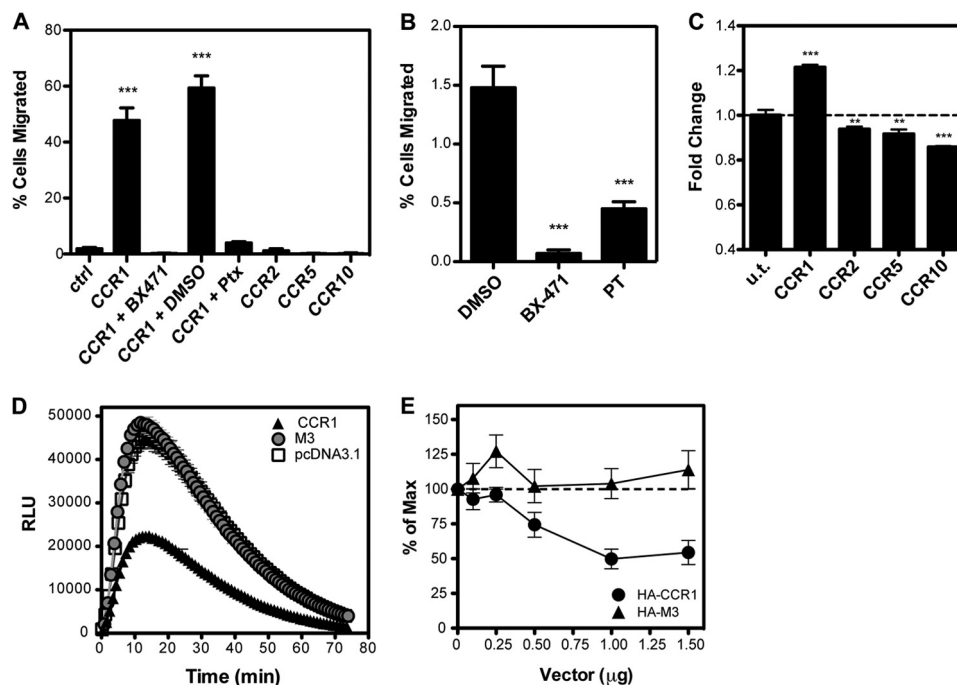
FIGURE 1. **CCR1 expression is sufficient to induce basal migration and inhibit cAMP formation.** Murine L1.2 cells stably transfected with either CCR1, CCR2, CCR5, or CCR10 (*A*) or human THP-1 cells endogenously expressing CCR1 (*B*) were placed in a microchemotaxis chamber, and the number of cells that spontaneously migrated into the lower chamber after 2 h at 37 °C was measured. The effect of the CCR1-specific inhibitor BX-471 and PT was also determined. The percentage of cells that migrated was calculated as the ratio of cells in the lower chamber in the microchemotaxis well to the number of cells initially added to the upper chamber. Data are the mean ± S.D. (*error bars*). *C,* basal F-actin content of L1.2 cells stably transfected with chemokine receptors or left untransfected (*u.t.*). Cells were permeabilized and stained with Alexa Fluor 488-phalloidin; results of a representative experiment performed in triplicate are plotted as -fold change over untransfected control. *D,* luminescence of pGloSensor™-22F/HEK293 cells transiently transfected with 1.0 μg of CCR1 (▲), M3 (◉), or empty pcDNA3.1 (□) vector. The signal from a representative experiment was measured in triplicate following incubation of cells with 4% GloSensor™ cAMP reagent for 1 h at 37 °C and stimulation with 10 μM forskolin. *E,* gene dosage experiment performed in triplicate in which increasing amounts of HA-CCR1 or HA-M3 in pcDNA3.1 vectors were transfected into pGloSensor™-22F/HEK293 cells and assayed as in *C.* Data were plotted as the mean ± S.D. using GraphPad Prism® (GraphPad Software), and the statistical significance was calculated using an unpaired Student's *t* test or one-way analysis of variance with Dunnett's post-test: **, $p < 0.01$; ***, $p < 0.0001$.

with serum-free DMEM with or without 1 μM CCL14 for specified periods of time at 37 °C. Cells were then washed with ice-cold PBS and lysed in RIPA buffer with protease inhibitor mixture and phosphatase inhibitor mixture II and III (Sigma-Aldrich) for 1 h at 4 °C. The supernatant fraction of the cell lysate was collected after 10 min of centrifugation at 20,000 × *g.* Anti-FLAG M2 affinity gel (Sigma-Aldrich) was added to the sample and incubated overnight at 4 °C on a rotating platform. The affinity gel was then washed 3–4 times in RIPA buffer, and elution was performed with 3X-FLAG peptide (Sigma-Aldrich). Eluted proteins were mixed with 5× Laemmli buffer and separated via SDS-PAGE in a 10% polyacrylamide gel. The presence of FLAG-CCR1 and β-arrestin-2-HA was measured by Western blotting and ECL Plus chemiluminescent detection (GE Healthcare) using anti-FLAG (Sigma-Aldrich), anti-mouse HRP (horseradish peroxidase, Promega), and anti-HA-HRP (Roche Applied Science) monoclonal antibodies.

Confocal Fluorescence Microscopy—HeLa, COS-7, or MEF cells were cultured on glass coverslips coated with 10 μg/ml human plasma fibronectin (Millipore) and transiently transfected with HA-CCR1, CCR1-mCherry, and/or β-arrestin-2-GFP using Lipofectamine™2000 (Invitrogen) as per the manufacturer's protocol. For cells transfected with CCR1-mCherry, 24 h post-transfection, the medium was replaced with serum-free DMEM, with or without 100 nM CCL14 for specified periods of time. For cells transfected with HA-CCR1, receptor was

prelabeled with anti-HA antibody directly conjugated to Alexa Fluor® 594 (Invitrogen) for 30 min on ice in wash buffer (DMEM, 0.5% BSA, 10 mM HEPES, pH 7.4) and washed with cold wash buffer, and then the medium was replaced with pre-warmed wash buffer with or without 1 μM CCL14 for specified periods of time. The cells were then fixed with 4% PFA for 5 min at room temperature. The coverslips with fixed cells were mounted onto microscope slides with FluoroSave (Calbiochem). Images were collected using an Olympus DSU spinning disk confocal microscope.

RESULTS

CCR1 Is a Constitutively Active Receptor—While testing the chemotactic ability of various mutants of CCR1 activating chemokines, it was observed that CCR1-expressing L1.2 cells consistently displayed a significant level of basal migration in transwell migration assays (Fig. 1*A*). Because constitutive activity had not previously been reported despite the extensive literature devoted to studies of CCR1, we set out to further characterize this phenomenon. The high levels of basal migration were specific to CCR1 because L1.2 cells expressing CCR2, CCR5, or CCR10 at comparable levels as CCR1 did not exhibit similar ligand-independent migration (Figs. 1*A* and 2*A*). Each receptor cell line remained capable of responding to chemokine agonist in a transwell migration assay (data not shown). The essential role of CCR1 in this constitutive process was demon-
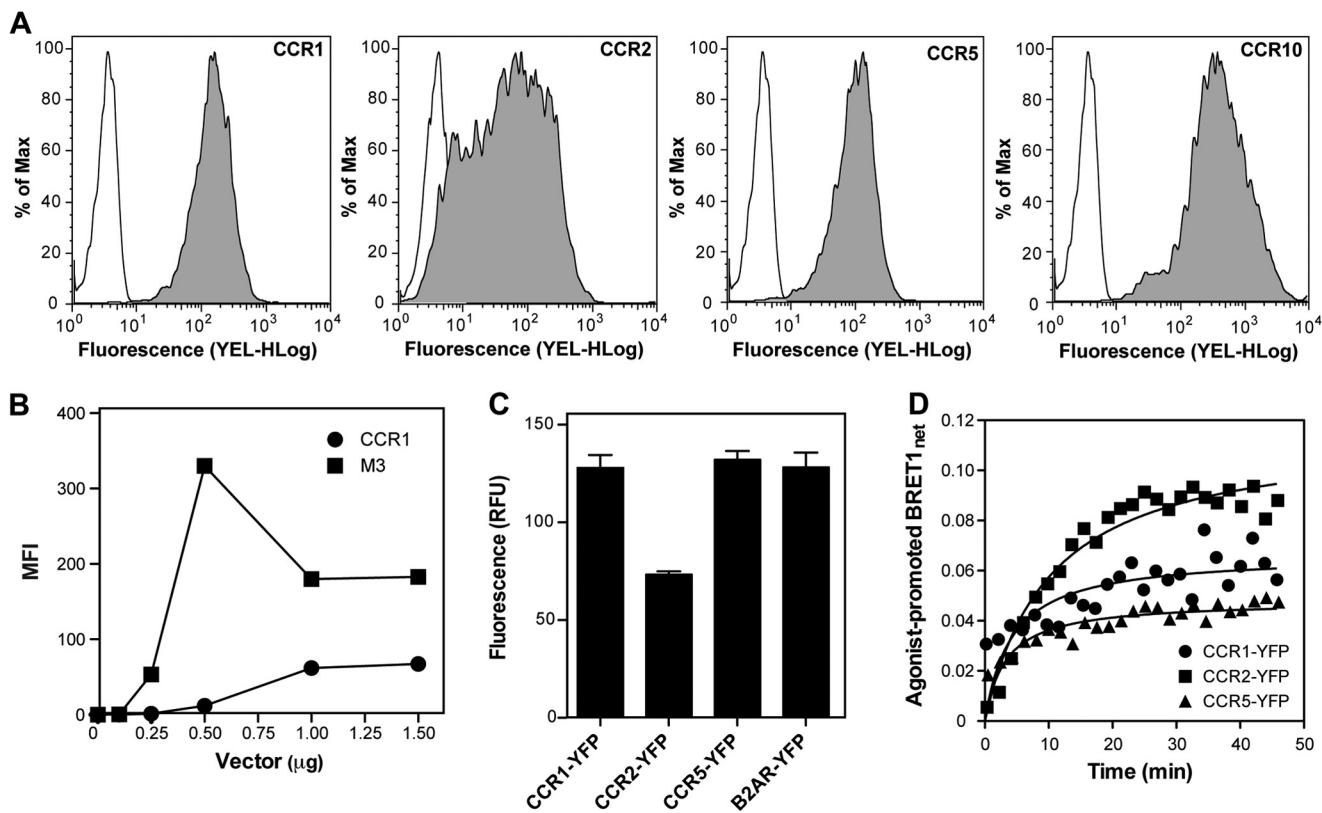
FIGURE 2. **Expression of chemokine receptors in various cell lines.** *A*, expression of CCR1, CCR2, CCR5, and CCR10 in stably transfected L1.2 cells. Cell surface receptor expression was determined using receptor-specific antibodies conjugated to PE and analyzed via flow cytometry. *Tinted areas* demonstrate receptor expression, and *unfilled areas* demonstrate isotype control antibody binding. *B*, expression of HA-CCR1 and HA-M3 transiently transfected into HEK293 cells stably expressing the pGloSensor-22F construct. Cell surface receptor levels were detecting using an antibody directed against the HA epitope tag conjugated to PE and analyzed via flow cytometry. Data shown from a representative experiment in triplicate as median fluorescence intensity (*MFI*). *C*, mean relative fluorescence values $\pm$ S.D. (*error bars*) of HEK293t cells transiently transfected in triplicate with CCR1-YFP, CCR2-YFP, CCR5-YFP, or β_2AR-YFP in the basal β-arrestin-2 BRET association assay. *D*, effect of chemokine agonist on the BRET1$_{net}$ signal over time between β-arrestin-2-Rluc and either CCR1-YFP ($\bullet$), CCR2-YFP ($\blacksquare$), or CCR5-YFP ($\blacktriangle$). CCR1- and CCR5-expressing HEK293t cells were treated with 100 nM CCL14, whereas CCR2-expressing cells were treated with 100 nM CCL7. Data shown are from a representative experiment performed in triplicate.

strated by the ability of the CCR1-specific antagonist BX-471 (50), a potent and competitive inhibitor of chemokine-mediated CCR1 activation, to ablate the basal migration (Fig. 1*A*). Treatment with PT also blocked migration, suggesting that it is driven by $G\alpha_{i/o}$ activation. To rule out the possibility that the constitutive activity is an artifact of heterologous overexpression of the receptor, the effect of endogenous CCR1 in untransfected cells was characterized. Human acute monocytic leukemia (THP-1) cells have been shown to express CCR1 and to respond to CCR1 chemokine agonists in various functional assays (51, 52). Although not as exaggerated as in L1.2 cells, THP-1 cells exhibited ligand-independent migration that represented a substantial fraction of the ligand-induced migration (data not shown) and could be attributed to a $G\alpha_{i/o}$-coupled receptor identified as CCR1 by the inhibitory effects of PT and BX-471, respectively (Fig. 1*B*). These observations were further supported by the effect of CCR1 expression on basal filamentous actin (F-actin) content in the L1.2 cells. These experiments showed elevated actin polymerization in cells expressing CCR1 compared with those expressing CCR2, CCR5, or CCR10 (Fig. 1*C*). One might hypothesize that the constitutive activity was a consequence of some unidentified agonist in the culture medium. However, basal migration was also observed in serum-free medium albeit at a lower level (data not shown). Further-

more, additional experiments carried out in the absence of serum (described below) were consistent with constitutive activity. Autocrine secretion of chemokine can also be ruled out as contributing to the observed constitutive activity because, of the 10 chemokine ligands known to activate CCR1, eight are also known agonists of CCR2 and/or CCR5 (CCL3, -5, -7, -8, -13, -14, and -16), leaving it highly unlikely that any potentially secreted chemokine would singularly activate CCR1.

To further demonstrate constitutive CCR1/$G\alpha_{i/o}$ signaling, the GloSensor™ assay (Promega) was used to measure forskolin-stimulated levels of intracellular cAMP in HEK293 cells stably expressing the pGloSensor™-22F construct and either CCR1, M3 muscarinic cholinergic receptor, or empty pcDNA3.1 vector control. M3 is known to couple to $G\alpha_{q/11}$ and activate phospholipase C (53); therefore, it serves as a negative control for the inhibition of adenylyl cyclase resulting from activation of a $G\alpha_{i/o}$-coupled receptor. CCR1 expression was shown to significantly lower the maximal forskolin-stimulated cAMP production compared with M3 expressed at similar levels or vector control (Fig. 1*D*). This effect was further demonstrated in a gene dosage experiment in which increasing levels of CCR1 expression resulted in significant reduction in maximal cAMP production (Fig. 1*E*). Expression of CCR1 was lower than that of M3 (Fig. 2*B*), yet it still had a significant inhibitory

effect on adenylyl cyclase activity. Together these data suggest that CCR1 is a constitutively active receptor that can activate G proteins and stimulate cell migration in an agonist-independent manner.

CCR1 Is Constitutively Internalized in Multiple Cell Types— Agonist binding to GPCRs typically results in G protein activation followed by receptor desensitization mediated by phosphorylation of intracellular domains and recruitment of arrestins. The arrestin proteins sterically occlude the receptor from further G protein coupling and connect the receptor to the internalization machinery of the cell to initiate endocytosis (54). Here it was tested whether CCR1 is internalized in the absence of agonist in various cell systems. HEK293 and L1.2 cells stably expressing CCR1 were labeled on ice with anti-CCR1 antibody and warmed to 37 °C for 1 h in the absence of exogenous agonist, and the subsequent amount of receptor remaining at the surface was determined by flow cytometry. In both cell types, CCR1 was constitutively internalized because a marked reduction in the cell surface levels of the receptor after 1 h of incubation was observed (Fig. 3*A*, *left* and *middle*). To confirm that constitutive internalization was not an artifact of heterologous overexpression of the receptor, the experiment was repeated with THP-1 cells, which endogenously express CCR1. A similar result was obtained demonstrating extensive down-regulation of CCR1 to the point where cell surface receptor was barely detectable (Fig. 3*A*, *right*). It remained possible, however, that CCR1 was being activated by secreted chemokine agonists in some autocrine fashion, thereby explaining the significant internalization of the receptor. To rule this out, the effect of treating the CCR1-expressing L1.2 cells with BX-471 in the constitutive internalization assay was tested. BX-471 was shown to have no effect on constitutive internalization of the receptor, making it highly improbable that a secreted agonist was responsible (Fig. 3*B*). Additionally, CCR1 constitutive internalization was independent of $G\alpha_{i/o}$ protein activation, because PT treatment did not inhibit basal down-regulation of the receptor (Fig. 3*B*), in line with previous studies of other chemokine receptors (55–57).

The effect of agonist-mediated CCR1 internalization over time in comparison with constitutive internalization was additionally measured. HEK293 cells expressing HA-CCR1 were prelabeled with CCR1 antibody and warmed as above in the presence or absence of 100 nM CCL14 (Fig. 3*C*). Whereas CCR1 exhibited rapid ligand-independent internalization, CCL14 stimulation significantly increased the extent of receptor removal from the cell surface. The same constitutive internalization assay was carried out without antibody prelabeling to measure whether CCR1 that is internalized in the absence or presence of agonist is recycled back to the cell surface. Over the time course of the assay, the amount of CCR1 on the cell surface at any given time point remained relatively constant, suggesting that CCR1 undergoes constitutive recycling to maintain consistent expression at the plasma membrane (Fig. 3*C*). However, overall levels of cell surface receptor were significantly down-regulated following CCL14 stimulation (Fig. 3*C*), suggesting that the receptor is being sent along a different internalization pathway following agonist binding compared with constitu-

tively internalized receptor and is not being recycled, which is in agreement with a previous report (58).

In order to observe localization of the receptor following constitutive internalization, the fate of CCR1 was measured via confocal immunofluorescence microscopy. HeLa and COS-7 cells were transiently transfected with HA-tagged CCR1, prelabeled with anti-HA antibody conjugated to Alexa Fluor 594®, and incubated for 1 h at 37 °C in the presence or absence of 1 μM CCL14. Use of an antibody against the N-terminal HA tag served as a control to ensure that the anti-CCR1 antibody utilized in the flow cytometry experiments above was not acting as an agonist and inducing internalization. Additionally, data from many internalization assays demonstrate that the anti-HA and anti-CCR1 antibodies do not preclude CCR1 activation by chemokines. Surface staining of HA-CCR1 was observed in HeLa and COS-7 cells, followed by significant internalization in both unstimulated and agonist-stimulated experimental conditions; however, CCL14-mediated activation of CCR1 appeared to induce more rapid receptor internalization compared with those cells left untreated (Fig. 3*D*). The majority of surface-stained HA-CCR1 was localized into discrete intracellular puncta in both cell types. These data again demonstrate that CCR1 is a constitutively active receptor and undergoes agonist-independent internalization in a variety of cell types from human, simian, and murine sources.
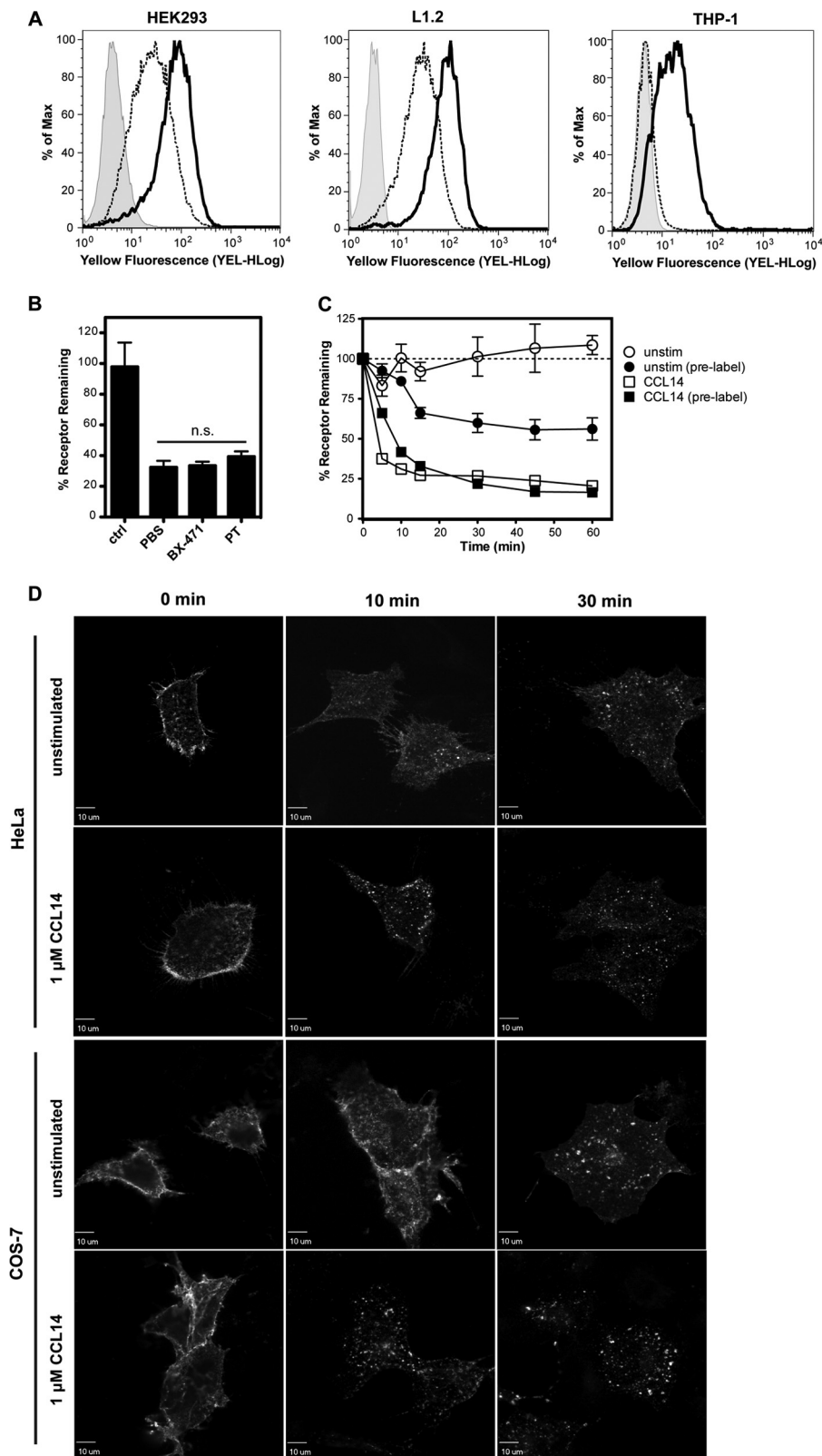
CCR1 Is Constitutively Phosphorylated, Leading to Basal Association with β-Arrestin-2— The findings above on the constitutive signaling and internalization of CCR1 suggest that the receptor may be basally phosphorylated, because phosphorylation is a canonical event that follows GPCR activation and mediates down-regulation. To determine whether CCR1 is basally phosphorylated, HEK293t cells expressing FLAG-tagged CCR1 were labeled with [³²P]orthophosphate and either left untreated or stimulated with 1 μM CCL14. FLAG-CCR1 was shown to be extensively labeled prior to agonist treatment compared with the untransfected control, with only a minor increase in phosphorylation following agonist addition (Fig. 4). Previous studies monitoring phosphorylation of related chemokine receptors CCR2 and CCR5 did not demonstrate this basal phosphorylation behavior (59, 60). The observation that CCR1 is constitutively phosphorylated conforms well with the data above indicating basal signaling activity and receptor down-regulation and suggests the involvement of β-arrestins in CCR1 constitutive internalization.

To investigate the agonist-independent association of CCR1 with β-arrestins, a BRET assay was initially employed. BRET has been extensively used to measure GPCR oligomerization as well as to monitor receptor interactions with intracellular proteins, including arrestins (47, 61, 62). HEK293t cells were transiently transfected with CCR1-YFP (energy acceptor) and β-arrestin-2-Rluc (energy donor) or with β-arrestin-2-Rluc alone as a control. The BRET signal was measured following the addition of the luciferase substrate coelenterazine-*h*. In the absence of CCR1 agonist, cells co-expressing CCR1-YFP and β-arrestin-2-Rluc exhibited a significantly higher BRET signal than cells expressing β-arrestin-2-Rluc alone, indicative of constitutive interaction (Fig. 5*A*). As with its effect on CCR1 constitutive internalization, treatment with BX-471 had no effect in

preventing basal association between CCR1 and β-arrestin-2 (Fig. 5B). In order to determine if this observation was unique to CCR1 or if it is common among other chemokine receptors, the BRET signal was evaluated between β-arrestin-2-Rluc and YFP-tagged chemokine receptors CCR2 and CCR5 as well as

the β₂AR. Under basal conditions and with similar levels of receptor expression (measured by YFP fluorescence; Fig. 2C), CCR1 was the only receptor tested that exhibited a basal $BRET1_{net}$ signal significantly above base line, indicative of constitutive association with β-arrestin-2-Rluc (Fig. 5B).
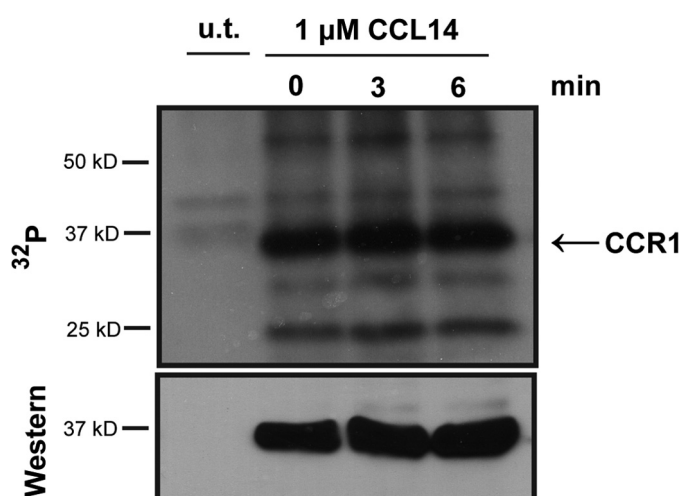
FIGURE 4. **CCR1 is constitutively phosphorylated.** HEK293t cells expressing FLAG-CCR1 were orthotopically labeled with the ^{32}P radioisotope and either left unstimulated or stimulated with 1 μM CCL14 for 3 or 6 min (*top*). The *arrow* indicates the band corresponding to the correct molecular weight for CCR1. Receptor levels were measured by Western blot using anti-FLAG-HRP (Sigma) and chemiluminescent detection (*bottom*). *u.t.*, untransfected.

This finding is also consistent with the observation that neither CCR2 nor CCR5 promotes constitutive migration *in vitro* (Fig. 1*A*). To ensure that the lack of basal association observed with CCR2 and CCR5 was not an artifact of non-functional receptors, the ability of the YFP-tagged chemokine receptors to form agonist-induced interactions with β-arrestin-2-Rluc was confirmed (Fig. 2*D*). Stimulation of CCR1 and CCR5 with CCL14 and CCR2 with CCL7 each led to a significant and time-dependent increase in the BRET signal, suggesting these receptors are functional and capable of engaging β-arrestin-2.

To determine if CCR1 exhibits preferential association with a particular arrestin isoform, the BRET assay was repeated with β-arrestin-1. Under non-stimulating conditions, CCR1-YFP exhibits a significantly higher BRET1$_{net}$ signal for β-arrestin-2-Rluc compared with that when β-arrestin-1-Rluc was used as the BRET donor (Fig. 5*C*). Importantly, CCR5-YFP did not exhibit preferential precoupling with either β-arrestin isoform under basal conditions. The specificity of the basal interaction between CCR1 and β-arrestin-2 was further demonstrated by co-transfecting increasing levels of β-arrestin-2-GFP into cells co-expressing CCR1-YFP and β-arrestin-2-Rluc and measuring the impact on the basal BRET signal. In the BRET1 assay, GFP expression does not significantly interfere with the reso-

nance energy transfer between Rluc and YFP because there is little spectral overlap between Rluc emission and GFP excitation. Thus, the dose-dependent decrease in the basal BRET$_{net}$ signal with the addition of β-arrestin-2-GFP is indicative of competition between β-arrestin-2-Rluc and β-arrestin-2-GFP for interaction with CCR1-YFP and provides further evidence that the constitutive interaction with CCR1 is specific to β-arrestin-2 (Fig. 5*D*).

The BRET measurements reflect interactions averaged throughout the cell. Therefore, to determine the subcellular localization of the interaction between CCR1 and β-arrestin-2, HeLa cells transiently transfected with CCR1-mCherry and β-arrestin-2-GFP were imaged by confocal fluorescence microscopy. CCR1 and β-arrestin-2 were shown to co-localize both at the cell surface and within intracellular compartments under basal conditions (Fig. 5*E*). Co-expression of CCR1-mCherry with β-arrestin-2-GFP brought about a significant translocation of β-arrestin-2-GFP from a homogenous distribution within the cell in the absence of CCR1 to localization within discrete puncta when co-expressed with the receptor (Fig. 5*E*), even in the absence of ligand stimulation.

To further confirm the observation from both the BRET and fluorescence microscopy experiments that CCR1 is constitutively associated with β-arrestin-2, the ability of β-arrestin-2 to co-immunoprecipitate with CCR1 was tested. HEK293t cells were transiently transfected with FLAG-CCR1 and increasing levels of β-arrestin-2-HA in order to ensure that a sufficient signal from β-arrestin-2 was obtained and that the interaction was not an artifact of overexpression. β-Arrestin-2-HA was shown to co-immunoprecipitate with FLAG-CCR1 at each level of detectable β-arrestin-2-HA expression (Fig. 5*F*). Interestingly, treatment with CCL14 did not appear to significantly increase the amount of β-arrestin-2-HA pulled down by the receptor, possibly in agreement with the previous data showing only a minor increase in CCR1 phosphorylation (Fig. 4). Although the BRET signal between CCR1-YFP and β-arrestin-2 is significantly increased upon the addition of CCL14 (Fig. 2*D*), this is probably due to an intermolecular conformational change between CCR1 and β-arrestin-2 that leads to a more favorable orientation for energy transfer between luciferase and YFP. To our knowledge, the data above represent the first demonstration of a wild-type G protein-signaling chemokine receptor that forms a stable and constitutive association with β-arrestin.

FIGURE 3. **CCR1 undergoes constitutive internalization in multiple cell types.** *A*, CCR1 constitutive internalization was measured in HEK293 cells stably expressing FLAG-CCR1 (*left*), L1.2 cells stably expressing CCR1 (*middle*), and THP-1 cells endogenously expressing CCR1 (*right*). Receptor initially present at the cell surface was labeled with CCR1 antibody on ice, and the amount of receptor remaining after 1 h of warming to 37 °C in serum-free medium was measured using a PE-conjugated secondary antibody and analyzed on a guava® easyCyte™ flow cytometer (Millipore). Representative flow cytometry plots from experiments conducted in triplicate normalized to the percentage of maximal fluorescent value are shown with initial cell surface CCR1 levels (*solid black line*), receptor remaining after 1 h of agonist-independent internalization (*dotted black line*), and unstained control cells (*line above gray-tinted area*). *B*, the effect of 1-h pretreatment with 1 μM BX-471 and 200 ng/ml PT on CCR1 constitutive internalization in L1.2 cells. The data are shown as mean $\pm$ S.D. (*error bars*) of the percentage of receptor remaining compared with control cells at the 0 h time point. The variance between the PBS-, BX-471-, and PT-treated cells was shown to be not significant (*n.s.*) by one-way analysis of variance with Tukey's post-test using GraphPad Prism® (GraphPad Software). *C*, constitutive and agonist-induced internalization and recycling of HA-CCR1 in stably expressing HEK293 cells over time. Cells were either prelabeled (*closed symbols*) or not (*open symbols*) with CCR1 antibody and stimulated with 100 nM CCL14 (*squares*) or left unstimulated (*circles*). The amount of receptor remaining on the cell surface afterward was measured at each time point in triplicate as described above. *D*, constitutive internalization of HA-CCR1 transiently transfected in HeLa (*top panels*) and COS-7 (*bottom panels*) cells. Cells with HA-CCR1 at their surface were prelabeled with anti-HA antibody conjugated to Alexa Fluor® 594 and then warmed in serum-free cell culture media with or without 1 μM CCL14 for 1 h. Cells were fixed and imaged using an Olympus DSU spinning disk confocal microscope.
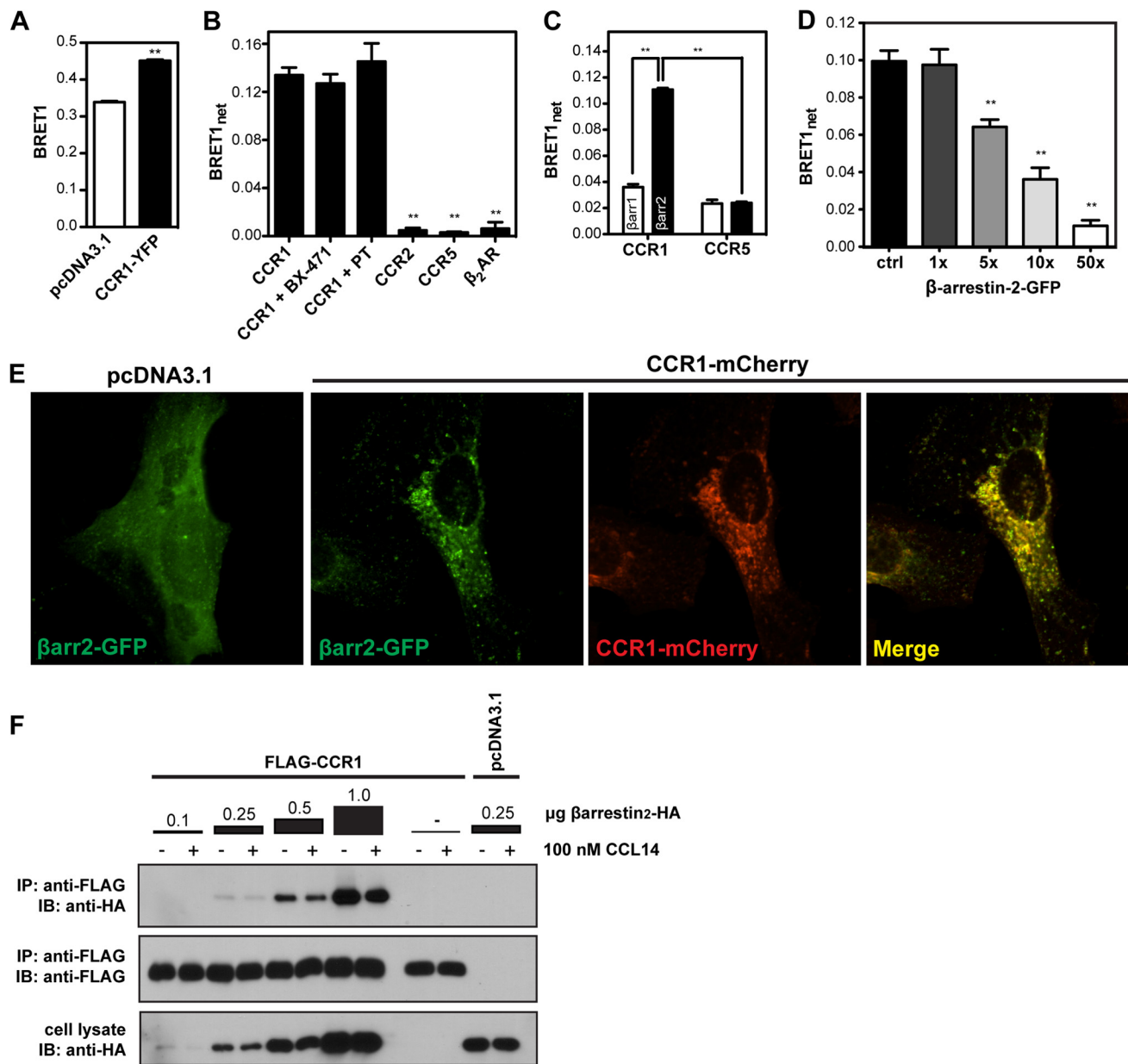
FIGURE 5. **CCR1 is constitutively associated with β-arrestin-2.** *A*, HEK293t cells were transiently transfected with β-arrestin-2-Rluc and pcDNA3.1 (*white bar*) or β-arrestin-2-Rluc and CCR1-YFP (*black bar*), and the BRET1 ratio was measured 48 h later at room temperature in the absence of ligand stimulation. *B*, comparison of the basal BRET1$_{net}$ signal from HEK293t cells co-expressing β-arrestin-2-Rluc and CCR1-, CCR2-, CCR5-, or β$_2$AR-YFP. The effect of CCR1 inhibition with 1 μM BX-471 and blockade of G$_{i/o}$ signaling with 200 ng/ml PT on the basal BRET1$_{net}$ signal is also shown. *C*, comparison of the basal BRET1$_{net}$ signal between β-arrestin-1-Rluc (*white bar*) and β-arrestin-2-Rluc (*black bar*) with either CCR1-YFP or CCR5-YFP. *D*, the basal BRET1$_{net}$ signal between CCR1-YFP and β-arrestin-2-Rluc with increasing levels of β-arrestin-2-GFP expression. The fold increase in β-arrestin-2-GFP levels on the *x* axis is the ratio of the micrograms of β-arrestin-2-GFP vector to β-arrestin-2-Rluc vector transfected into cells co-expressing CCR1-YFP. The data above (*A–D*) are plotted as the mean ± S.D. (*error bars*) of a representative experiment done in triplicate. Statistical significance was calculated using an unpaired *t* test (*A*) or one-way analysis of variance with Dunnett's multiple comparisons test (*B–D*) (*, $p < 0.05$; **, $p < 0.01$). *E*, HeLa cells were cultured on fibronectin-coated coverslips and transfected with β-arrestin-2-GFP alone (*far left panel*) or with co-transfected with CCR1-mCherry (*three right panels*). Cells were washed, fixed, and imaged using a confocal microscope. Co-localization is indicated as *yellow* in the *merged image. F*, HEK293t cells were transiently transfected with FLAG-CCR1 or pcDNA3.1 (*far right lanes*) and increasing levels of β-arrestin-2-HA and treated with PBS or 100 nM CCL14 for 15 min at 37 °C. CCR1 and any associated proteins were immunoprecipitated (*IP*), and the presence of receptor and β-arrestin-2-HA was measured by Western blot (*IB*).

Constitutive Internalization of CCR1 Is Mediated by β-Arrestin-2—To investigate the role of β-arrestin-2 in the constitutive internalization of CCR1, HA-tagged receptor was expressed in MEFs isolated from wild-type and β-arrestin-2$^{-/-}$ mice. Cell surface receptor was prelabeled with anti-HA-Alexa Fluor 594® and incubated at 37 °C as described above. CCR1 underwent constitutive internalization in wild-type MEF cells, as shown by the formation of discrete intracellular puncta after incubation for 30 min compared with the plasma membrane localization of the receptor at the 0 min time point (Fig. 6, *top panels*). By contrast, HA-CCR1 expressed in β-arrestin-2-deficient MEF cells was significantly impaired in its ability to be basally down-regulated (Fig. 6, *bottom panels*). These data, together with the previous observations of constitutive association, demonstrate that β-arrestin-2 is an important mediator of agonist-independent CCR1 internalization.
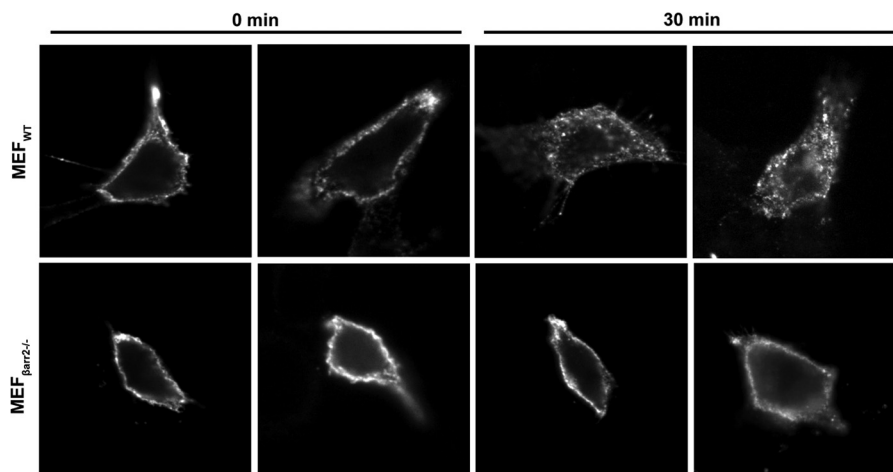
FIGURE 6. **Constitutive internalization of CCR1 is mediated by β-arrestin-2.** Wild-type and β-arrestin-2-deficient MEFs were transiently transfected on coverslips with HA-CCR1, and cell surface receptor was prelabeled with anti-HA Alexa Fluor® 594. Cells were then warmed with serum-free medium without agonist for 30 min, and the extent of receptor internalization was observed using a confocal microscope.

CCR1 Constitutively Interacts with both Gα$_i$ and β-Arrestin-2—The observation of constitutive G protein-mediated migration and PT-insensitive β-arrestin-2-mediated internalization suggests the possibility that CCR1 can coordinate both signaling and regulatory molecules. The BRET assay was therefore used to demonstrate whether CCR1 exhibited ligand-independent coupling with Gα$_i$. HEK293 cells expressing CCR1-YFP and one of two variants of Gα$_i$-Rluc (with the Rluc sequence inserted into one of two loops of Gα$_i$ at position 60 or 122, neither of which affects G protein function (63)) exhibited a basal BRET1$_{net}$ signal significantly above background (Fig. 7A). Both Gα$_i$-60-Rluc and Gα$_i$-122-Rluc were tested to ensure that the positioning of the luciferase did not yield an artificially positive signal. M3-YFP, which does not couple to Gα$_i$, did not yield an appreciable BRET1$_{net}$ signal when expressed at similar levels as CCR1-YFP in cells with either Gα$_i$-Rluc construct. To further confirm the specificity of the basal Gα$_i$ association with CCR1, a BRET saturation assay was carried out in which CCR1-YFP expression was steadily increased in cells expressing a constant level of Gα$_i$-Rluc or Gα$_{12/13}$-Rluc, which was included as a negative control. When the BRET1$_{net}$ signal is plotted against the YFP:Rluc expression level and the result is a hyperbolic saturation curve as opposed to a quasilinear and non-saturable one, the interaction is believed to be specific (46). Indeed, the result of the BRET saturation curve between CCR1-YFP and Gα$_i$-Rluc indicates that the interaction is specific with a slightly higher signal resulting from the CCR1-YFP/Gα$_i$-122-Rluc pair compared with CCR1-YFP/Gα$_i$-60-Rluc (Fig. 7B). As a negative control, CCR1-YFP and Gα$_{12/13}$-Rluc were examined and exhibited a linear, non-saturable BRET curve indicative of a nonspecific interaction (Fig. 7B).

Recent reports involving the intermolecular interactions of constitutively active GCPRs have brought into question the dogmatic idea that G protein coupling to and β-arrestin association with the same receptor are mutually exclusive (64). Given the finding that CCR1 is basally associated with both Gα$_i$ and β-arrestin-2, the question arose whether it associates with Gα$_i$ and β-arrestin-2 in a multiprotein assembly or as functionally distinct CCR1 populations. Accordingly, the effect of CCR1

expression on the interaction between Gα$_i$ and β-arrestin-2 was examined. HEK293 cells stably transfected with CCR1 under a doxycycline-inducible expression system were co-transfected with Gα$_i$-122-Rluc and β-arrestin-2-YFP. In the absence of significant CCR1 expression, Gα$_i$ and β-arrestin-2 exhibited a small basal BRET1$_{net}$ signal (Fig. 7C). However, when CCR1 expression was up-regulated by exogenous doxy-cycline addition, the agonist-independent BRET1$_{net}$ signal between Gα$_i$ and β-arrestin-2 was significantly increased. Additionally, activation of these cells with 1 μM CCL14 resulted in a relatively rapid and stable increase in the BRET1$_{net}$ signal, suggesting either closer proximity of the two proteins or a conformational change that places the YFP and Rluc into a more permissive orientation for energy transfer (Fig. 7D). Together, these data suggest the constitutive formation of a receptor·G protein·β-arrestin complex that remains stably associated upon receptor activation. However, one cannot exclude the presence of functionally distinct subpopulations of receptor as well (*e.g.* CCR1·G protein and CCR1·β-arrestin-2).

CCR1 Forms a Constitutive Homo-oligomer—A question immediately arises as to how CCR1 is able to structurally accommodate concurrent association with G protein and β-arrestin. Previous studies have suggested that GPCR oligomerization may account for the formation of multiprotein complexes at the cytoplasmic surfaces of the receptors (27, 65, 66), and indeed, many chemokine receptors have been shown to homo- and hetero-oligomerize in cells (67–69). Furthermore, five crystal structures of CXCR4 from different space groups all revealed similar dimers (70). However, the propensity of CCR1 to homo- or hetero-oligomerize has not yet been reported. In a BRET saturation assay with cells expressing CCR1-Rluc and CCR1-YFP, M3-YFP, or γ-aminobutyric acid B2 receptor-YFP (GABA-YFP), only the CCR1-Rluc/CCR1-YFP pair yielded a hyperbolic saturation curve indicative of a specific BRET signal, whereas M3 and GABA yielded quasilinear association curves indicating nonspecific associations (Fig. 8A). To further confirm the specificity of the homo-oligomerization of CCR1, a variant of the BRET saturation assay was used in which the expression ratio between the YFP- and Rluc-tagged proteins is
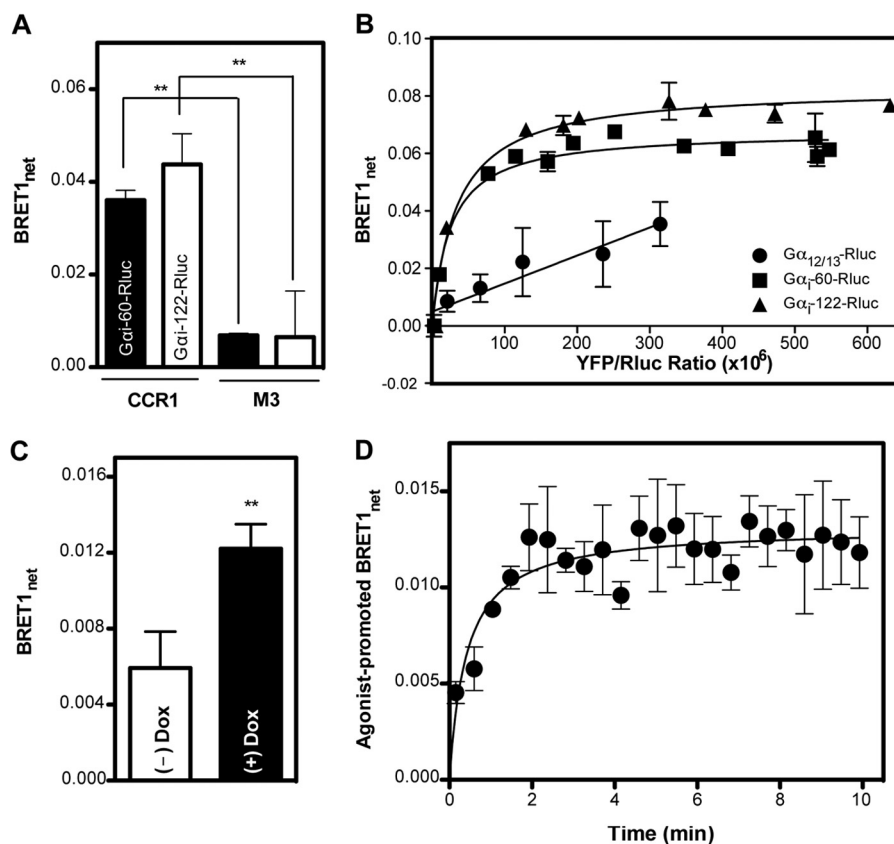
FIGURE 7. **CCR1 is constitutively associated with Gα$_i$ and forms a basal complex with G protein and β-arrestin.** *A*, HEK293 cells were transiently co-transfected with CCR1-YFP or M3-YFP and Gα$_i$-Rluc with insertion of the luciferase at position 60 (Gα$_i$-60-Rluc, *black bar*) or 122 (Gα$_i$-122-Rluc, *white bar*) to test for effects of Rluc orientation. Forty-eight hours post-transfection, the basal BRET1$_{net}$ value was measured. *B*, BRET saturation assay in which the expression level of Gα$_i$-60-Rluc (■), Gα$_i$-122-Rluc (▲), or Gα$_{12/13}$-Rluc (●) was kept constant while the expression of CCR1-YFP was continually increased. The respective *curves* indicate a specific association between CCR1-YFP and either of the Gα$_i$-Rluc constructs but not for Gα$_{12/13}$-Rluc. *C*, FLAG-CCR1/HEK293/TetO cells in which CCR1 expression can be induced with doxycycline were transfected with both Gα$_i$-122-Rluc and β-arrestin-2-YFP. The change in the BRET1$_{net}$ signal between Gα$_i$ and β-arrestin-2 in the absence (*white bar*) and presence (*black bar*) of 2 μg/ml doxycycline is shown. The statistical significance was calculated using an unpaired *t* test (**, $p < 0.01$) (GraphPad Prism®). *D*, the same cells as in *C* were stimulated with 1 μM CCL14, and the effect of the agonist on the BRET1$_{net}$ signal over time is shown. *Error bars*, S.D.

kept constant while the overall density of the receptors in the cell is increased. In this assay, a specific interaction is demonstrated by a linear curve with a non-zero *y*-axis intercept (71), as was observed for the CCR1-Rluc/CCR1-YFP interaction as opposed to CCR1-Rluc/M3-YFP (Fig. 8*B*). Therefore, it seems that CCR1 is capable of forming either receptor clusters or more well defined oligomeric complexes, minimally containing two subunits. Although the role of oligomerization in GPCR signaling and regulation remains unclear, such complexes may provide the surface area needed to overcome the steric hindrance of concurrent G protein and β-arrestin binding to CCR1 (72, 73).

CCR1 Internalizes Chemokine Independently of G Protein Activation—Based on the above data, it appears that CCR1 possesses significant constitutive activity. However, as for many other constitutive GPCRs, the functional relevance of this behavior is not known. Several atypical chemokine receptors (*e.g.* D6, DARC, and CXCR7), classified as such because they do not couple to G proteins, have been shown to exhibit chemokine scavenging activity defined as receptor-mediated internalization of chemokine without G protein-mediated signaling (74–76). Scavenging activity of G protein-coupled chemokine receptors has also been suggested on the basis of elevated levels of ligands in the serum and central nervous system tissue of chemokine receptor knock-out mice (77). Finally, a direct demonstration of the ability of CCR2 on human monocytes to scavenge CCL2 in the presence of PT was recently reported (55). To test whether CCR1 could be acting in a similar manner, CCR1/L1.2 cells were tested for their ability to internalize Cy3B-labeled CCL7 in the presence and absence of PT. The effect of CCR1 inhibition with BX-471 was also tested, and the results with CCR1/L1.2 cells were compared with results with untransfected and CCR5-expressing L1.2 cells. Non-treated CCR1/L1.2 cells showed significant internalization of CCL7-Cy3B over time, whereas CCR5/L1.2 and untransfected L1.2 cells exhibited only minimal background binding to the chemokine, suggesting specificity of the CCL7-Cy3B interaction with CCR1 (Fig. 9). The specificity was further evidenced by the significant reduction of CCL7-Cy3B uptake by CCR1/L1.2 cells in the presence of BX-471. Critically, CCL7-Cy3B uptake was only minimally affected by PT treatment, demonstrating that CCR1 is capable of internalizing chemokine independent of G protein activation and consistent with a potential scavenging behavior.

DISCUSSION

In this report, we demonstrated that heterologous or endogenous expression of CCR1 is sufficient to induce basal migration of both murine and human leukocytic cell lines and
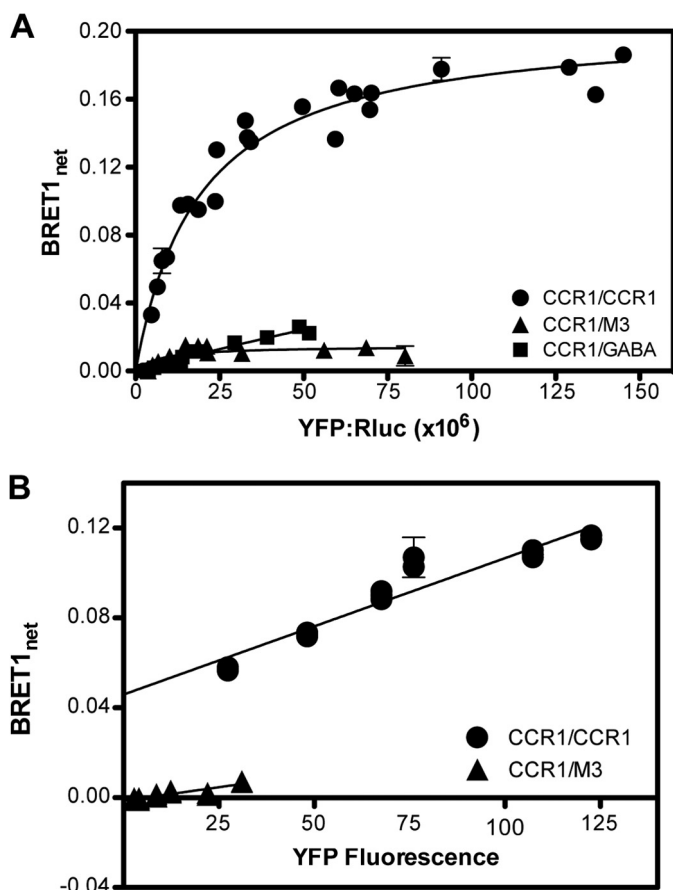
FIGURE 8. **CCR1 forms a specific homo-oligomer.** *A*, BRET saturation assay in which energy donor (CCR1-Rluc) expression levels are kept constant while the expression of energy acceptor (receptor-YFP) is continually increased in order to compare the homo-oligomerization of CCR1 (●) with hetero-oligo-merization with the M3 (▲) or GABA(B2) (■) receptor. A non-linear and satu-rable curve is indicative of a specific interaction between the two proteins being studied. *B*, type II BRET saturation assay in which the expression ratio between the energy donor (CCR1-Rluc) and energy acceptor (receptor-YFP) is kept constant while the overall expression of both proteins is continually increased in order to compare homo-oligomerization of CCR1 (●) with hetero-oligomerization with the M3 (▲) receptor. A linear relationship with a non-zero intercept is indicative of a specific interaction. *Error bars*, S.D.

agonist-independent G protein signaling in HEK293 cells. Constitutive activity among members of the chemokine receptor family has been reported previously; however, the data are limited, and this study represents the first to identify CCR1 as a constitutively active receptor. The wild-type sequence of the related CCR3 receptor has been shown to exhibit constitutive activity as measured by GTPγS binding in CCR3-expressing CHO membranes (35). In this study, the CCR3-specific small molecule inhibitor Banyu (I), whose previous inverse agonist efficacy was not known, inhibited basal GTPγS binding to membranes. Similarly, in the present study, the CCR1-specific inhibitor BX-471, also previously thought to be a competitive antagonist, acted as an inverse agonist to inhibit the basal migration activity of CCR1. CCR4 also showed evidence of constitutive activity on the basis of ligand-independent increases in F-actin content correlating directly with increasing CCR4 expression in primary human $CD4^+$ T cells (36). Interestingly, in this case, none of the several CCR4 inhibitors tested was able to reduce the constitutive activity of the receptor. Among the

CXC-class of chemokine receptors, only CXCR1 has been shown to possess constitutive activity (39); an analysis of the intensely studied CXCR4 in Sf9 insect cell membranes demonstrated no basal signaling (78). Although GPCR constitutive activity is sometimes criticized as being dependent upon the cell line being used, the expression level of the receptor, or the functional assay employed to identify constitutive activity, it is noteworthy that CCR1 exhibited considerable basal activity in the context of multiple cell lines from different mammalian species, in all signaling and physical interaction assays tested, and at endogenous levels of expression.

The observation that CCR1 is constitutively active motivated subsequent studies on the regulation and trafficking of the receptor and its interactions with intracellular proteins. Recent studies have suggested that careful regulation of chemokine receptor activity may be more important than the activation of the receptor itself (14). Classically, agonist activation of a GPCR initiates a G protein-signaling cascade followed by phosphorylation of receptor intracellular domains by GPCR kinases or second messenger kinases (such as PKA or PKC) (79). Phosphorylation uncouples the receptor from G proteins and allows recruitment of β-arrestin. In turn, β-arrestin sterically occludes the receptor from further coupling to G proteins and facilitates its association with clathrin-coated pits, leading to internalization (80). Many subsequent factors determine whether the receptor is recycled back to the membrane or destined for degradation. The regulation of constitutively active receptors in the absence of agonist is less clear. Beginning with the discovery that a constitutively active $β_2$-adrenergic receptor mutant is basally phosphorylated and continually internalized (81), a variety of scenarios have since been reported. For example, a constitutively active mutant of CXCR4 was shown to be basally phosphorylated and internalized (43, 82), whereas the D6 decoy chemokine receptor is constitutively internalized in a phosphorylation-independent manner (83). Additionally, the dopamine D4 receptor is constitutively phosphorylated but not internalized (84). Therefore, whether or not the constitutively active CCR1 was continually internalized was addressed first, followed by whether basal phosphorylation was evident.

In multiple cell systems using either heterologously or endogenously expressed protein, CCR1 exhibited significant constitutive internalization. These systems included human HEK293, HeLa, and THP-1, simian COS-7, and murine L1.2 cells labeled with antibodies directed against the receptor itself or an epitope tag on the N terminus. Therefore, CCR1 constitutive activity appears to be an intrinsic property allowing the receptor to adopt an internalization-competent conformation or set thereof in multiple cellular environments. Agonist stimulation increased the rate of internalization, as evidenced by more rapid removal of CCR1 from the cell surface in HEK293 cells and increased localization of CCR1 within intracellular vesicles in HeLa and COS-7 cells. Interestingly, inhibition of CCR1 with the specific antagonist BX-471 had no effect on constitutive internalization, suggesting that it is a neutral antagonist, whereas it acted as an inverse agonist toward constitutive cell migration. Constitutive internalization was also shown to be independent of $Gα_{i/o}$ activation because PT treatment did not affect the amount of CCR1 remaining at the cell
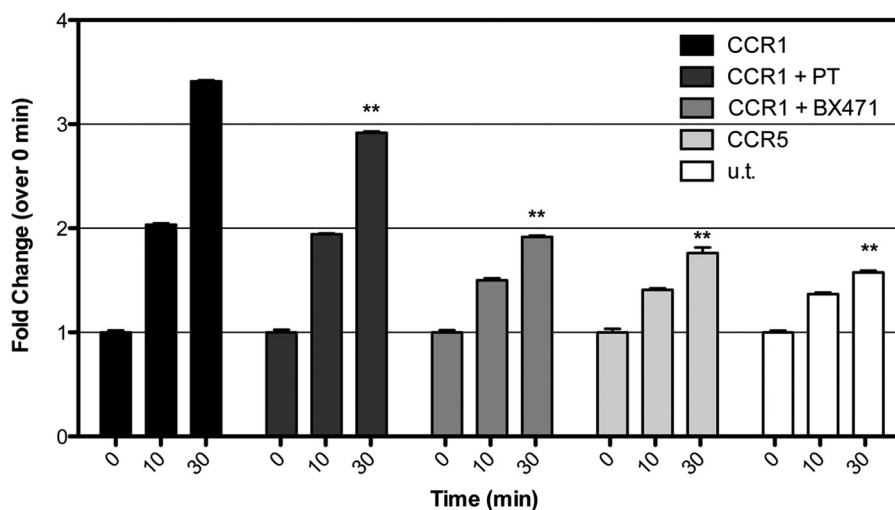
FIGURE 9. **CCR1-mediated internalization of CCL7-Cy3B and effect of G protein inactivation.** CCR1/L1.2 cells were treated in the absence or presence of 0.2 μg/ml PT or 100 μM BX-471 for 1 h prior to incubation with CCL7-Cy3B for up to 30 min at 37 °C. CCR5/L1.2 and untransfected (*u.t.*)/L1.2 cells were included as controls. Internalization of CCL7-Cy3B was measured in triplicates as the median fluorescence intensity (*MFI*) of cells analyzed on a guava® easyCyte™ flow cytometer. The data are displayed as -fold change of median fluorescence intensity over unstimulated cells. The statistical significance of the difference in -fold change in median fluorescence intensity is displayed for the 30 min time point and compared with untreated CCR1/L1.2 using a two-way analysis of variance with Bonferroni post-tests. **, $p < 0.01$ (GraphPad Prism®). *Error bars*, S.D.

surface. This result is similar to previous studies of CCR2, where Gα_i activation was necessary for leukocyte migration but not for β-arrestin recruitment and receptor internalization (55, 85). Finally, it was demonstrated that constitutively internalized CCR1 is recycled back to the plasma membrane to maintain a constant level of receptor at the cell surface.

Consistent with its propensity to constitutively internalize, CCR1 exhibited significant basal phosphorylation. In fact, only a minor increase in phosphorylation was observed following CCL14 addition. Furthermore, the constitutive phosphorylation was correlated with the ability of CCR1 to constitutively associate with β-arrestins, as demonstrated in multiple assays (BRET, co-immunoprecipitation, and confocal microscopy). Following from the lack of a significant increase in receptor phosphorylation, stimulation of CCR1 with CCL14 led to only minor increases in co-immunoprecipitation and co-localization with β-arrestin-2. Basal phosphorylation has been shown to occur in many constitutively active wild-type and mutant GPCRs, including CXCR4 (43), the HCMV-encoded GPCR US28 (86), lutropin/choriogonadotropin receptor (87), and vasopressin 2 receptor (88), among others. Although receptor phosphorylation is not absolutely necessary for recruiting β-arrestins (62), many of these studies demonstrated a reliance upon Ser/Thr phosphorylation for basal or agonist-induced β-arrestin association, as seems to be the case for CCR1. However, although atypical chemokine receptors that do not exhibit the capacity to signal through G proteins have previously been shown to form agonist-independent complexes with β-arrestins (27, 83), this behavior has not previously been observed for any other G protein-coupled chemokine receptor. This non-canonical interaction was specific for CCR1 compared with the other chemokine receptors tested in this study, suggesting that CCR1 has unique structural traits that set it apart from other receptors that require agonist binding to drive β-arrestin association. As with constitutive internalization, BX-471 had no effect on the agonist-independent association between CCR1

and β-arrestin-2, suggesting that it is a functionally selective ligand permissive to basal phosphorylation of CCR1 and β-arrestin-2 association while antagonistic to G protein activation. The precoupling of CCR1 and β-arrestin-2 was also shown to be required for constitutive internalization because CCR1 expressed in MEFs lacking β-arrestin-2 remained at the cell surface, whereas receptor expressed in wild-type MEFs exhibited significant constitutive internalization. Thus, in the absence of agonist, CCR1 adopts a conformation (or set thereof) resulting in basal phosphorylation of the receptor, interaction with β-arrestin-2, and removal of CCR1 from the plasma membrane by a β-arrestin-2-dependent, G protein-independent mechanism.

The question remained as to how CCR1 could apparently induce cell migration while being phosphorylated and associated with β-arrestins. A recent study on the signaling and regulatory proteins associated with the constitutively active relaxin family peptide 1 (RXFP1) receptor challenged the canonical view that G protein coupling and binding of β-arrestin are mutually exclusive (64, 89). RXFP1 was shown to constitutively form a multiprotein "signalsome" complex at its intracellular surface that contained both positive and negative modulators of cAMP production, including G protein, β-arrestin, a protein kinase, a phosphodiesterase, and a scaffolding protein. This complex was hypothesized to allow for rapid and fine tuned regulation of RXFP1 signaling and led our laboratory to consider the possibility that CCR1 could functionally coordinate more than one protein at a time. Using the BRET assay, CCR1 was shown to exhibit significant basal precoupling to Gα_i in a specific manner. Thus, taken together with its constitutive coupling with β-arrestin-2, it appears that CCR1 associates with (or is in close proximity to) both Gα_i and β-arrestin-2. Furthermore, direct proximity between Gα_i-122-Rluc and β-arrestin-2-YFP was demonstrated via BRET in cells with or without co-expression of unlabeled CCR1. Although a small basal signal was observed in cells lacking CCR1, induction
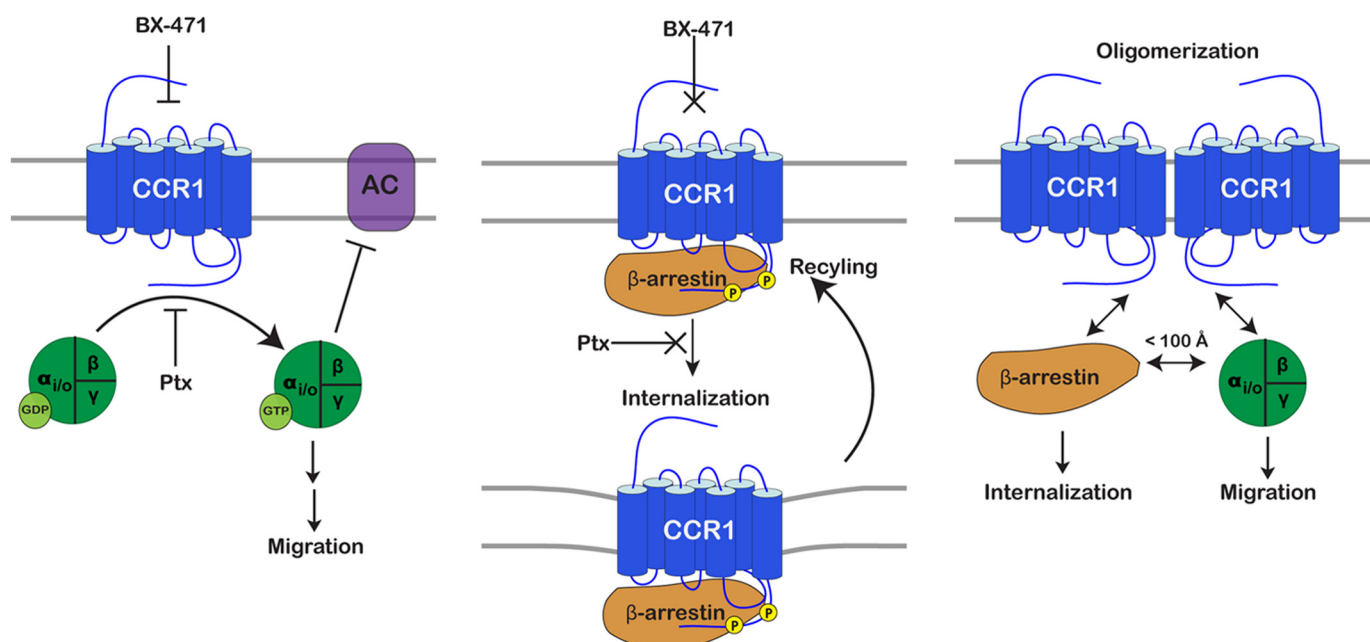
FIGURE 10. **Model of CCR1 constitutive activity.** CCR1 expression is sufficient for inducing basal migration and G protein signaling, which can be blocked with a CCR1-specific inhibitor or PT treatment (*left*). At the same time, CCR1 is also constitutively phosphorylated, leading to β-arrestin-2 recruitment, receptor internalization, and recycling (*middle*). The fate of the internalized receptor and whether it is sent for degradation or eventually recycled back to the cell surface in the presence of ligand stimulation remains to be determined. CCR1 inhibition with BX-471 was unable to block constitutive internalization or prevent basal association with β-arrestin-2. Additionally, a preformed complex that brings CCR1, Gα$_i$, and β-arrestin-2 into close proximity may provide precise regulation of signal transduction by a constitutively active or agonist-activated receptor. The observation that CCR1 forms a homo-oligomer may also explain how the receptor is physically able to form concurrent interactions with these intracellular proteins (*right*). *AC*, adenylyl cyclase.

of CCR1 expression significantly increased the BRET signal between Gα$_i$ and β-arrestin-2. The specificity of this CCR1-mediated complex was further demonstrated by activation with CCL14, which further increased the BRET signal over time. Because co-immunoprecipitation experiments suggested that CCL14 does not induce additional β-arrestin-2 recruitment, this result probably represents either a decrease in the distance separating Gα$_i$ and β-arrestin-2 or a conformational rearrangement of YFP and luciferase that places them into a more favorable orientation for energy transfer. Either way, the signal reached a plateau ~2 min after CCL14 stimulation and remained stable for the remainder of the assay, suggesting that Gα$_i$ and β-arrestin-2 do not dissociate following receptor activation but instead remain complexed with CCR1 as it presumably undergoes internalization. This finding is not unprecedented because other GPCRs (*e.g.* dopamine D4, calcium sensing receptor, and mutant vasopressin 2 receptor) have exhibited constitutive association with β-arrestins that is undiminished following receptor activation despite clear evidence of G protein signaling (90–92), indicating stable complex formation.

How CCR1 is physically able to interact with both G protein and β-arrestin was then considered. Structural analyses and experimental modeling of β-arrestin binding to the intracellular domains of a GPCR indicate an extensive surface area of contact that would effectively preclude G protein coupling (93). On the other hand, the crystal structure of the β2 adrenergic receptor in complex with G$_s$ did not reveal contacts between helices VII and VIII (the latter previously implicated in β-arrestin binding), opening the possibility of association with other proteins (94). Although it remains unclear whether a mono-

meric GPCR can accommodate concurrent association with multiple proteins, receptor oligomerization could provide sufficient intracellular domain surface area to support the formation of a multiprotein complex. Indeed, dimeric receptors have been reported as functional units for β-arrestin binding, including the CXCR4/CXCR7 heterodimer recently shown to constitutively recruit β-arrestin-2 (27). Therefore, the propensity for CCR1 to form homo-oligomers was tested. Using two types of BRET saturation assays, CCR1 was shown to clearly form homo-oligomeric complexes, thereby providing a plausible explanation for concurrent G protein/β-arrestin association with the receptor. Despite their apparent proximity in a multiprotein complex, it is still unclear whether CCR1·G protein and CCR1·β-arrestin or CCR1·β-arrestin·G protein complexes act as functionally distinct units. For example, there may be CCR1 populations that are only coupled to β-arrestin and involved in constitutive internalization and recycling and other assemblies of receptor·G protein·β-arrestin that are involved in canonical agonist-dependent signaling and internalization.

The functional relevance and mechanism of CCR1 constitutive activity remains to be more thoroughly investigated. However, the data presented here suggests that constitutive internalization and recycling of CCR1 could be associated with a chemokine scavenging function of the receptor. Mechanistically, internalization of CCL7 was not inhibited by PT, providing evidence that it is capable of internalizing ligand separate from activation of canonical Gα$_i$ signaling. Moreover, constitutive CCR1 internalization required β-arrestin. This mechanism shows parallels with that of the "professional" scavenging chemokine receptor, D6, which also utilizes a G protein-independent and β-arrestin-dependent pathway (95). Moreover, in

this same publication, it was noted that uncoupling of CCR5 from a G protein-dependent pathway was insufficient to convert this chemotactic receptor into a chemokine scavenger and that other specific structural/signaling features are required. CCR1 constitutive activity and its persistent association with β-arrestin could very well be that missing signaling feature that allows a canonical chemokine receptor to convert to a scavenging modality. However, in contrast to D6, CCR1 possesses the ability to signal both through a canonical G protein pathway and through a G protein-independent/β-arrestin-dependent internalization/recycling pathway. Given that this receptor is activated by at least 10 proinflammatory chemokines, consumption of ligand without activation of G protein could represent a means by which CCR1 can remodel the local concentration of the chemoattractant gradient while maintaining receptor responsiveness. However, further experiments are necessary to determine whether CCR1 can mediate leukocyte migration along an increasing gradient of chemokine without desensitization while continuously consuming ligand.

In summary, this report has shown for the first time that CCR1 exhibits constitutive activity sufficient to induce agonist-independent migration of multiple CCR1-expressing cell types. The receptor undergoes continual internalization mediated by constitutive phosphorylation and association with β-arrestin-2. Surprisingly, CCR1 was also shown to be concurrently engaged in a complex with both β-arrestin-2 and $G\alpha_i$, which can possibly be explained by the formation of CCR1 homo-oligomers. Although it is possible that separate subpopulations of CCR1 are selectively engaged with either G protein or β-arrestin, the data provide a model of a CCR1 "signalsome" that facilitates close proximity of the receptor with signaling and regulatory proteins enabling agonist-independent signal transduction from some receptors and continuous down-modulation from others (Fig. 10). Chemokine scavenging by non-signaling CCR1 may serve as a functional explanation for this behavior. The constitutive activity of CCR1 also suggests a new pharmacological axis for drug development. In principle, the non-canonical behavior of CCR1 could be exploited in the context of inflammatory diseases with drugs that block G protein activation but are permissive or agonistic for the non-canonical β-arrestin-mediated receptor internalization and chemokine scavenging.

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