0026-895X/05/6706-1966–1976$20.00
MOLECULAR PHARMACOLOGY
Vol. 67, No. 6
9779/3033781
Mol Pharmacol 67:1966–1976, 2005
Printed in U.S.A.

Mutation of the DRY Motif Reveals Different Structural Requirements for the CC Chemokine Receptor 5-Mediated Signaling and Receptor Endocytosis

Bernard Lagane, Sébastien Ballet, Thierry Planchenault, Karl Balabanian, Emmanuel Le Poul, Cédric Blanpain, Yann Percherancier, Isabelle Staropoli, Gilbert Vassart, Martin Oppermann, Marc Parmentier, and Françoise Bachelerie

Unité d'Immunologie Virale, Institut Pasteur, Paris, France (B.L., T.P., K.B., Y.P., I.S., F.B.); Institut de Recherche Interdisciplinaire en Biologie Humaine et Moléculaire (S.B., C.B., G.V., M.P.), Service de Génétique Médicale (G.V.), Université Libre de Bruxelles, Campus Erasme, Brussels, Belgium; Euroscreen, Gosselies, Belgium (E.L.P.); and Department of Immunology, Georg-August-University, Göttingen, Germany (M.O.)

Received November 26, 2004; accepted March 10, 2005

ABSTRACT

CC chemokine receptor 5 (CCR5) is a G protein-coupled receptor that governs migration of leukocytes and serves as a coreceptor for the R5 tropic strains of human immunodeficiency virus (HIV). CCR5-mediated signaling in response to CC chemokines relies on G protein activation. Desensitization, which rapidly turns off G protein-dependent signaling, involves phosphorylation of CCR5 that promotes interaction of the receptor with β-arrestins for endocytosis. Whether coupling to G proteins, desensitization, and endocytosis of CCR5 require the same structural determinants remains a matter of investigation. Here, we show that CCR5 displayed agonist-independent coupling to G proteins. This constitutive activity of the receptor was abrogated by TAK779 (*N*,*N*-dimethyl-*N*-[4-[[[2-(4-methylphenyl)-6,7-dihydro-5*H*-benzocyclohepten-8-yl]carbonyl]amino]benzyl]tetrahydro-2*H*-pyran-4-aminium chloride), a nonpeptidic CCR5 ligand that inhibits HIV infection and was found to depend on the integrity of the Asp-Arg-Tyr (DRY) motif. Changing Arg-126 by the neutral residue Asn (R126N-CCR5 mutant) abolished CCR5-mediated activation of G proteins, either constitutively or in response to agonists. In contrast, R126N-CCR5 not only retained agonist-promoted phosphorylation and β-arrestin-dependent endocytosis but also displayed a higher basal phosphorylation than wild-type CCR5. Expression of β-arrestin in R126N-CCR5-expressing cells resulted in receptor down-regulation, thereby suggesting that R126N-CCR5 spontaneously interacts with β-arrestins. However, although expression of β-arrestin favored wild-type CCR5-mediated chemotaxis, it failed to promote migration of cells expressing R126N-CCR5. Overall, these data indicate that structural requirements for CCR5-mediated activation of G proteins, albeit not involved in receptor desensitization and internalization, are needed for β-arrestin-mediated chemotaxis. These results have implications for how distinct biological responses of CCR5 might rely on a different set of receptor conformations.

This work was supported by the Agence Nationale de Recherche sur le SIDA, Ensemble Contre le SIDA, the Actions de Recherche Concertées of the Communauté Française de Belgique, the Belgian program on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming, the Fonds de la Recherche Scientifique Médicale of Belgium, and the Fondation Médicale Reine Elizabeth. B.L., K.B., and Y.P. were supported by fellowships from the Agence Nationale de Recherche sur le SIDA and Ensemble Contre le SIDA.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.104.009779.

CCR5 is a G protein-coupled heptahelical receptor (GPCR) that initiates intracellular signaling in response to CC chemokines, including CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, and CCL8/monocyte chemoattractant protein 2. CCR5 is involved in the recruitment of leukocytes to the site of inflammation and is also associated to a number of pathological disorders, from inflammatory diseases to AIDS (Proudfoot, 2002). In the present work, we deal with the molecular mechanisms that underlie CCR5 conformational changes and functions.

ABBREVIATIONS: CCR5, CC chemokine receptor 5; GPCR, G protein-coupled heptahelical receptor; RANTES, regulated on activation of normal T cell expressed and secreted; GRK, G protein-coupled receptor kinase; TM, transmembrane; DRY, Asp-Arg-Tyr; MIP, macrophage inflammatory protein; HIV, human immunodeficiency virus; EGFP, enhanced green fluorescent protein; CHO, Chinese hamster ovary; HEK, human embryonic kidney; mAb, monoclonal antibody; TAK779, *N*,*N*-dimethyl-*N*-[4-[[[2-(4-methylphenyl)-6,7-dihydro-5*H*-benzocyclohepten-8-yl]carbonyl]amino-]benzyl]tetrahydro-2*H*-pyran-4-aminium chloride; PE, phycoerythrin; FCS, fetal calf serum; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; wt, wild-type; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; sCD4, soluble CD4; AM, acetoxymethyl ester; ELISA, enzyme-linked immunosorbent assay; AU, arbitrary unit; MFI, mean fluorescence intensity; PTX, pertussis toxin.

Transduction of signals by GPCRs depends on the interaction of the receptors with distinct families of proteins, including heterotrimeric G proteins, G protein-coupled receptor kinases (GRKs), second messenger-dependent protein kinases, and β-arrestins. After agonist binding, GPCRs adopt an active conformation that interacts with G proteins (Seifert and Wenzel-Seifert, 2003). This event catalyzes the exchange of GTP for GDP in the nucleotide-binding pocket of G protein α-subunits, so that the G proteins become activated and Gα-GTP as well as the Gβγ dimer can activate effector proteins. The waning of G protein-dependent signaling (i.e., desensitization of GPCRs) rapidly takes place with the phosphorylation of agonist-occupied GPCRs, which then interact with β-arrestins (Shenoy and Lefkowitz, 2003). β-Arrestins not only target desensitized receptors to clathrin-coated pits for endocytosis but also act as scaffolding proteins that link GPCRs to the stimulation of additional signaling pathways (Shenoy and Lefkowitz, 2003).

Activation of GPCRs by agonists involves conformational changes that permit intracellular sequences to interact with G proteins. Relative motions of transmembrane (TM) helices 3 and 6 are reported after activation of rhodopsin-related GPCRs (Farrens et al., 1996; Gether et al., 1997), a family to which belongs the chemokine receptor CCR5 (Proudfoot, 2002). Studies on the molecular mechanisms that underneath the transitions between inactive and active conformations of these GPCRs point to a key role for the highly conserved E/DRY motif lying at the interface between TM3 and the second intracellular loop. When the first residue of the E/DRY motif is mutated, numerous receptors display an agonist-independent activation (i.e., a constitutive activity) (Scheer et al., 1997; Rasmussen et al., 1999), whereas mutation of the Arg residue generally impairs G protein-dependent signaling (Ballesteros et al., 1998; Scheer et al., 2000; Chung et al., 2002; Amara et al., 2003). It was proposed that, in inactive states of rhodopsin-related GPCRs, the anionic form of the Glu/Asp residue allows for the adjacent Arg to be buried with respect to the cytosol (Scheer et al., 1997; llesteros et al., 1998; Greasley et al., 2002). Upon activation, protonation of the Glu/Asp residue would shift Arg out of the helix bundle. This event was thought to promote rearrangements of TMs and to trigger a conformation of receptors that can activate G proteins.

Whether the receptor conformational changes that lead to activation of G proteins are required for desensitization and internalization is currently a matter of debate (Vilardaga et al., 2001). In fact, constitutively active receptors often display increased agonist-independent phosphorylation and internalization (Pei et al., 1994), and efficiency of agonists in eliciting G protein-dependent signaling generally parallels their abilities in promoting receptor phosphorylation (Benovic et al., 1988). In contrast, our present data show that mutating the DRY motif within CCR5 and antagonist binding to the receptor have contrary effects on G protein coupling, desensitization, and endocytosis. It is noteworthy that replacement of Arg-126 in the DRY motif abrogates activation of G proteins but preserves phosphorylation and interactions with β-arrestins for endocytosis. We show that β-arrestins are involved in CCR5-mediated chemotaxis but that this process is abolished after mutation of Arg-126. Overall, our results provide evidence that some of the structural determinants needed for CCR5-mediated activation of G proteins are also required for β-arrestin-mediated chemotaxis but not for receptor desensitization and internalization.

Materials and Methods

Materials. The recombinant human chemokine CCL5/RANTES was obtained from R&D Systems Europe (London, UK) and CCL4/MIP-1β was provided by Dr. F. Baleux (Institut Pasteur, Paris, France). Ten micromolar CCL5 and 100 μM CCL4 stock solutions were prepared in sterile water. TAK779 was obtained from the AIDS Research and Reference Reagent Program catalog of National Institutes of Health (Bethesda, MD). ^{125}I-CCL4 (specific activity 2000 Ci/mmol) was purchased from Amersham Biosciences Inc. (Orsay, France). [^{35}S]gp140 from the Bx08 human immunodeficiency virus (HIV) strain was prepared as described previously (Staropoli et al., 2000). The pβ-arrestin2-EGFP and pN1-EGFP plasmids described previously (Scott et al., 2002) were provided by Dr. S. Marullo (Institut Cochin, Paris, France). Transient transfections of these plasmids in CHO and HEK 293T cells were performed using the FuGENE 6 transfection system (Roche Diagnostics, Mannheim, Germany) and the calcium phosphate-DNA coprecipitation method, respectively. Pertussis toxin (PTX) was purchased from Sigma-Aldrich (St. Louis, MO).

Flow Cytometry Analysis. Cell surface expression of receptors was determined as described previously (Amara et al., 2003). Staining was performed using monoclonal antibodies (mAbs) recognizing epitopes within the N-terminal domain (MC-5 and CTC5) or the second extracellular loop [phycoerythrin (PE)-conjugated 2D7, 45531] of CCR5. MC-5 (from Dr. M. Mack, Medizinische Poliklinik, University of Munich, Munich, Germany), CTC5 (from Dr. R. W. Doms, University of Pennsylvania, Philadelphia, PA) and 45531 (R&D Systems Europe) were subsequently labeled with a secondary, PE-conjugated goat anti-mouse IgG antibody (Rockland, Gilbertsville, PA). PE-coupled mAb 2D7 was from BD Biosciences (San Diego, CA). Analysis was carried out on a BD Biosciences FACSCalibur.

CCR5 Constructs and Expression. Plasmids encoding the CCR5 mutants (R126N-CCR5 and D125V-CCR5) were constructed by site-directed mutagenesis using the QuikChange procedure (Stratagene, La Jolla, CA) in the pcDNA3 plasmid. The mutated coding sequences were subcloned into the bicistronic expression vector pEFIN3 as described previously (Blanpain et al., 2002). CHO-K1 cells were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Cergy Pontoise, France). CCR5 constructs were transfected using FuGENE 6 in the CHO-K1 variant cell line that is cultured with 250 μg/ml Zeocin (Invitrogen) for maintenance of an apo-aequorin encoding plasmid (Blanpain et al., 2002). Selection of receptor-expressing cells was obtained after a 14-day culture with 400 μg/ml neomycin (Invitrogen). The populations of mixed cell clones expressing the CCR5 variants were used for functional assays ([^{35}S]GTPγS binding, endocytosis, and calcium mobilization). Clones expressing the highest amounts of receptors at the cell surface were used for chemokine binding assays. Clones were selected after cell limit dilution and screened for CCR5 expression by flow cytometry analysis and ^{125}I-CCL4 saturation binding experiments (B_{max} are equal to 206 ± 34 fmol/mg of protein for wt-CCR5 and 80 ± 24 fmol/mg of protein for D125V-CCR5). Receptor expression into HEK 293T cells was conducted using a previously described lentivirus-based strategy (Amara et al., 2003). HEK 293T cells were maintained in DMEM (Invitrogen) supplemented with 10% FCS and antibiotics. Cell surface expression of receptors was determined by flow cytometry analysis.

Membrane Preparation. For [^{35}S]GTPγS and iodinated chemokine binding experiments, membranes from HEK 293T or CHO-K1 cells were prepared as follows. Cells were grown near to confluence and detached from plates in EDTA-containing PBS. The cells were incubated for 30 min at 4°C in buffer A (15 mM Tris-HCl, pH 7.5, 2

mM $MgCl_2$, 0.3 mM EDTA, and 1 mM EGTA) and then passed in a glass homogenizer. The supernatants resulting from a 5-min centrifugation at 500*g* were then centrifuged at 40,000*g* for 30 min at 4°C. The pellets were suspended in buffer A and centrifuged at 40,000*g* for 30 min. The membrane pellets were then suspended in buffer B (75 mM Tris-HCl, pH 7.5, 12.5 mM $MgCl_2$, 0.3 mM EDTA, 1 mM EGTA, and 250 mM sucrose) at a protein concentration >1 mg/ml. Protein content was quantified using the bicinchoninic acid protein assay reagent (Pierce Chemical, Brebières, France) with bovine serum albumin (BSA) as a standard. Crude membrane fractions were stored at −80°C until use.

[^{35}S]GTPγS Binding. [^{35}S]GTPγS binding to crude membrane preparations was performed in 96-well microplates (basic flashplates; PerkinElmer Life and Analytical Sciences, Boston, MA). Membranes (10–15 μg of proteins/well) were incubated for 15 min at 30°C in 20 mM HEPES, pH 7.4, containing 100 mM NaCl, 10 μg/ml saponin, 1 μM GDP and 3 mM $MgCl_2$, in the presence or absence (basal [^{35}S]GTPγS binding) of ligands. Then, 0.1 nM [^{35}S]GTPγS (Amersham Biosciences Inc.) was added to membrane-containing mixes, which were incubated for 30 min at 30°C. The incubation was stopped by centrifugation (800*g* for 10 min) at 4°C and removal of supernatants. Microplates were counted 2 min per well in a PerkinElmer Wallac 1450 Microbeta Trilux or in a Topcount counter (PerkinElmer Life and Analytical Sciences).

Binding Experiments. Saturation binding experiments of CCL4 to membranes from CHO-K1 cells expressing wt-CCR5 or D125V-CCR5 were performed using Minisorb tubes (Nunc, Rochester, NY). Samples containing 2 μg of membrane proteins and increasing concentrations of ^{125}I-CCL4 in 0.1-ml final volume of assay buffer (50 mM HEPES, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5% BSA) were incubated for 90 min at 27°C. Nonspecific binding was measured in the presence of a 200-fold excess of unlabeled CCL4. Bound CCL4 was separated by filtration through GF/B filters presoaked in 0.5% polyethylenimine (Sigma-Aldrich). Filters were counted in a beta scintillation counter. Binding of [^{35}S]gp140 from the Bx08 HIV-1 strain to CCR5-expressing HEK 293T cells was conducted as follows. Cells (5×10^5) were suspended in 0.1 ml of assay buffer (50 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 5% BSA, and 0.1% NaN_3) containing a saturating concentration of soluble CD4 (sCD4, 180 nM; Progenics, Tarrytown, NY). Increasing concentrations of [^{35}S]gp140 were added for the saturation binding experiments. For the competition binding assays, 5 nM [^{35}S]gp140 was used as a tracer in the presence of increasing concentrations of unlabeled CCL4. Binding was performed at room temperature for 1 h. Nonspecific binding was determined either in the absence of sCD4 or using 1 μM TAK779. To remove unbound radioactivity, cell suspensions were diluted with 1 ml of ice-cold wash buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, and 1 mM $CaCl_2$) and pelleted at 16,000*g* for 10 s. Pellets were then washed once and suspended in wash buffer before addition of Optiphase Supermix solution (PerkinElmer Life and Analytical Sciences). Bound radioactivity was counted in a 1450 PerkinElmer Wallac Microbeta Trilux γ counter. Binding parameters were determined with the Prism software (GraphPad Software Inc., San Diego, CA) using nonlinear regression applied to a one-site model.

Intracellular Calcium Mobilization. Intracellular calcium measured in CCR5-expressing HEK 293T cells loaded with fluo-3-AM (Sigma-Aldrich) was conducted in a VICTOR multilabel counter (PerkinElmer Life and Analytical Sciences). In brief, loading of cells with fluo-3-AM was accomplished after two washes in buffer A (100 mM HEPES, pH 7.0, 140 mM NaCl, 5 mM KCl, 5 mM glucose, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM Na_2HPO_4, 1.4 mM $MgSO_4$, and 0.3 mM KH_2PO_4). Cells were then suspended in buffer A (5.10^6 cells/ml) and were incubated at 37°C for 30 min in the presence of 8 μM fluo-3-AM. Cells were incubated for a further 30 min after addition of 1 volume of buffer B (i.e., buffer A supplemented with 5% FCS, pH 7.4). Cells were then washed twice, suspended in buffer B (5×10^6 cells/ml), and stored at 4°C. For measurements, aliquots of cells were preincubated at 37°C for 1 min and then further diluted up to 0.2 ml with buffer B, in a 96-well flat bottom plate, in the presence or absence of the indicated CCL4 concentrations. Intracellular calcium release was recorded by monitoring fluorescence emission at 530 nm (with λ_{ex} = 485 nm) every 2 s. Maximum (F_{max}) and minimum (F_{min}) fluorescence values were determined after addition of Triton X-100 and EGTA, respectively. Calculation of intracellular calcium concentration (Ca_i) was performed as described previously (McColl and Naccache, 1997), using the equation $Ca_i = 400\ [(F - F_{min})/(F_{max} - F)]$, where 400 refers to the nanomolar equilibrium dissociation constant of Ca^{2+} for fluo-3.

Chemotaxis Assays. Migration of CCR5-expressing HEK 293T cells was evaluated using a Transwell system (Corning Costar, Brumath, France) as follows. Before the assay, HEK 293T cells were detached from plates in EDTA-containing PBS, washed twice in DMEM, and resuspended at 2×10^6 cells/ml in buffer A (DMEM supplemented with 20 mM HEPES and 1% BSA). Cells (3×10^5 in 0.15 ml of buffer A) were added to the upper chamber of a 6.5-mm-diameter, 8-μm-pore polycarbonate Transwell culture insert, and 0.6 ml of the same medium with or without CCL4 was added to the lower chamber. Chemotaxis proceeded for 4 h at 37°C in humidified air with 5% CO_2. At the end of the incubation period, the underside of the polycarbonate membrane was vigorously washed with EDTA-containing PBS to recover transmigrated cells in the lower chamber. The fraction of cells migrating across the polycarbonate membrane was assessed by flow cytometry and the chemotaxis index was calculated as follows: (number of cells that migrated toward CCL4)/(number of cells that migrated spontaneously).

Receptor Phosphorylation. Determination of receptor phosphorylation after metabolic labeling with [^{32}P]orthophosphate was performed as follows. HEK 293T cells detached from plates in EDTA-containing PBS were washed twice in medium A [phosphate-free DMEM (Invitrogen), with 8% (v/v) dialyzed FCS (Sigma-Aldrich) and 1 mM sodium-pyruvate) and then incubated for 90 min at 37°C in medium A containing [^{32}P]orthophosphate (125 μCi/ml; Amersham Biosciences Inc.). Incubations performed in six-well dishes (10^7 cells/2 ml/well) under gentle stirring were continued for the indicated times in the absence (basal) or presence of ligands. Labeled cells were washed twice in ice-cold HEPES-based buffer (20 mM HEPES, pH 7.4, and 120 mM NaCl) before incubation for 1 h at 4°C on a rotating wheel into lysis buffer [10^7 cells in 1 ml of lysis buffer [20 mM Tris-HCl, pH 7.4, 100 mM $(NH_4)_2SO_4$, 10% (v/v) glycerol, and 1% (w/v) Cymal-5], supplemented with phosphatase (5 mM NAF, 10 mM *p*-nitrophenyl phosphate, 10 mM β-glycerophosphate, and 1 mM orthovanadate) and protease (Roche Diagnostics, Basel, Switzerland) inhibitors]. After centrifugation, supernatants were measured for their protein contents according to the Bradford procedure. One milligram of solubilized proteins was further diluted up to 1 ml with lysis buffer and then incubated overnight at 4°C on a rotating wheel with 3 μg of 2D7 anti-CCR5 mAb (BD Biosciences). The formed receptor/antibody complexes were captured with 35 μl of protein G plus/protein A agarose beads (Calbiochem, San Diego, CA) for 3 h at 4°C. After three washes and homogenization in 1 ml of lysis buffer, bead-bound complexes were divided into two identical 0.5-ml samples to allow quantification of both receptor phosphorylation and immunoprecipitated receptors by Western blot analysis. After electrophoresis on two separate 12% SDS-polyacrylamide gels overnight at 4°C under reducing conditions, one gel was dried and analyzed for ^{32}P labeling using an Amersham Biosciences PhosphorImager. The other gel, after electroblotting onto a polyvinylidene difluoride membrane, was immunoblotted using the MC-5 mAb. Immobilized antigen-antibody complexes were detected with a mixture of horseradish peroxidase-coupled protein A/protein G (1:1; Sigma-Aldrich), developed by enhanced chemiluminescence (ECL$^+$; Amersham Biosciences Inc.), and quantified using a LAS-1000 charge-coupled device camera (Image Gauge 3.4 software; Fuji Photo Film Co., Tokyo, Japan). In addition, receptor amounts in whole cell lysates (4 μg of proteins) were determined by immunoblotting with the MC-5 mAb

to compare wt-CCR5 and R126N-CCR5 immunoprecipitation efficiencies.

Phosphorylation of serine residues at position 337 and 349 was determined using an enzyme-linked immunosorbent assay (ELISA), as described previously (Pollok-Kopp et al., 2003). In brief, 2×10^6 HEK 293T cells that express wt- or R126N-CCR5 were incubated for 10 min at 37°C in the absence (basal) or presence of CCL4, washed once with ice-cold PBS, and then scraped in 0.7 ml of lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 0.05% SDS with the aforementioned phosphatase and protease inhibitors). After centrifugation (3000*g* for 10 min), supernatants were assessed for their protein contents using the Bradford procedure. Supernatants (0.1 ml) were then applied for 1 h at room temperature either directly or after a 2-fold dilution in lysis buffer into wells of microtiter plates coated with the anti-CCR5 mAb T21/8. The biotinylated mAb E11/19 and V14/2, which recognize phosphorylated Ser-349 and Ser-337, respectively, were then added for a 1-h incubation at 37°C. Detection was performed using streptavidin-horseradish peroxidase and ortho-phenylenediamine-HCl as substrate (PerkinElmer Life and Analytical Sciences). The assays were calibrated with a standard protein, which was obtained by the conjugation of BSA with synthetic N-terminal and C-terminal CCR5 peptides at 1:5:5 M ratios using succinimidyl 4-(*n*-maleimidomethyl)-cyclohexane-1-carboxylate as a cross-linking reagent. Results were expressed in arbitrary units (AU) (1 AU = 1 ng of BSA-peptide per milliliter), and normalized for the protein contents in the cell lysates.

Receptor Down-Modulation. Receptor down-modulation was studied as described previously (Amara et al., 1997). In brief, cells were incubated at 37°C for 45 min in DMEM (HEK 293T cells) or Ham's F-12 (CHO cells) containing 20 mM HEPES and 1% BSA (2×10^6 cells/ml), in the presence or absence of CCL4. Once treated, cells were placed on ice and then washed twice with ice-cold PBS containing 1% BSA. To remove receptor-bound CCL4, cells were incubated for 2 min in 50 mM glycine, pH 2.7, containing 100 mM NaCl, and subsequently diluted up to 1 ml with ice-cold PBS/1% BSA buffer. Cells were washed twice with the same buffer before staining with PE-conjugated 2D7 mAb and analysis by flow cytometry. No receptor down-modulation was found when cells were incubated at 4°C in the presence of ligand. Receptor expression in treated cells was calculated as follows: [(receptor geometric mean fluorescence intensity (MFI) of treated cells)/(receptor MFI of untreated cells)] × 100; 100% corresponds to receptor expression at the surface of cells incubated in the medium alone.

Results

Coupling of CCR5 to G Proteins: The Role of Arg-126. To assess the role of the DRY motif in the coupling of CCR5 to G proteins, we first substituted Arg-126 by the neutral Asn residue and stably expressed the resulting R126N-CCR5 mutant or its wild-type counterpart in HEK 293T cells. We then selected cell populations showing similar cell surface expressions for both receptors using the 2D7 mAb that recognizes a conformational epitope within the second extracellular loop (ECL-2) of CCR5 (Fig. 1A). Results were confirmed with other mAbs targeted against epitopes within the N-terminal domain of CCR5 (MC-5 and CTC5) or ECL-2 (45531) (data not shown), thus suggesting that both receptors share similar abilities to bind these antibodies. Saturation binding experiments using increasing concentrations of ^{35}S-labeled gp140 from the Bx08 HIV-1 strain also confirmed that wt-CCR5 and R126N-CCR5 expressed at similar levels at the surface of the selected HEK 293T cells (B_{max} = 50,000 and 47,000 receptors per cell for wt- and R126N-CCR5, respectively, with K_D = 10–11 nM for both receptors). As reported previously (Amara et al., 2003), we found that R126N-CCR5 failed to promote

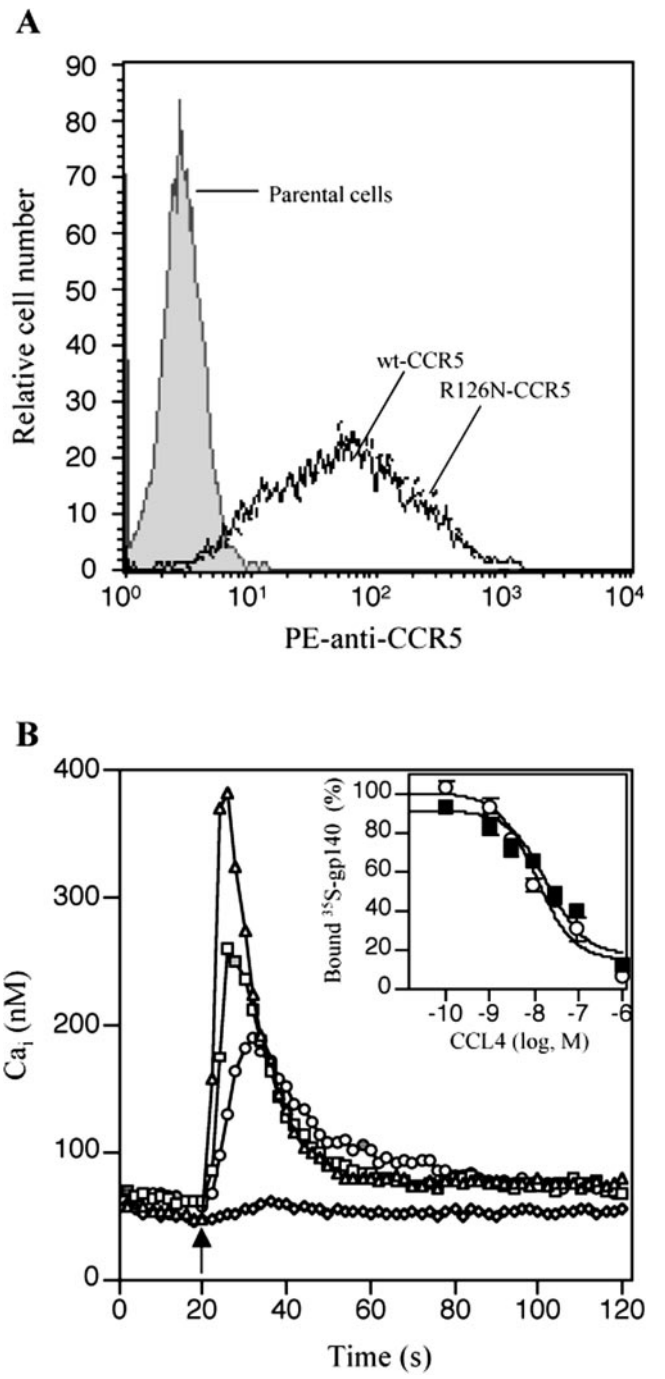

Fig. 1. wt-CCR5 and R126N-CCR5 cell surface expressions and functions in HEK 293T cells. A, cell surface expressions of wt-CCR5 (regular line) and R126N-CCR5 (dotted line) were determined using the PE-conjugated anti-CCR5 mAb 2D7 by flow cytometry. Nearly equal geometric MFIs, which relate to receptor levels at the cell surface, for wt- (MFI = 48) and R126N-CCR5 (MFI = 55) were deduced as illustrated in this representative experiment. Staining of parental cells with PE-conjugated 2D7 (filled peak) was used as a negative control. B, intracellular calcium mobilization is shown in wt-CCR5-expressing cells loaded with fluo-3-AM in response to 50 nM (circles), 150 nM (squares), and 500 nM (triangles) CCL4. CCL4 (500 nM) failed to trigger intracellular calcium release in cells expressing R126N-CCR5 (diamonds). The arrow indicates CCL4 stimulation. Results are representative from three independent experiments. Inset, competition by CCL4 for [^{35}S]gp140 binding to wt-CCR5- (open symbols) or R126N-CCR5 (closed symbols)-expressing cells. Experiments were carried out in the presence of 180 nM sCD4. Results were normalized for nonspecific (0%) and specific binding in the absence of competitor (100%). A representative experiment of two performed in duplicate is shown.

intracellular calcium mobilization in response to CCL4, in contrast to wt-CCR5 (Fig. 1B). Together with our observations that R126N-CCR5 conserves an affinity for CCL4 nearly similar to that of its wt counterpart (Fig. 1B, inset; $IC_{50} = 12 \pm 4$ and 22 ± 3 nM for wt-CCR5 and R126N-CCR5, respectively), these data suggest that charge-neutralizing mutation of Arg-126 prevents CCR5 from activating G proteins.

We then directly investigated this possibility using a [^{35}S]GTPγS binding-based assay to measure activation of G proteins mediated by wt- or R126N-CCR5. Exposure of membranes from wt-CCR5-expressing cells to CCL4 promoted a robust, dose-dependent, [^{35}S]GTPγS binding (Fig. 2A, circles; $EC_{50} = 4.5$ nM). In contrast, CCL4 had marginal effects on [^{35}S]GTPγS binding to R126N-CCR5-expressing membranes (Fig. 2A, triangles). Similar results were obtained using CCL5 as a ligand (not shown). This finding emphasized the pivotal role played by Arg-126 in allowing CCR5 to activate G proteins.

CCR5 Spontaneously Interacts with G Proteins: The Role of the DRY Motif and Identification of TAK779 as an Inverse Agonist. Expression of wt-CCR5 in HEK 293T cells resulted in a substantial increase in basal [^{35}S]GTPγS binding (Fig. 2, A–C). This suggests that a fraction of this receptor spontaneously activates G proteins. It has been proposed that G protein availability as well as plasma membrane organization (Ostrom et al., 2000; Nasman et al., 2001), which differ from one cell type to another, influence

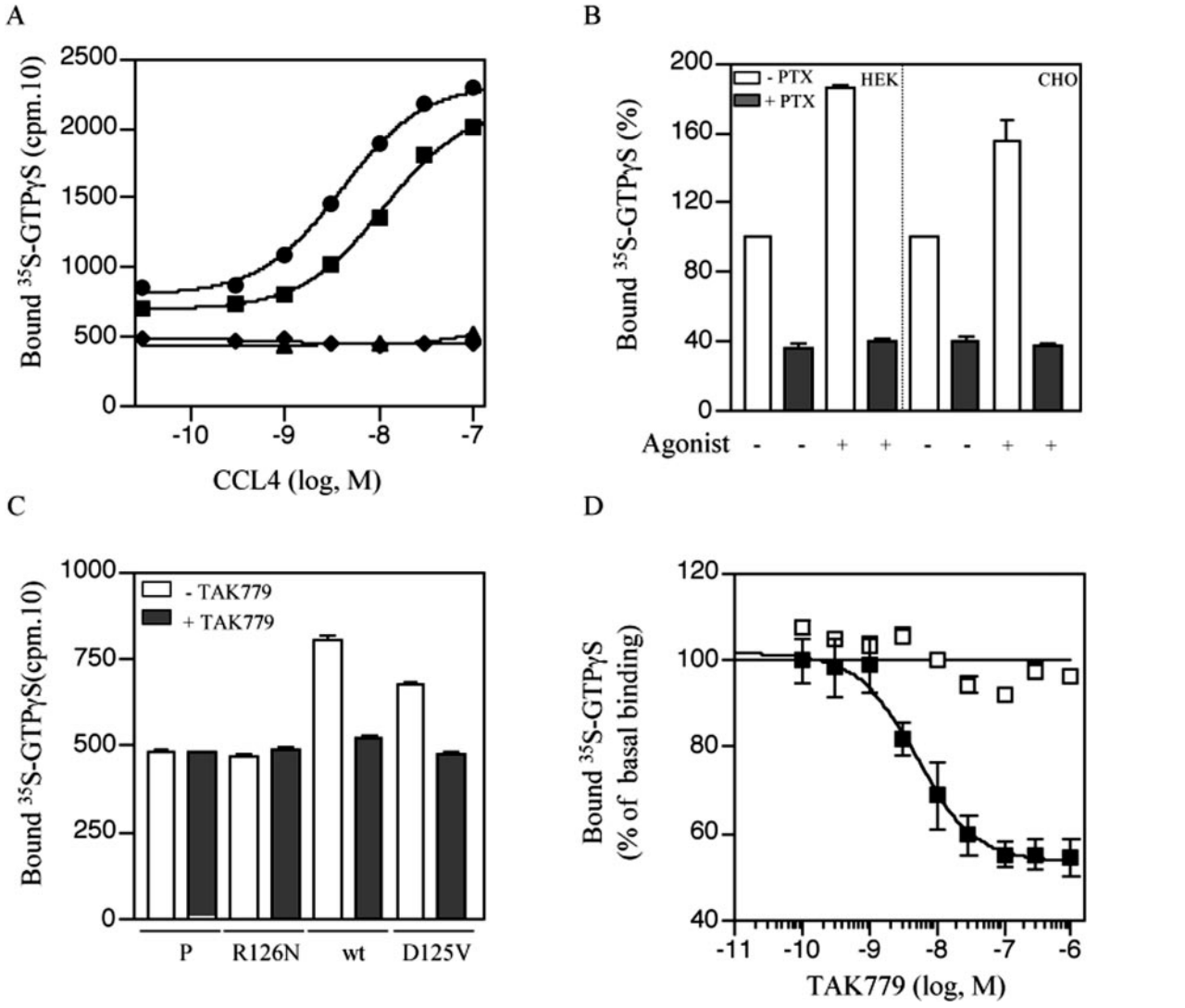

Fig. 2. Spontaneous and agonist-induced coupling of CCR5-derived receptors to G proteins. A, CCL4-induced [^{35}S]GTPγS binding to membranes from parental HEK 293T cells (diamonds) or expressing wt-CCR5 (circles), D125V-CCR5 (squares), or R126N-CCR5 (triangles). Membranes were incubated in assay buffer containing 0.1 nM [^{35}S]GTPγS, 1 μM GDP, and 3 mM $MgCl_2$, and the indicated concentrations of CCL4. Results are representative from two to five independent determinations performed in triplicate. Similar cell surface expressions of receptors were controlled by flow cytometry analysis. B, [^{35}S]GTPγS binding to membranes from HEK 293T (left) and CHO-K1 (right) cell populations expressing wt-CCR5 and cultured for 15h in the absence (open columns) or presence (closed columns) of 100 ng/ml PTX. Results in the absence or presence of agonist (30 nM CCL4 for HEK 293T cells and 60 nM CCL5 for CHO-K1 cells) are shown. Values from two to three determinations are expressed as the percentage of basal [^{35}S]GTPγS binding to membranes from wt-CCR5-expressing cells cultured in the absence of PTX (100%). C, [^{35}S]GTPγS binding to membranes from parental (P) HEK 293T cells or expressing wt-CCR5, D125V-CCR5, or R126N-CCR5 in the absence (open columns) or presence (closed columns) of 1 μM TAK779. D, dose-dependent effects of TAK779 on basal [^{35}S]GTPγS binding to membranes from populations of CHO-K1 cells expressing wt-CCR5 (closed squares) or R126N-CCR5 (open squares). The data were normalized for basal binding in the absence of TAK-779 (100%). All points were run in triplicate (means $\pm$ S.E.M.). A representative experiment of three performed independently is shown.

the efficiency of receptor to G protein coupling. In addition, coupling of some receptors to G proteins, including chemokine receptors, has been found to be cell type-specific (Arai and Charo, 1996). We rule out this possibility by reproducing the enhanced basal [^{35}S]GTPγS binding to membranes from CHO-K1 cells stably expressing wt-CCR5 (Fig. 2, B and D). Pertussis toxin (PTX) treatment was found to abolish basal binding of [^{35}S]GTPγS to membranes from both HEK 293T and CHO-K1 cells expressing wt-CCR5 (Fig. 2B), thus demonstrating that this binding results from spontaneous activation of G_i/G_o type α-subunits.

Whether constitutive activity of CCR5 relies on intrinsic structural properties of the receptor was next investigated. We first found that replacement of Arg-126 by Asn fully abrogated basal [^{35}S]GTPγS binding (Fig. 2C). The anionic charge of the Arg-adjacent acidic residue of the DRY motif is believed to lock rhodopsin-related receptors in an inactive state, and thus to prevent coupling to G proteins (Ballesteros et al., 1998). We were surprised to find that replacing Asp-125 in CCR5 by the bulky hydrophobic residue Val (D125V-CCR5) neither increases constitutive activity nor favors agonist-induced coupling to G proteins (Fig. 2A, squares). Even, we consistently observed that D125V-CCR5 displayed slightly reduced spontaneous (Fig. 2C) and agonist-induced coupling to G proteins relative to its wild-type counterpart (Fig. 2A; EC_{50} = 11 nM). We controlled that D125V-CCR5 behaves as wt-CCR5 in calcium mobilization (data not shown) and binding assays (K_D = 0.39–0.41 nM for CCL4).

Finally, the existence of basal coupling of CCR5 to G proteins prompted us to search for inverse agonists of the receptor. TAK779 is a low molecular weight, nonpeptidic molecule, which binds to CCR5 and then blocks HIV entry into cells (Dragic et al., 2000). TAK779 inhibits agonist-induced signaling and is thus considered as a specific CCR5 antagonist (Baba et al., 1999). We confirmed this effect (data not shown), but additionally, we unraveled that TAK779-reduced basal [^{35}S]GTPγS binding to CCR5-expressing membranes (Fig. 2, C and D) in a dose-dependent manner (IC_{50} = 4.9 nM). In the presence of TAK779, we controlled that high concentrations of CCL4 fully restored [^{35}S]GTPγS binding (not shown), thus ruling out nonspecific effects of TAK779 on nucleotide binding to G protein α subunits. Together, these findings indicate that TAK779 is a potent inverse agonist of CCR5.

CCR5 Endocytosis Is Preserved upon Arg-126 Substitution. Whether activation of G proteins and endocytosis of CCR5 requires the same structural elements remains a matter of investigations. We thus explored whether replacement of Arg-126 also impaired endocytosis of CCR5 in response to chemokines. For these experiments, we preferred CHO-K1 cells stably expressing wt-CCR5 or R126N-CCR5 (Fig. 3) over HEK 293T cells that show limited CCR5 down-modulation (our data in Fig. 5; Aramori et al., 1997). Figure 3, A and B, show that a 45-min exposure to 100 nM CCL4 resulted in a robust decrease of cell surface expression of wt-CCR5 and R126N-CCR5, respectively. A time-dependent analysis of CCL4-induced down-modulation of receptors confirmed that R126N-CCR5 disappeared from the cell surface with even higher efficiency compared with wt-CCR5 (Fig. 3C). Removal of CCL4 and further incubation of cells in the absence of the chemokine resulted in the reexpression of both receptors (64% within 15 min; data not shown), thereby suggesting that R126N-CCR5 recycling to the cell surface is preserved. These results indicate that activation of G proteins is not required for endocytosis of CCR5 to occur in response to chemokines.

The Effects of Arg-126 Replacement on CCR5 Phosphorylation. We further investigated the molecular mechanisms that underneath down-modulation of R126N-CCR5. Phosphorylation of the CCR5 C terminus that promotes β-ar-

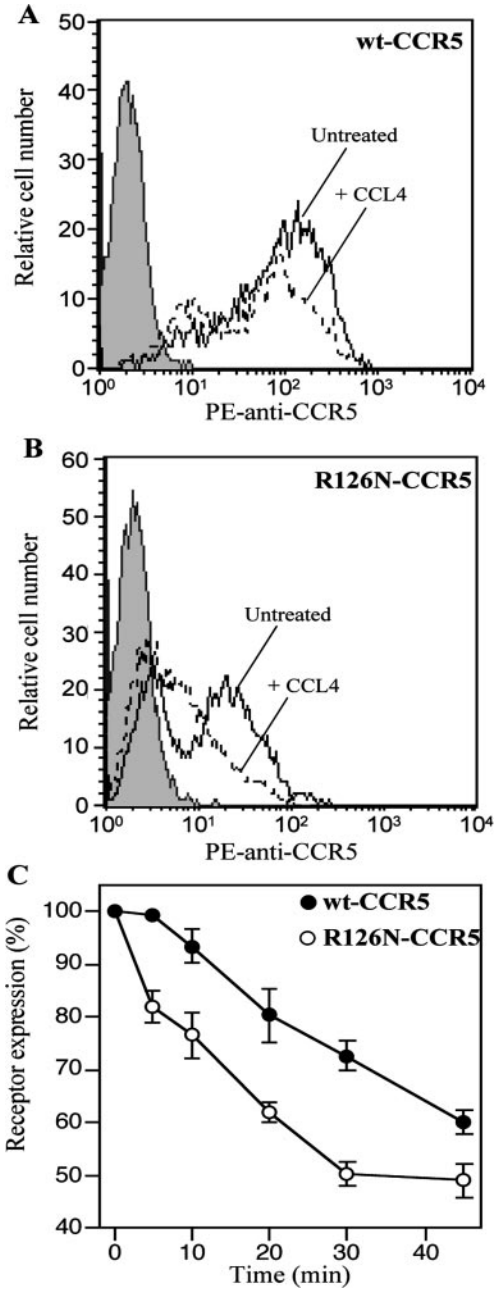

Fig. 3. wt-CCR5 and R126N-CCR5 are subjected to CCL4-induced endocytosis. Representative cell surface expressions of wt-CCR5 (A) and R126N-CCR5 (B) in CHO cells are shown, after stimulation (dotted line) or not (regular line) by 100 nM CCL4 for 45 min at 37°C. Analysis was performed by flow cytometry using the anti-CCR5 mAb PE-2D7. The filled peaks represent the signals using an isotype control mAb (PE-conjugated IgG2a). C, time-dependent endocytosis of wt-CCR5 (closed circles) and R126N-CCR5 (open circles) induced by 100 nM CCL4 at 37°C. The results, presented as the means ± S.D. of three independent determinations, indicate the amount of receptors, expressed in percentage, that remains at the cell surface after CCL4 stimulation, compared with untreated cells.

restin binding to the receptor is required for endocytosis through clathrin-coated pits (Pollok-Kopp et al., 2003). Investigation of the phosphorylation status of wt-CCR5 and R126N-CCR5 was thus carried out by metabolic labeling with [^{32}P]orthophosphate of HEK 293T cells expressing similar amounts of receptors (Fig. 1A). After incubation in the absence (i.e., basal phosphorylation) or presence of CCL4, receptors were immunoprecipitated with the 2D7 mAb. We found that both wt-CCR5 and R126N-CCR5 were subjected to phosphorylation after chemokine stimulation (Fig. 4, A and B). Under basal conditions, phosphorylation of the mutant receptor was unexpectedly increased relative to that of its wt counterpart (Fig. 4, A and B). For radioactivity to be normalized for the amount of receptors, we performed quantitative detection of immunoprecipitated receptors using the MC-5 mAb that targets a linear epitope within the N-terminal domain of CCR5 (Blanpain et al., 2002). We consistently noticed a diminished recovery of R126N-CCR5 upon immunoprecipitation (Fig. 4C, IP), whereas this receptor remained as efficiently detected in whole cell lysates (Fig. 4C, L), compared with the wt receptor. These observations are suggestive of a decreased ability of the 2D7 mAb to recognize the solubilized form of R126N-CCR5 [but not its native (i.e., unsolubilized) structure; Fig. 1A], which may indicate a conformation for R126N-CCR5 that differs from that of wt-CCR5. When normalized for the amount of immunoprecipitated receptors, the radioactivity values shown in Fig. 4D indicate that exposure of wt-CCR5-expressing cells to CCL4 resulted in a 5-fold increase of receptor phosphorylation respective to the basal level. For R126N-CCR5, CCL4 seemed less potent in increasing phosphorylation of R126N-CCR5 (1.5-fold), but the mutant receptor displayed a magnified

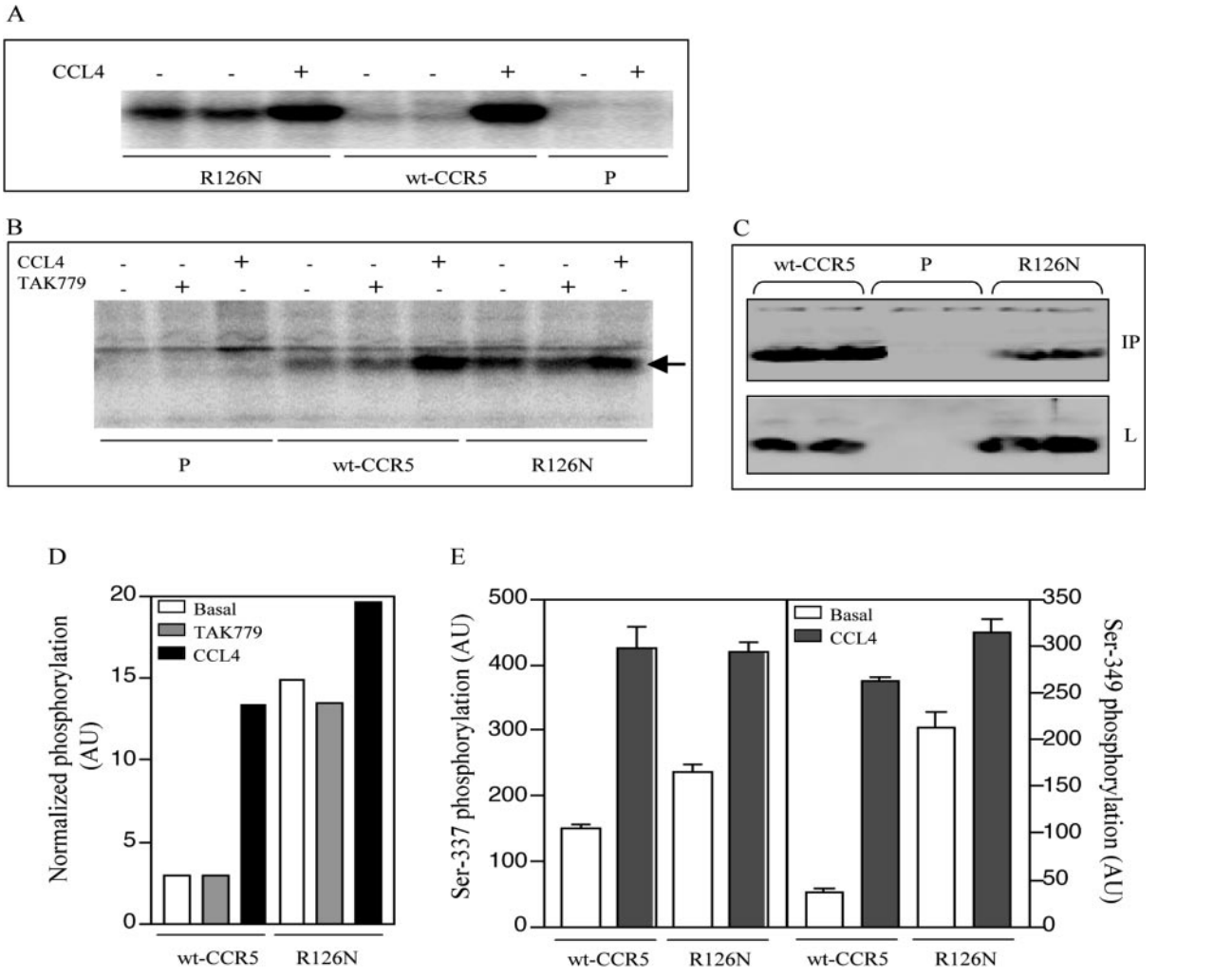

Fig. 4. wt-CCR5 and R126N-CCR5 are subjected to phosphorylation. Basal and CCL4-induced phosphorylations of wt-CCR5 and R126N-CCR5 were compared in HEK 293T cells expressing similar amounts of receptors (see Fig. 1). A, ^{32}P-Labeled cells were incubated in the absence or presence of 500 nM CCL4 for 30 min at 37°C. After solubilization of cells, 1 mg of proteins was immunoprecipitated overnight and the labeling of immunoprecipitated receptors was assessed using an Amersham Biosciences PhosphorImager. Control experiments performed with parental HEK 293T cells (P) are also shown. B, the experiment was carried out as in A. Basal phosphorylation of receptors in the presence of 1 μM TAK779 for 4 h is shown. C, immunodetections using the MC-5 mAb of precipitated wt-CCR5 and R126N-CCR5 (IP) versus those of receptors in whole cell lysates (L). D, phosphorylation amounts of receptors depicted in figure (B) are normalized for the amounts of immunoprecipitated receptors, which were determined as illustrated in C (IP). Open columns, basal; gray columns, 1 μM TAK779; closed columns, 500 nM CCL4. E, phosphorylation of wt-CCR5 and R126N-CCR5 at Ser-337 (left) and Ser-349 (right), in the absence (open columns) or presence (closed columns) of 100 nM CCL4. The histograms are derived from sandwich ELISA experiments using phospho–site-specific mAbs and calibrated with a BSA-phosphopeptide standard protein as detailed under *Materials and Methods*. An experiment representative of three independent determinations performed in triplicate (means ± S.D.) is shown. The results are expressed in AUs normalized for 1 mg of cell protein.

basal phosphorylation. In some cases, reversion of basal phosphorylation by inverse agonists has been reported (Pei et al., 1994). However, we found here that incubation of cells with the inverse agonist TAK779 consistently failed to reverse basal phosphorylation of wt-CCR5 or R126N-CCR5 (Fig. 4, B and D).

Among the four distinct serine residues that are targeted for phosphorylation within the CCR5 C terminus, we recently quantified phosphorylations of Ser-337 and Ser-349 in intact cells using an ELISA (Pollok-Kopp et al., 2003). Following this approach, we confirmed that stimulation of wt-CCR5-expressing HEK 293T cells with CCL4 caused phosphorylation of serine residues 337 and 349 (Fig. 4E, left and right, respectively). Within R126N-CCR5, both serines were also phosphorylated after CCL4 stimulation (Fig. 4E). In keeping with the experiments from metabolic labeling, we observed that basal phosphorylation of R126N-CCR5 at position 349 was enhanced compared with the wt receptor (Fig. 4E, right).

Overall, these results indicate that R126N-CCR5 retains the ability to be phosphorylated, both spontaneously and in response to chemokines. It is noteworthy that this phosphorylation targets Serine residues that are required for β-arrestin-dependent endocytosis (Pollok-Kopp et al., 2003), which highlights that mutation of Arg-126 in CCR5 has diverging effects on G protein activation and desensitization.

The Effects of β-Arrestins on Cell Surface Expression of R126N-CCR5. To assess functional interactions of β-arrestins with wt-CCR5 and R126N-CCR5, we evaluated their ability to modulate cell surface expression of receptors in CHO (Fig. 5A) and HEK 293T (Fig. 5B) cells. Cells were transfected with β-arrestin2-EGFP (βarr2) or the pN1-EGFP control vector (N1), and cell surface expression of receptors was determined in green fluorescent protein-positive-gated cells by flow cytometry analysis, in the presence (Fig. 5, closed columns) of absence (Fig. 5, open columns) of CCL4. We found that β-arrestin2 first caused the down-regulation of up to 20% wt-CCR5 at the surface of CHO cells (Fig. 5A, left; $p = 0.027$). Second, it strongly enhanced the extent of receptor down-modulation in response to CCL4 (75%; $p = 0.002$; Fig. 5A, left). Basal down-regulation of wt-CCR5 suggested that a part of the receptor spontaneously interacts with β-arrestin2 (i.e., in an agonist-independent manner), which may rely on the constitutive activity of the receptor we report in these cells. It was striking that β-arrestin2 also down-regulated R126N-CCR5, with even a higher efficiency (60%, $p < 0.001$; Fig. 5A, right). CCL4-mediated down-modulation of R126N-CCR5 was moderately modified by β-arrestin2 expression (80%, $p = 0.030$). Likewise, β-arrestin2 expression was found to promote basal down-regulation of R126N-CCR5 (30%, $p = 0.002$; Fig. 5B, right) and to enhance CCL4-dependent down-modulation (50%, $p = 0.007$) in HEK 293T cells (Fig. 5B). In these cells, wt-CCR5 was impaired in its ability to undergo agonist-promoted down-modulation as mentioned previously (Aramori et al., 1997), but β-arrestin2 partly restored this function of the receptor. Overall, these results indicate that R126N-CCR5 preserves its ability to interact with β-arrestins, either spontaneously as revealed by β-arrestin-dependent down-regulation of the receptor, or in the presence of CCL4.

***β*-Arrestins Regulate CCR5-Mediated Chemotaxis.** β-Arrestins have been implicated as playing a key role in cellular chemotaxis mediated by some receptors (Shenoy and Lefkowitz, 2003). Yet, involvement of β-arrestins in CCR5-mediated chemotaxis is still a matter of debate. Indeed, overexpression of β-arrestins augmented CCR5-mediated chemotaxis (Sun et al., 2002), but recent works suggested that CCR5 desensitization negatively regulates chemotaxis (Vroon et al., 2004). In fact, the molecular mechanisms that underlie β-arrestin-dependent chemotaxis, and especially whether this process requires G protein activation, are currently unclear.

We directly examined this possibility taking advantage of the fact that R126N-CCR5, which is unable to trigger G protein-dependent signaling, efficiently interacts with β-arrestins for endocytosis. The impact of β-arrestin2 expression on the ability of HEK 293T cells expressing wt-CCR5 or R126N-CCR5 to migrate toward CCL4 was thus assessed

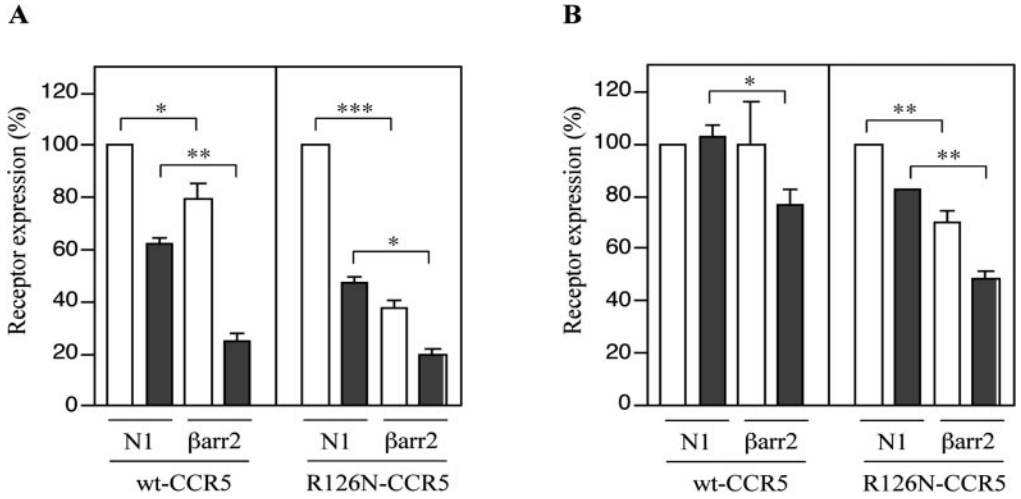

Fig. 5. Effects of β-arrestin2 on cell surface expressions of wt-CCR5 and R126N-CCR5. Expression of wt-CCR5 or R126N-CCR5 at the surface of CHO-K1 (A) and HEK 293T (B) cells transiently transfected either with pN1-EGFP (N1) or pβ-arrestin2-EGFP (βarr2), in the absence (open columns) or presence of 100 nM CCL4 for 45 min at 37°C (closed columns), was assessed by flow cytometry using the anti-CCR5 mAb PE-2D7. The results (means ± S.E.M. of three to five independent determinations) represent receptor expression at the surface of GFP-positive-gated cells, expressed as percentage of the values in GFP-positive, pN1-EGFP-transfected cells in the absence of CCL4 (100%). p values in Student's t test are *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

(Fig. 6). In wt-CCR5-expressing cells, we found that β-arrestin2 expression strongly reduced the maximal effective chemotactic concentration of CCL4 (i.e., increased CCL4 potency) from 5×10^{-1} to 10^{-1}–10^{-2} nM. In striking contrast, we observed that R126N-CCR5-expressing cells remain dramatically impaired in their ability to migrate toward CCL4, even upon expression of β-arrestin2. These results indicate that recruitment of β-arrestins to R126N-CCR5 is not sufficient, per se, to promote chemotaxis. This highlights the possibility that the structural features for CCR5-mediated activation of G proteins, albeit not needed for desensitization and internalization, are required for β-arrestin-mediated regulation of chemotaxis.

Discussion

According to the ternary complex model for receptor activation (Seifert and Wenzel-Seifert, 2003), GPCRs exist in equilibrium between an inactive conformation (R) and an active conformation (R*). In the R* state, GPCRs activate G proteins. Agonists increase the proportion of R*, whereas inverse agonists are predicted to stabilize the R state. In natural system where the receptor/G protein ratio is low (Ostrom et al., 2000), the extent of constitutive activity is often not high enough to be measured (Kenakin, 2001). However, ectopic expression of GPCRs is instrumental to attest that constitutive activity is a property shared by GPCRs (Burford et al., 2000). We report here that CCR5 is subjected to agonist-independent coupling to G_i/G_o proteins, thus highlighting that it spontaneously isomerizes from a R state to a R* state. This constitutive activity is unlikely to be a peculiarity of a cell system, because it was observed in different cell types, here (Fig. 2) and elsewhere (Chen et al., 2000). We have reported previously that residues within the second and the third TMs are critical for the conformational changes of

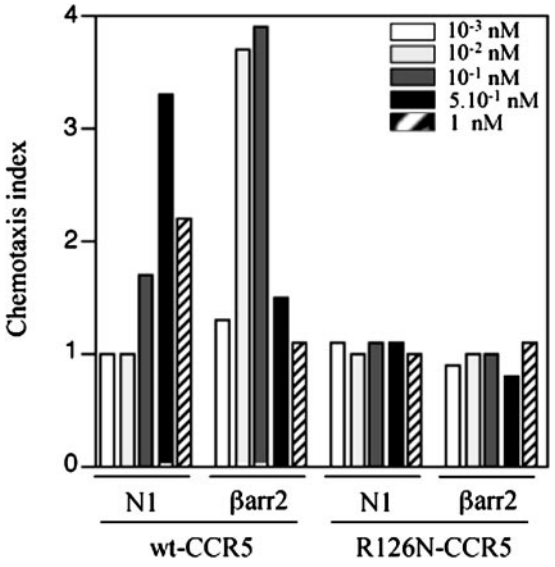

Fig. 6. Effects of β-arrestin2 on chemotaxis of wt-CCR5- or R126N-CCR5-expressing HEK 293T cells. CCL4-induced-chemotaxis of wt-CCR5- or R126N-CCR5-expressing cells, transiently transfected either with N1 or βarr2, was evaluated using a Transwell system. CCL4 was used at the indicated concentrations. The data are from a representative experiment of two and represent chemotaxis indexes of GFP-positive-gated cells. Nontransfected cells and pN1-EGFP-expressing cells respond similarly to CCL4 stimulation (data not shown). Spontaneous migrations (dotted line) were nearly equal for all cell populations.

CCR5 during activation (Govaerts et al., 2003). Because TAK779 is reported to interact with some of these residues (Dragic et al., 2000), this may explain why it behaves as an inverse agonist for CCR5. TAK779 inhibits HIV entry into cells by preventing the viral glycoprotein gp120 from binding to CCR5 (Dragic et al., 2000). However, how it acts as an antiviral molecule remains unclear, because it does not promote CCR5 internalization (data not shown; Baba et al., 1999) and binds to regions of the receptor that are distinct from those interacting with gp120 (Dragic et al., 2000). Our data demonstrating that TAK779 modifies CCR5 conformations make it likely that TAK779-induced inhibition of viral entry results from allosteric mechanisms rather than from sterically hindering gp120 binding.

It is proposed for rhodopsin-related GPCRs that activation depends on the equilibrium between the deprotonated and protonated forms of the first residue within the E/DRY motif (Scheer et al., 1997; Ballesteros et al., 1998). Thus, charge-neutralizing mutations of this residue generally result in the constitutive activation of these GPCRs (Rasmussen et al., 1999). The natural occurrence of a VRY sequence in the Kaposi's sarcoma herpesvirus-GPCR instead of the prevalent E/DRY motif is suggested to underlie the strong constitutive activity of this receptor (Burger et al., 1999). Indeed, in CXCR2 that is the closest homolog of the Kaposi's sarcoma herpesvirus-GPCR, replacement of Asp-138 by the bulky hydrophobic Val residue in the DRY motif resulted in its constitutive activation (Burger et al., 1999). In contrast, we show here that substitution of Val for Asp-125 within CCR5 does not increase constitutive activity of the receptor, and even diminishes it. In addition, it does not affect chemokine affinities and alters modestly CCL4-induced activation of G proteins. This indicates that among GPCR, the first residue of the E/DRY motif plays distinct, and somewhat opposite, roles in the equilibrium between active and inactive conformations of GPCRs.

We found that mutation of Arg-126 by the neutral residue Asn disrupted chemokine-induced G protein-dependent signaling of CCR5 and abolished constitutive activity of the receptor. Our results extend to CCR5 previous observations from other GPCRs (Ballesteros et al., 1998; Chung et al., 2002) indicating the crucial role of Arg in G protein activation. The molecular mechanisms that underlie disruption of receptor-mediated activation of G proteins after replacement of Arg are a matter of debate. It has been proposed that activation of G proteins by receptors relies in part on ionic interactions between the Arg guanidinium group and a conserved Asp residue within the α5 helix of the G protein α subunit (Oliveira et al., 1999). According to this model, replacement of the cationic Arg-126 by the neutral Asn residue would prevent CCR5 from interacting with G proteins. On the other hand, R126N-CCR5 might bind to G proteins, but it would be deficient in catalyzing GDP release, as proposed for other Arg mutants of GPCRs (Scheer et al., 2000). Finally, inability of receptors carrying mutations of Arg to activate G proteins was suggested to arise from increased agonist-independent desensitization (Barak et al., 2001; Wilbanks et al., 2002). For example, the vasopressin type II receptor Arg mutant V2R R137H is unable to couple to G proteins because of its constitutive association with β-arrestins (Barak et al., 2001). Our observations that R126N-CCR5 undergoes enhanced basal phosphorylation have been made for others Arg

mutant receptors that fail to activate G proteins (Scheer et al., 2000). We showed that R126N-CCR5 phosphorylation targets some of the canonical Ser residues within the C-terminal domain of the receptor that are needed for β-arrestin binding. Thus, it seems likely that constitutive phosphorylation of R126N-CCR5 causes this receptor to associate with β-arrestins in the absence of agonist, thereby impairing receptor to G protein coupling efficiency. Favored interactions of R126N-CCR5 with β-arrestins may also account for the receptor down-regulation we observed in cells transfected with β-arrestin2 (Fig. 5), as it is known that β-arrestin binding to phosphorylated receptors triggers them to clathrin-coated pits for endocytosis (Shenoy and Lefkowitz, 2003). Finally, these constitutive interactions with β-arrestins could also cause the low cell surface expression of R126N-CCR5 that we consistently observed in CHO cells (Fig. 3), in contrast to HEK 293T cells (Fig. 1), which were described to contain cytosolic levels of GRKs and β-arrestins not high enough to promote efficient endocytosis of CCR5 (Aramori et al., 1997). Together, our results indicate that the Arg residue of the DRY motif in CCR5, beyond its role in G protein activation, is of importance for the stability of the receptor at the plasma membrane.

Until recently, the prevailing model postulated that receptor coupling to G proteins was required for phosphorylation and internalization. It was also inferred that G protein-dependent signaling and desensitization resulted from the same agonist-induced conformation, based on the fact that constitutively active mutants of GPCRs displayed increased constitutive internalization (Pei et al., 1994) and that partial agonists led to only modest receptor phosphorylation (Benovic et al., 1988). In contrast to this model, we observed that although CCR5 constitutively interacts with G proteins, R126N-CCR5, which is impaired in its ability to activate G proteins, is more efficiently internalized in response to CCL4 (Fig. 3) and is subjected to higher constitutive phosphorylation (Fig. 4) and β-arrestin-dependent down-modulation (Fig. 5). This indicates that G protein activation is not a prerequisite for CCR5 desensitization and internalization, in accordance with works in other receptor systems, which have reported that these functions are independent processes (Vilardaga et al., 2001; Azzi et al., 2003; Wei et al., 2003; Olli-Lahdesmaki et al., 2004). Our data also strongly suggest that CCR5 assumes distinct conformational states for coupling to G proteins and β-arrestin-dependent desensitization and internalization. The differential effects that distinct ligands have on these functions of CCR5 strengthen this hypothesis. Here, we show that TAK779 abolishes the spontaneous coupling of CCR5 to G proteins (Fig. 2) but has little effect on receptor phosphorylation (Fig. 4). On the other hand, some CCR5 ligands do not alter coupling to G proteins but have effects on desensitization or internalization of the receptor. For example, RANTES (9-68), a CCR5 ligand that blocks HIV infection, is deficient in promoting G protein-dependent signaling, albeit being fully potent in internalizing CCR5 (Arenzana-Seisdedos et al., 1996). The fact that different ligands stabilize distinct conformations of the same receptor, which in turn differ in their ability to promote signaling has been reported previously. For example, CCL21 and CCL19 that bind to the chemokine receptor CCR7 activate G proteins with equal potency, but only CCL19 can trigger a receptor conformation that is subjected to desensitization and internalization (Kohout et al., 2004). Likewise, some agonists of the μ-opioid receptor that have similar effects on receptor-mediated signaling are known to substantially differ in their capacity to promote endocytosis (Keith et al., 1996). Using a panel of chemically related ligands of the β2-adrenergic receptor and fluorescence lifetime spectroscopy, Swaminath et al. (2004) have described a model for the β2-adrenergic receptor activation where agonist binding promotes a succession of conformational states with at least two distinct functions: the first is coupling to G protein and the second refers to internalization. It is interesting that β-arrestins, which bind to the activated receptor for endocytosis through clathrin-coated pits, can induce signaling in a G protein-independent manner (Azzi et al., 2003; Wei et al., 2003); this also strongly supports the notion that GPCRs share different active conformations with distinct cellular responses.

It has been shown that two regions of CCR5, namely, the C terminus and the second intracellular loop, interact with β-arrestin and that interaction with the second intracellular loop requires an intact DRY motif (Huttenrauch et al., 2002). It is somewhat intriguing that mutation of Arg-126 does not result in impaired β-arrestin-dependent endocytosis. It is therefore likely that interaction of β-arrestin with the DRY motif does not much contribute to the overall CCR5 endocytosis. However, it has been reported for other GPCRs that distinct functions of β-arrestins depend on interactions with independent sites of the receptor (Cheng et al., 2000; Stalheim et al., 2005). For the V2 vasopressin receptor and the angiotensin II receptor, phosphorylation at different sites on the receptor by different GRKs has been recently found to promote recruitment of β-arrestins with distinct functional potentials (Kim et al., 2005; Ren et al., 2005). Likewise, one can assume that β-arrestin regulates distinct functions of CCR5 depending on its interaction with the C terminus or the second intracellular loop of the receptor. We extend previous results indicating that β-arrestins modulate CCR5-mediated chemotaxis (Sun et al., 2002), and we show that replacement of Arg-126 fully disrupts this process. This suggests that chemotaxis primarily requires G protein activation and that β-arrestin recruitment to the receptor, per se, is not sufficient to promote chemotaxis. As a consequence, these data highlight the possibility that CCR5-mediated activation of G proteins, albeit not required for desensitization and internalization, is needed for β-arrestin-mediated regulation of chemotaxis. On the other hand, but not exclusively, our data are compatible with the hypothesis that β-arrestin-dependent chemotaxis, in contrast to β-arrestin-dependent endocytosis, requires interactions with an intact DRY motif. For the chemokine receptor CXCR4, β-arrestin-dependent stimulation of extracellular signal-regulated kinases also requires interactions of β-arrestins with regions of the receptor apart from the C terminus (Cheng et al., 2000). Further studies will be needed to delineate this possibility that the DRY motif of GPCRs, in addition to control G protein-dependent signaling, regulates β-arrestin-mediated scaffolding of signaling pathways.

Acknowledgments

We are grateful to Drs. F. Baleux, S. Marullo, M. Mack, and R. W. Doms for providing CCL4, the pβ-arrestin2-EGFP and pN1-EGFP plasmids, and the MC-5 and CTC5 mAbs, respectively.

References

Amara A, Gall SL, Schwartz O, Salamero J, Montes M, Loetscher P, Baggiolini M, Virelizier JL, and Arenzana-Seisdedos F (1997) HIV coreceptor downregulation as antiviral principle: SDF-1alpha-dependent internalization of the chemokine receptor CXCR4 contributes to inhibition of HIV replication. *J Exp Med* **186:**139–146.

Amara A, Vidy A, Boulla G, Mollier K, Garcia-Perez J, Alcami J, Blanpain C, Parmentier M, Virelizier JL, Charneau P, et al. (2003) G protein-dependent CCR5 signaling is not required for efficient infection of primary T lymphocytes and macrophages by R5 human immunodeficiency virus type 1 isolates. *J Virol* **77:** 2550–2558.

Arai H and Charo IF (1996) Differential regulation of G-protein-mediated signaling by chemokine receptors. *J Biol Chem* **271:**21814–21819.

Aramori I, Ferguson SS, Bieniasz PD, Zhang J, Cullen B, and Cullen MG (1997) Molecular mechanism of desensitization of the chemokine receptor CCR-5: receptor signaling and internalization are dissociable from its role as an HIV-1 coreceptor. *EMBO (Eur Mol Biol Organ) J* **16:**4606–4616.

Arenzana-Seisdedos F, Virelizier JL, Rousset D, Clark-Lewis I, Loetscher P, Moser B, and Baggiolini M (1996) HIV blocked by chemokine antagonist. *Nature* (*Lond*) **383:**40019.

Azzi M, Charest PG, Angers S, Rousseau G, Kohout T, Bouvier M, and Pineyro G (2003) beta-Arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. *Proc Natl Acad Sci USA* **100:**11406–11411.

Baba M, Nishimura O, Kanzaki N, Okamoto M, Sawada H, Iizawa Y, Shiraishi M, Aramaki Y, Okonogi K, Ogawa Y, et al. (1999) A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity. *Proc Natl Acad Sci USA* **96:**5698–5703.

Ballesteros J, Kitanovic S, Guarnieri F, Davies P, Fromme BJ, Konvicka K, Chi L, Millar RP, Davidson JS, Weinstein H, et al. (1998) Functional microdomains in G-protein-coupled receptors. The conserved arginine-cage motif in the gonadotropin-releasing hormone receptor. *J Biol Chem* **273:**10445–10453.

Barak LS, Oakley RH, Laporte SA, and Caron MG (2001) Constitutive arrestin-mediated desensitization of a human vasopressin receptor mutant associated with nephrogenic diabetes insipidus. *Proc Natl Acad Sci USA* **98:**93–98.

Benovic JL, Staniszewski C, Mayor F Jr, Caron MG, and Lefkowitz RJ (1988) β-Adrenergic receptor kinase. Activity of partial agonists for stimulation of adenylate cyclase correlates with ability to promote receptor phosphorylation. *J Biol Chem* **263:**3893–3897.

Blanpain C, Vanderwinden JM, Cihak J, Wittamer V, Le Poul E, Issafras H, Stangassinger M, Vassart G, Marullo S, Schlndorff D, et al. (2002) Multiple active states and oligomerization of CCR5 revealed by functional properties of monoclonal antibodies. *Mol Biol Cell* **13:**723–737.

Burford NT, Wang D, and Sadee W (2000) G-protein coupling of mu-opioid receptors (OP3): elevated basal signalling activity. *Biochem J* **348:**531–537.

Burger M, Burger JA, Hoch RC, Oades Z, Takamori H, and Schraufstatter IU (1999) Point mutation causing constitutive signaling of CXCR2 leads to transforming activity similar to Kaposi's sarcoma herpesvirus-G protein-coupled receptor. *J Immunol* **163:**2017–2022.

Chen G, Way J, Armour S, Watson C, Queen K, Jayawickreme CK, Chen WJ, and Kenakin T (2000) Use of constitutive G protein-coupled receptor activity for drug discovery. *Mol Pharmacol* **57:**125–134.

Cheng ZJ, Zhao J, Sun Y, Hu W, Wu YL, Cen B, Wu GX, and Pei G (2000) β-Arrestin differentially regulates the chemokine receptor CXCR4-mediated signaling and receptor internalization and this implicates multiple interaction sites between β-arrestin and CXCR4. *J Biol Chem* **275:**2479–2485.

Chung DA, Wade SM, Fowler CB, Woods DD, Abada PB, Mosberg HI, and Neubig RR (2002) Mutagenesis and peptide analysis of the DRY motif in the alpha2A adrenergic receptor: evidence for alternate mechanisms in G protein-coupled receptors. *Biochem Biophys Res Commun* **293:**1233–1241.

Dragic T, Trkola A, Thompson DA, Cormier EG, Kajumo FA, Maxwell E, Lin SW, Ying W, Smith SO, Sakmar TP, et al. (2000) A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. *Proc Natl Acad Sci USA* **97:**5639–5644.

Farrens DL, Altenbach C, Yang K, Hubbell WL, and Khorana HG (1996) Requirement of rigid-body motion of transmembrane helices for light activation of rhodopsin. *Science (Wash DC)* **274:**768–770.

Gether U, Lin S, Ghanouni P, Ballesteros JA, Weinstein H, and Kobilka BK (1997) Agonists induce conformational changes in transmembrane domains III and VI of the beta2 adrenoceptor. *EMBO (Eur Mol Biol Organ) J* **16:**6737–6747.

Govaerts C, Bondue A, Springael JY, Olivella M, Deupi X, Le Poul E, Wodak SJ, Parmentier M, Pardo L, and Blanpain C (2003) Activation of CCR5 by chemokines involves an aromatic cluster between transmembrane helices 2 and 3. *J Biol Chem* **278:**1892–1903.

Greasley PJ, Fanelli F, Rossier O, Abuin L, and Cotecchia S (2002) Mutagenesis and modelling of the α_{1b}-adrenergic receptor highlight the role of the helix 3/helix 6 interface in receptor activation. *Mol Pharmacol* **61:**1025–1032.

Huttenrauch F, Nitzki A, Lin FT, Honing S, and Oppermann M (2002) β-Arrestin binding to CC chemokine receptor 5 requires multiple C-terminal receptor phosphorylation sites and involves a conserved Asp-Arg-Tyr sequence motif. *J Biol Chem* **277:**30769–30777.

Keith DE, Murray SR, Zaki PA, Chu PC, Lissin DV, Kang L, Evans CJ, and von Zastrow M (1996) Morphine activates opioid receptors without causing their rapid internalization. *J Biol Chem* **271:**19021–19024.

Kenakin T (2001) Inverse, protean and ligand-selective agonism: matters of receptor conformation. *FASEB J* **15:**598–611.

Kim J, Ahn S, Ren XR, Whalen EJ, Reiter E, Wei H, Lefkowitz RJ, and Chen W (2005) Functional antagonism of different G protein-coupled receptor kinases for β-arrestin-mediated angiotensin II receptor signaling. *Proc Natl Acad Sci USA* **102:**1442–1447.

Kohout TA, Nicholas SL, Perry SJ, Reinhart G, Junger S, and Struthers RS (2004) Differential desensitization, receptor phosphorylation, β-arrestin recruitment and ERK1/2 activation by the two endogenous ligands for the CC chemokine receptor 7. *J Biol Chem* **279:**23214–23222.

McColl SR and Naccache PH (1997) Calcium mobilization assays. *Methods Enzymol* **288:**301–309.

Nasman J, Kukkonen JP, Ammoun S, and Akerman KE (2001) Role of G-protein availability in differential signaling by alpha 2-adrenoceptors. *Biochem Pharmacol* **62:**913–922.

Oliveira L, Paiva AC, and Vriend G (1999) A low resolution model for the interaction of G proteins with G protein-coupled receptors. *Protein Eng* **12:**1087–1095.

Olli-Lahdesmaki T, Tiger M, Vainio M, Scheinin M, and Kallio J (2004) Ligand-induced alpha2-adrenoceptor endocytosis: relationship to Gi protein activation. *Biochem Biophys Res Commun* **321:**226–233.

Ostrom RS, Post SR, and Insel PA (2000) Stoichiometry and compartmentation in G protein-coupled receptor signaling: implications for therapeutic interventions involving G_s. *J Pharmacol Exp Ther* **294:**407–412.

Pei G, Samama P, Lohse M, Wang M, Codina J, and Lefkowitz RJ (1994) A constitutively active mutant β2-adrenergic receptor is constitutively desensitized and phosphorylated. *Proc Natl Acad Sci USA* **91:**2699–2702.

Pollok-Kopp B, Schwarze K, Baradari VK, and Oppermann M (2003) Analysis of ligand-stimulated CC chemokine receptor 5 (CCR5) phosphorylation in intact cells using phosphosite-specific antibodies. *J Biol Chem* **278:**2190–2198.

Proudfoot AE (2002) Chemokine receptors: multifaceted therapeutic targets. *Nat Rev Immunol* **2:**106–115.

Rasmussen SG, Jensen AD, Liapakis G, Ghanouni P, Javitch JA, and Gether U (1999) Mutation of a highly conserved aspartic acid in the β2 adrenergic receptor: constitutive activation, structural instability and conformational rearrangement of transmembrane segment 6. *Mol Pharmacol* **56:**175–184.

Ren XR, Reiter E, Ahn S, Kim J, Chen W, and Lefkowitz RJ (2005) Different G protein-coupled receptor kinases govern G protein and {beta}-arrestin-mediated signaling of V2 vasopressin receptor. *Proc Natl Acad Sci USA* **102:**1448–1453.

Scheer A, Costa T, Fanelli F, De Benedetti PG, Mhaouty-Kodja S, Abuin L, Nenniger-Tosato M, and Cotecchia S (2000) Mutational analysis of the highly conserved arginine within the Glu/Asp-Arg-Tyr motif of the α_{1b}-adrenergic receptor: effects on receptor isomerization and activation. *Mol Pharmacol* **57:**219–231.

Scheer A, Fanelli F, Costa T, De Benedetti PG, and Cotecchia S (1997) The activation process of the alpha1B-adrenergic receptor: potential role of protonation and hydrophobicity of a highly conserved aspartate. *Proc Natl Acad Sci USA* **94:**808–813.

Scott MG, Benmerah A, Muntaner O, and Marullo S (2002) Recruitment of activated G protein-coupled receptors to pre-existing clathrin-coated pits in living cells. *J Biol Chem* **277:**3552–3559.

Seifert R and Wenzel-Seifert K (2003) The human formyl peptide receptor as model system for constitutively active G-protein-coupled receptors. *Life Sci* **73:**2263–2280.

Shenoy SK and Lefkowitz RJ (2003) Multifaceted roles of beta-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling. *Biochem J* **375:**503–515.

Stalheim L, Ding Y, Gullapalli A, Paing MM, Wolfe BL, Morris DR, and Trejo J (2005) Multiple independent functions of arrestins in the regulation of protease-activated receptor-2 signaling and trafficking. *Mol Pharmacol* **67:**78–87.

Staropoli I, Chanel C, Girard M, and Altmeyer R (2000) Processing, stability and receptor binding properties of oligomeric envelope glycoprotein from a primary HIV-1 isolate. *J Biol Chem* **275:**35137–35145.

Sun Y, Cheng Z, Ma L, and Pei G (2002) β-Arrestin2 is critically involved in CXCR4-mediated chemotaxis and this is mediated by its enhancement of p38 MAPK activation. *J Biol Chem* **277:**49212–49219.

Swaminath G, Xiang Y, Lee TW, Steenhuis J, Parnot C, and Kobilka BK (2004) Sequential binding of agonists to the β2 adrenoceptor. Kinetic evidence for intermediate conformational states. *J Biol Chem* **279:**686–691.

Vilardaga JP, Frank M, Krasel C, Dees C, Nissenson RA, and Lohse MJ (2001) Differential conformational requirements for activation of G proteins and the regulatory proteins arrestin and G protein-coupled receptor kinase in the G protein-coupled receptor for parathyroid hormone (PTH)/PTH-related protein. *J Biol Chem* **276:**33435–33443.

Vroon A, Heijnen CJ, Lombardi MS, Cobelens PM, Mayor F Jr, Caron MG, and Kavelaars A (2004) Reduced GRK2 level in T cells potentiates chemotaxis and signaling in response to CCL4. *J Leukoc Biol* **75:**901–909.

Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM, and Lefkowitz RJ (2003) Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc Natl Acad Sci USA* **100:**10782–10787.

Wilbanks AM, Laporte SA, Bohn LM, Barak LS, and Caron MG (2002) Apparent loss-of-function mutant GPCRs revealed as constitutively desensitized receptors. *Biochemistry* **41:**11981–11989.

Address correspondence to: Dr. Françoise Bachelerie, Institut Pasteur, Unité d'Immunologie Virale, 28 rue du Dr Roux, 75015 Paris, France. E-mail: fbachele@pasteur.fr.