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Site-specific Phosphorylation of CXCR4 Is Dynamically Regulated by Multiple Kinases and Results in Differential Modulation of CXCR4 Signaling*[S]

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The chemokine receptor CXCR4 is a widely expressed G protein-coupled receptor that has been implicated in a number of diseases including human immunodeficiency virus, cancer, and WHIM syndrome, with the latter two involving dysregulation of CXCR4 signaling. To better understand the role of phosphorylation in regulating CXCR4 signaling, tandem mass spectrometry and phospho-specific antibodies were used to identify sites of agonist-promoted phosphorylation. These studies demonstrated that Ser-321, Ser-324, Ser-325, Ser-330, Ser-339, and two sites between Ser-346 and Ser-352 were phosphorylated in HEK293 cells. We show that Ser-324/5 was rapidly phosphorylated by protein kinase C and G protein-coupled receptor kinase 6 (GRK6) upon CXCL12 treatment, whereas Ser-339 was specifically and rapidly phosphorylated by GRK6. Ser-330 was also phosphorylated by GRK6, albeit with slower kinetics. Similar results were observed in human astroglia cells, where endogenous CXCR4 was rapidly phosphorylated on Ser-324/5 by protein kinase C after CXCL12 treatment, whereas Ser-330 was slowly phosphorylated. Analysis of CXCR4 signaling in HEK293 cells revealed that calcium mobilization was primarily negatively regulated by GRK2, GRK6, and arrestin3, whereas GRK3, GRK6, and arrestin2 played a primary role in positively regulating ERK1/2 activation. In contrast, GRK2 appeared to play a negative role in ERK1/2 activation. Finally, we show that arrestin association with CXCR4 is primarily driven by the phosphorylation of far C-terminal residues on the receptor. These studies reveal that site-specific phosphorylation of CXCR4 is dynamically regulated by multiple kinases resulting in both positive and negative modulation of CXCR4 signaling.

CXCR4 is a widely expressed chemokine receptor that is essential for development, hematopoiesis, organogenesis, and vascularization (1). CXCR4 also plays a prominent role in a number of diseases including WHIM syndrome (2), human immunodeficiency virus-1 entry (3), and cancer progression and metastasis (4). Interestingly, WHIM syndrome is the direct result of C-terminal truncations of CXCR4 that result in enhanced receptor function (5). CXCR4 is also overexpressed in at least 23 different types of cancer (6), and accumulating evidence suggests that there is dysregulation of CXCR4 transcription, signaling, and trafficking (1).

Protein phosphorylation is the most prevalent post-translational modification and plays a major role in regulating protein function (7, 8). Importantly, phosphorylation is one of the earliest events in regulating G protein-coupled receptor (GPCR)[3] signaling, initiating a process known as desensitization (9, 10). Agonist-promoted desensitization is primarily mediated by members of the GPCR kinase (GRK) family, which specifically phosphorylates agonist-occupied GPCRs (9–11). This promotes the recruitment and high affinity binding of arrestins, which function to uncouple the receptor from G protein, target receptors for internalization, and promote G protein-independent signaling (12, 13). Studies with the prototypical GPCR, rhodopsin, have shown that the receptor is phosphorylated on multiple residues *in vitro* and *in vivo* by GRK1 (14, 15), resulting in the recruitment of visual arrestin and light adaptation (16). Subsequent work has demonstrated how alterations in these regulatory mechanisms have direct pathophysiological consequences (17). Germ line mutations in either GRK1 or visual arrestin result in a lack of receptor desensitization and the onset of Oguchi disease (18, 19). Although the specific protein kinases that mediate phosphorylation of other GPCRs have not been well defined, site-specific and tissue-specific phosphorylation of GPCRs likely have distinct effects on signaling (20).

CXCR4 is rapidly phosphorylated within its 45-amino acid serine/threonine-rich C-terminal tail upon activation. Previous studies have suggested a number of potential phosphorylation sites critical for agonist (CXCL12)- and PKC-mediated recep-

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[3] The abbreviations used are: GPCR, G protein-coupled receptor; BRET, bioluminescence resonance energy transfer; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; GRK, GPCR kinase; LC/MS/MS, liquid chromatography-tandem mass spectrometry; PBS, phosphate-buffered saline; PKC, protein kinase C; TBS-T, Tris-buffered saline with Tween; WHIM syndrome, warts, hypogammaglobulinemia, infection, and myelokathexis syndrome; Bis, bisindolylmaleimide; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; siRNA, small interfering RNA.

tor internalization (21, 22) and degradation (23). In addition, GRK2 (22, 24, 25), GRK3 (26), GRK6 (27, 28), and PKC (22, 29) have been implicated in CXCR4 regulation, although the sites of phosphorylation, the kinases involved in the phosphorylation of specific sites, and the functional role of site-specific phosphorylation remain largely unknown.

To better understand the role of phosphorylation in regulating CXCR4 function, we sought to identify agonist-promoted sites of phosphorylation and the kinases that mediate site-specific phosphorylation. Using liquid chromatography-tandem mass spectrometry (LC/MS/MS) and phospho-specific antibodies, we identified seven serine residues that are phosphorylated in response to CXCL12 stimulation. We show that phosphorylation of these sites occurs with distinct kinetics and kinase specificity; namely, Ser-324/5 phosphorylation is rapid, transient, and primarily mediated by PKC and GRK6, Ser-330 phosphorylation is delayed and is mediated by GRK6, and Ser-339 is phosphorylated rapidly by GRK6. Finally, we show that GRK-mediated phosphorylation of CXCR4 has distinct effects on arrestin recruitment and conformation leading to differential effects on calcium mobilization and ERK1/2 activation after CXCR4 activation.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—HEK293 cells (Microbix, Toronto, Canada) were maintained in complete Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 25 mM HEPES, pH 7.4. Cells stably expressing human CXCR4 were selected and maintained in complete DMEM supplemented with 0.8 mg/ml G418 and penicillin/streptomycin. HEK293 cells were plated in fresh complete DMEM 24 h before RNAi transfection. Normal human astrocytes derived from neural precursor cells were purchased from ScienCell Research Laboratories (San Diego, CA). Cells were cultured in Astrocyte Medium supplemented with fetal bovine serum (2%) and Astrocyte Growth Supplement provided by ScienCell Research Laboratories. As previously reported (30), these cells express functional CXCR4 and maintain an astroglial phenotype during the entire culture period, as assessed by glial fibrillary acidic protein staining. Cells were used for 2–3 passages and serum-starved for 24 h before experimental treatments. All siRNAs were synthesized by Dharmacon (Lafayette, CO) with the ON-TARGET plus modification. Four separate siRNAs were reconstituted and pooled at a final concentration of 15 pmol/μl. GRKs were targeted against the following sense strands: GRK2, 5′-GGGACGUGUUCCAGAAAUU-3′, 5′-GCUCGCAUCCCUUCUCGAA-3′, 5′-GGAAUCAAGUUACUGGACA-3′, 5′-GCAAUAAGUUCACACGGUU-3′; GRK3, 5′-GGAGUGUGAUGCAGAAGUA-3′, 5′-GAGGAUACCAAAGGGAUUA-3′, 5′-GGGAAGGACUGUAUUAUGC-3′, 5′-GAACACGUACAAAGUCAUU-3′; GRK5, 5′-CCAACACGGUCUUGCUGAA-3′, 5′-GGGAGAACCAUUCCACGAA-3′, CAAACCAUGUCAGCUCGAA-3′, 5′-GAUUAUGGCCACAUUAGGAUU-3′; GRK6, 5′-GGUGAAGAAUGAACGGUAC-3′, 5′-GAGCUUGGCCUACGCCUAU-3′, GCACGUAACGCAGAAUUUU-3′, 5′-CGCCAAGAUUGCUGUGGAA-3′. PKCδ was targeted against the following sense strands: 5′-CCAUGAGUUUAUCGCCACC-3′ and 5′-CAGCACAGAGCGUGGGAAA-3′. The arrestin and PKCα siRNAs have been previously described (31, 32). The PKC siRNAs were reconstituted individually at 60 pmol/μl before transfection, and 300 pmol of each were combined for a total of 600 pmol/transfection. Non-targeting siRNA pooled control modified with the ON-TARGET plus modification was used as the control in all siRNA experiments. HEK293 cells ~65–70% confluent were transfected with 600 pmol of siRNA using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen) per the manufacturer's instructions. Cells were maintained in low serum media for 4 h, at which point an equal volume of 2× complete media (20% fetal bovine serum and 25 mM HEPES) was added. Cells were split 48 h post-transfection for assay the following day.

Site-specific mutagenesis was carried out using the QuikChange site-directed mutagenesis kit (Stratagene) per the manufacturer's guidelines. All mutations were verified by sequencing.

Purification of FLAG CXCR4—Purification of FLAG-tagged CXCR4 was performed as previously described for the β_2-adrenergic receptor (33) with minor modifications. Cells grown to ~90% confluency in five 15-cm plates were washed twice with phosphate-buffered saline (PBS). Cells were then incubated in serum-free DMEM for 6 h, and the media were aspirated and replaced with fresh serum-free DMEM containing 50 nM CXCL12 for 10 min. Cells were scraped into 10 ml of hypotonic lysis buffer (20 mM Tris-HCl, pH 7.5, 2.5 mM $CaCl_2$, 10 mM NaF, and one Complete Protease inhibitor tablet (EDTA-free)) and lysed by 10 strokes in a Dounce homogenizer (tight pestle). Membranes were pelleted by centrifugation at 40,000 × *g* for 20 min at 4 °C. Pellets were resuspended in 10 ml of Buffer A (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM NaF, 1% dodecyl maltoside, 2.5 mM $CaCl_2$, and one Complete protease inhibitor tablet (EDTA-free)) and homogenized by 20 strokes in a Dounce homogenizer. Cellular debris was cleared by centrifugation at 40,000 × *g* for 20 min at 4 °C. The resulting supernatant was passed over an M1 anti-FLAG affinity column (Sigma) (0.4 ml resin/10 ml of lysate, flow rate of 5 ml/h) equilibrated in Buffer B (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% dodecyl maltoside, 2.5 mM $CaCl_2$) at room temperature. The column was washed once with 5 ml of Buffer C (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.1% dodecyl maltoside, 2.5 mM $CaCl_2$) at a rate of 10 ml/h followed by an additional wash with 5 ml of Buffer B. Bound receptor was then eluted from the column in Buffer D (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% dodecyl maltoside, 200 μM FLAG peptide, 1 μM AMD 3100 (Sigma-Aldrich) 10 mM EDTA) in 1-ml fractions and immediately snap-frozen in liquid nitrogen and stored at −80 °C. Fractions containing CXCR4 (by Western blot) were concentrated using Microcon ultracell YM-10 concentrators (Millipore Corp., Billerica, MA). Purified CXCR4 was electrophoresed on a 4–20% SDS-glycine gradient gel (Invitrogen), stained with Coomassie Blue, destained, excised, and shipped on ice to the University of Virginia Biomedical Research Facility for LC/MS/MS analysis.

LC/MS/MS Procedure—Gel pieces were transferred to siliconized tubes, washed, and destained overnight in 200 μl of 50% methanol. The gel pieces were dehydrated in acetonitrile and then rehydrated in 30 μl of 10 mM dithiothreitol in 0.1 M

ammonium bicarbonate and incubated at room temperature for 30 min. The solution was removed, and the sample was alkylated in 30 μl of 50 mM iodoacetamide in 0.1 M ammonium bicarbonate at room temperature for 30 min. The gel pieces were dehydrated in 100 μl of acetonitrile, rehydrated in 100 μl of 0.1 M ammonium bicarbonate, dehydrated in 100 μl of acetonitrile, completely dried by vacuum centrifugation, and then rehydrated in 20 μl of 50 mM ammonium bicarbonate containing 20 ng/μl Lys-C or chymotrypsin and incubated overnight at 37 °C. Peptides were extracted from the polyacrylamide in two 30-μl aliquots of 50% acetonitrile, 5% formic acid, and the extracts were combined and evaporated to 15 μl for mass spectrometry analysis.

The LC/MS system consisted of a Thermo Electron LTQ-FT mass spectrometer system with a Protana nanospray ion source interfaced to a self-packed 8 cm × 75 μm id Phenomenex Jupiter 10 μm C18 reversed-phase capillary column. Extract (1–5 μl) was injected, and peptides were eluted from the column using an acetonitrile, 0.1 M acetic acid gradient at a flow rate of 0.25 μl/min. The nanospray ion source was operated at 2.8 kV. The digest was analyzed using the double play capability of the instrument acquiring full scan mass spectra (ion cyclotron resonance; 100,000 resolution) to determine peptide molecular weights and five product ion spectra (ion trap) to determine the amino acid sequence in sequential scans. This mode of analysis produces ~1500 collision-activated dissociation spectra of ions ranging in abundance over several orders of magnitude. The data were analyzed by data base searching using the Sequest search algorithm against CXCR4. Putative phosphorylated peptides were confirmed by manual analysis.

Generation of Polyclonal Antibodies Specific for Ser(P)-324/5 and Ser(P)-330—Polyclonal antibodies specific for Ser(P)-324/5 and Ser(P)-330 were generated by Open Biosystems (Huntsville, AL). Briefly, peptides corresponding to phosphorylated CXCR4 at Ser-324/5 (C-Ahx-RGpSpSLKIL, where Ahx is amino hexonic acid, pS is phosphoserine, and C is Cys) and Ser-330 (CLKILpSKGKRGGH) were synthesized, coupled to hemocyanin, and used to immunize rabbits following a standard immunization protocol. Crude sera from each rabbit was collected at days 28, 56, and 72 after primary immunization and tested for immuno-reactivity. Antibody was purified from pooled bulk sera (days 56 and 72) from animal E5199 (Ser(P)-324/5) and E5198 (Ser(P)-330) using the immunizing peptide and concentrated to 1 mg/ml, and aliquots were stored at −80°C. Antibody specificity was evaluated by preincubating 10 μg of purified antibody with vehicle (PBS), 10 μg of the immunizing peptide, or 10 μg of the nonphosphorylated peptide.

Calcium Mobilization and ERK1/2 Activation—Calcium mobilization was performed as previously described (31). For analyzing ERK1/2 activation, cells were plated into 6-well plates 24 h before stimulation. Confluent cells were washed twice with PBS and maintained in 1 ml of serum-free DMEM for 6 h at 37 °C before stimulation. After stimulation, media was aspirated on ice, and cells were lysed by the addition of 300 μl of 2× SDS sample buffer and stored at −80 °C until processed. Lysates were thawed on ice, sonicated for 10 s (10% amplitude), and allowed to sit at room temperature for 30 min before electrophoresis. Equal volumes were separated by 10% SDS-PAGE, transferred to nitrocellulose, blocked with ODYSSEY® blocking buffer (Li-Cor® Biosciences), and blotted overnight with a mixture of anti-phospho-p42/44 (Cell Signaling Technologies, Boston, MA) and anti-ERK2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The following day blots were washed extensively with Tris-buffered saline containing 0.1% Tween 20 (TBS-T), incubated with a mixture of goat anti-rabbit Alexa® Fluorophore 680 conjugated (Molecular Probes) and goat anti-mouse IRDye 800 conjugated secondary (Rockland Immunochemicals) antibodies (1:5000) for 1 h at room temperature. Blots were washed extensively with TBS-T and visualized using the ODYSSEY® infrared imaging system (Li-Cor® Biosciences).

Detection of Ser(P)-324/5, Ser(P)-330, and Ser(P)-339—HEK293 cells stably expressing FLAG CXCR4 were stimulated and processed as described for ERK1/2 activation. An equal volume of cell lysate (FLAG CXCR4) or 20 μg of whole cell extract (astroglia) was separated by 10% SDS-PAGE, transferred to nitrocellulose, and blocked for 1 h in 0.25% gelatin. Blots were incubated overnight at 4°C with a mixture of anti-CXCR4 (BD Bioscience) and anti-Ser(P)-324/5, anti-Ser(P)-330, or anti-Ser(P)-339 primary antibodies. Blots were extensively washed with TBS-T and incubated with a mixture of goat anti-rabbit Alexa® Fluorophore 680-conjugated (Molecular Probes) and goat anti-rat IRDye 800-conjugated secondary (Rockland Immunochemicals) antibodies for 1 h at room temperature. Blots were developed as described above for phospho-ERK. Phospho-CXCR4 was then normalized to total CXCR4 and represented as percent maximum. For assays with PKC inhibition, cells were pretreated with vehicle (DMSO) or appropriate inhibitor for 30 min before stimulation with CXCL12.

Electrophoretic Mobility Shift Assay—An equal volume of cell lysate was separated by 10% SDS-PAGE for 1 h 40 min at ~135 V, transferred to nitrocellulose, and blocked for 1 h in 5% milk in TBS-T. Blots were subsequently probed for CXCR4 as described above for phospho-CXCR4.

Bioluminescence Resonance Energy Transfer (BRET) Assay—For BRET studies, HEK293T cells were maintained in DMEM supplemented with 5% fetal bovine serum, 100 units/ml penicillin and streptomycin, all from Wisent. Twenty-four hours before transfection, cells were seeded at a density of 500,000 cells per well in 6-well dishes. Transient transfections were performed using the linear polyethyleneimine (M_r 25000, Polysciences, Warrington, PA) method with a DNA:polyethyleneimine ratio of 2:7. Twenty ng of CXCR4-*R*LucII, 120 ng of GRK6SA CXCR4-*R*LucII, or 90 ng of 5Atail CXCR4-*R*LucII were co-transfected with either 500 ng of GFP2-arrestin2 or GFP2-arrestin3. The total amount of DNA transfected in each well was adjusted to 2 μg with salmon sperm DNA. After overnight incubation, transfection medium was replaced with fresh DMEM for 3 h to allow cell recovery. Transfected cells were then detached, seeded in pretreated poly-L-ornithine hydrobromide (Sigma, P3655) 96-well white plates at 100,000 cells per well, and left in culture for an additional 24 h before being processed for the BRET assay.

The expression level of the energy acceptor (GFP2)-tagged proteins was measured as total fluorescence using a FlexStationII (Molecular Device) with an excitation filter at 395 nm and an

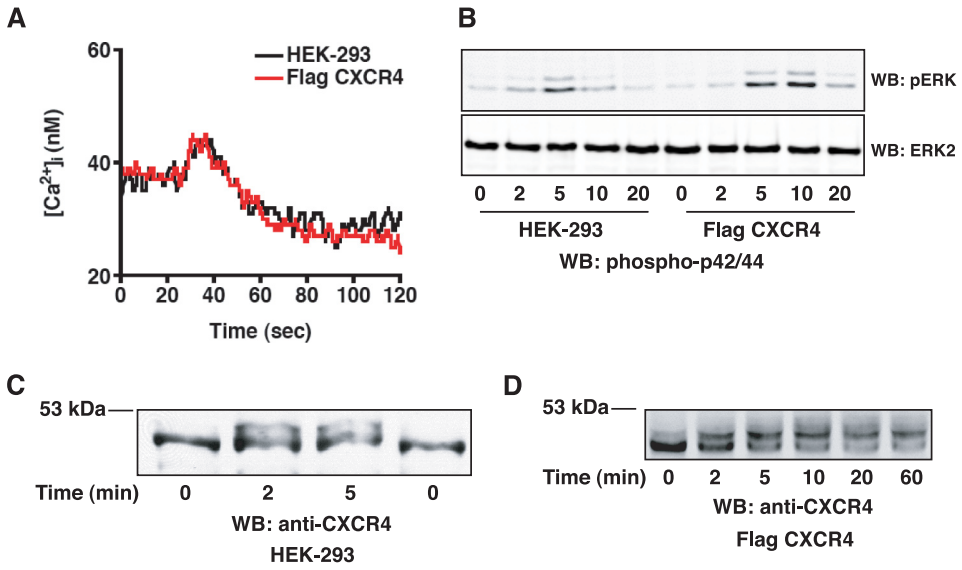

FIGURE 1. **Establishing and characterizing HEK293 cells that stably express CXCR4.** *A*, HEK293 cells or cells stably expressing FLAG CXCR4 were loaded with the ratiometric calcium indicator Fura-2/AM before stimulation with CXCL12 (100 nM). The change in intracellular calcium was calculated by monitoring the change in fluorescence of Fura-2/AM. Shown is a representative trace of calcium mobilization from three independent experiments. *B*, after a 6-h serum starvation, HEK293 cells or FLAG CXCR4 cells were stimulated with CXCL12 for the times indicated. pERK2 was normalized to total ERK2 by LiCor imaging to determine that overexpression of CXCR4 led to a 2.5-fold increase in ERK activation. Shown are representative Western blots (*WB*) of phospho-ERK1/2 and total ERK2 from three independent experiments. CXCL12-promoted retardation of electrophoretic mobility of endogenous CXCR4 (*C*) or FLAG tagged CXCR4 (*D*). After a 6-h serum starvation, cells were stimulated with 100 nM CXCL12 for the indicated times. Crude membranes were prepared, and 50 μg of solubilized protein (*endogenous*) or an equal volume of whole cell lysate (*Flag CXCR4*) was separated by 10% SDS-PAGE. Shown is a representative Western blot from four independent experiments.

emission filter at 510 nm. The expression level of the energy donor (*R*LucII)-tagged protein was measured using a Mithras LB940 multidetector plate reader (Berthold Technologies, Bad Wildbad, Germany) in the presence of 5 μM coelenterazine 400A (Biotium, Hayward, CA) after 5 min of incubation.

For BRET measurements, cells were washed once 36–48 h after transfection with PBS, and coelenterazine 400A was added to a final concentration of 5 μM in PBS 5 min before reading. Readings were then collected using a MITHRAS LB940 multidetector plate reader allowing the sequential integration of the signals detected in the 480 ± 20- and 530 ± 20-nm windows for luciferase and GFP2 light emissions, respectively. The BRET signal was determined by calculating the ratio of the light intensity emitted by the GFP2-arrestin over the light intensity emitted by the Receptor-*R*LucII. The values were corrected by subtracting the background BRET signal detected when the Receptor-*R*LucII construct was expressed alone. To assess the effects of ligand, CXCL12 was added at 100 nM just before reading.

Statistical Analysis—All data are represented as the means ± S.E. Data were analyzed using a two-tailed Student's *t* test with significance set at $p \leq 0.05$.

RESULTS

Phospho-site Mapping of CXCR4 by Mass Spectrometry—CXCR4 is rapidly phosphorylated and internalized after agonist activation (22, 29, 34). Truncation of the C-terminal tail of CXCR4, which contains 15 serine and 3 threonine residues, eliminates agonist-promoted phosphorylation, attenuates internalization, and enhances receptor activity (29, 34). Because alanine scanning mutagenesis suggested that multiple regions of the C-tail may be phosphorylated after CXCL12 stimulation (22), we used mass spectrometry to better define the specific sites phosphorylated in CXCR4. We initially made a cell line stably expressing FLAG-tagged CXCR4 to enable rapid purification of the receptor, as previously demonstrated for the β_2-adrenergic receptor (33). HEK293 cells were chosen as a model cell because they express CXCR4 endogenously, although at very low levels (~20 fmol/mg of membrane protein). A clonally selected HEK293 cell line expressing FLAG-tagged CXCR4 at ~0.5 pmol/mg (termed FLAG CXCR4 cells) was chosen for further study. CXCL12 stimulation of endogenous CXCR4 in HEK293 cells resulted in robust calcium mobilization (Fig. 1*A*) and ERK1/2 activation (Fig. 1*B*), although due to low expression of endogenous CXCR4 we were unable to detect CXCL12-mediated inhibition of cAMP production or activation of p38 or AKT (data not shown). Stable expression of CXCR4 did not enhance CXCL12-mediated calcium mobilization (Fig. 1*A*) but did lead to an ~2.5-fold increase in activation of ERK1/2 (Fig. 1*B*). In addition, as an indirect measure of receptor phosphorylation, we looked at the ability of CXCL12 to induce an electrophoretic mobility shift of CXCR4 on SDS-PAGE. Stimulation of either HEK293 cells (Fig. 1*C*) or FLAG CXCR4 cells (Fig. 1*D*) resulted in a rapid retardation of electrophoretic mobility, consistent with receptor phosphorylation. Thus, the FLAG CXCR4 cells appear to be a good model to further characterize CXCR4 phosphorylation.

Mass spectrometry has become a valuable tool for identifying amino acids that are post-translationally modified (35), a strategy recently employed for the β_2-adrenergic receptor (33). Because phosphorylation adds ~80 Da to the molecular mass of a peptide, peptides with changes of 80 Da (or multiples thereof) from the theoretical mass can be identified, trapped, and subsequently fragmented by MS/MS to provide site-specific information on phosphorylation (35). To identify sites of phosphorylation on CXCR4, FLAG CXCR4 cells were treated with CXCL12 for 10 min, and the receptor was then affinity-purified on an anti-FLAG column (Fig. 2*A*). This procedure resulted in an ~80% recovery of the receptor and yielded ~0.5 μg of purified CXCR4 per preparation (Fig. 2*B*). Duplicate samples of purified CXCR4 were then digested with either Lys-C or chymotrypsin, and the resulting peptides were subjected to LC/MS/MS. This identified peptides containing 38 of the 45 C-terminal residues of CXCR4 including residues 310–328 and

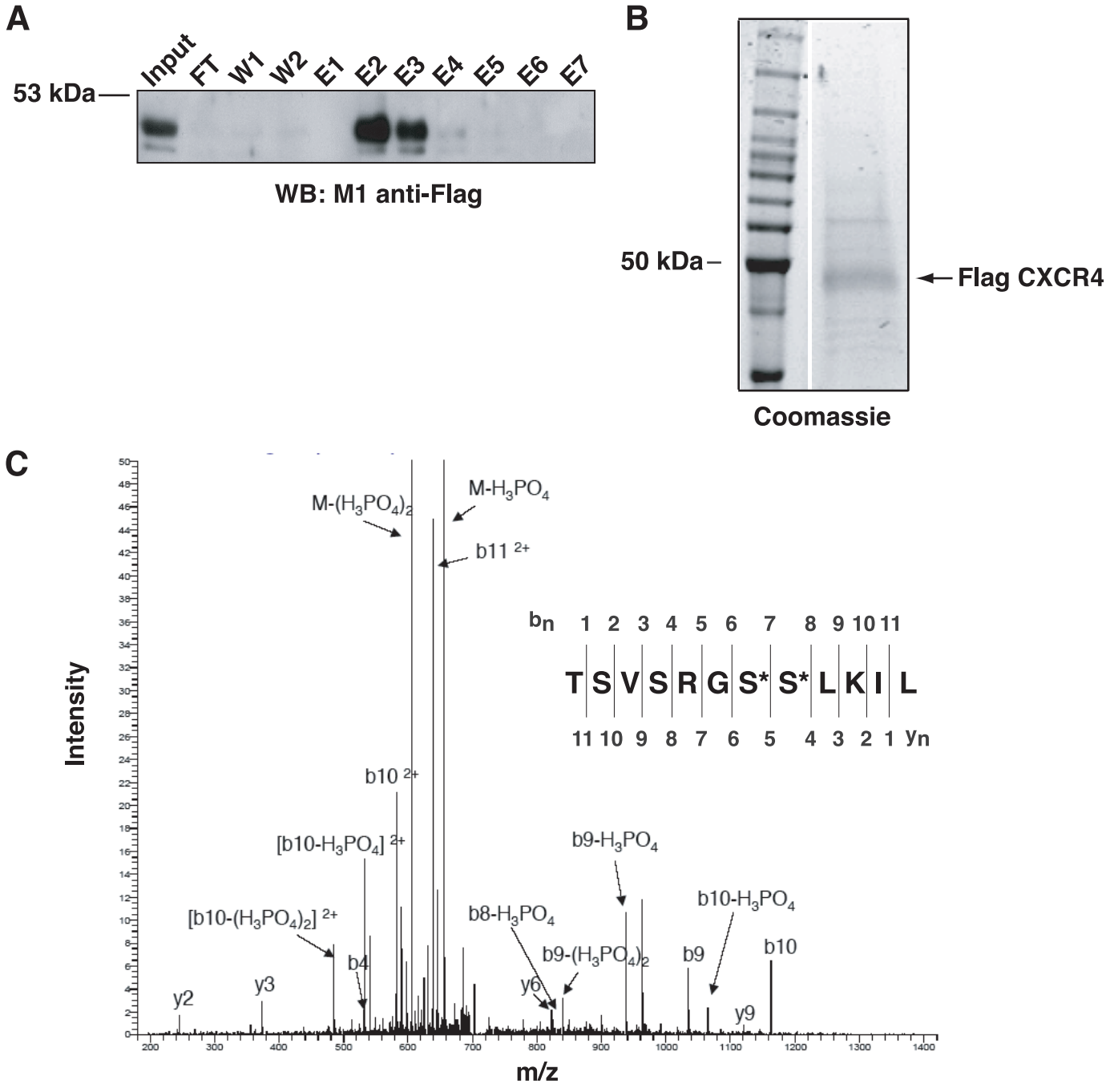

FIGURE 2. **Purification and mass spectrometry analysis of CXCR4.** *A*, FLAG CXCR4 was purified from five 15-cm plates after a 10-min stimulation with 50 nM CXCL12. The bulk of the receptor (~80%) eluted in fractions 2 and 3 and was highly purified as shown by Coomassie Blue staining (*B*). *WB*, Western blot. *FT*, flow through. *C*, shown is a representative mass spectrum of the peptide Thr-318 to Leu-328 after a chymotrypsin digest demonstrating that CXCR4 is phosphorylated on Ser-324 and Ser-325. *D*, shown is an amino acid sequence of the C-terminal tail of CXCR4. Residues highlighted in *red* are those that are predicted to be phosphorylated by mass spectrometry. *Brackets* under Ser-338-Ser-341, Ser-346-Ser-348, and Ser-351/Ser-352 indicate that one residue in each cluster is phosphorylated, although the exact residue was not identified by mass spectrometry.

334–352, and several of the peptides were phosphorylated (Table 1). Fig. 2*C* shows a representative mass spectrum of a peptide (Thr-318 to Leu-328) obtained from a chymotryptic digest that contains five potential phosphorylation sites (Thr-318, Ser-319, Ser-321, Ser-324, and Ser-325). The peak occurring with a mass ratio of ~600 demonstrates that this peptide

TABLE 1

Phosphorylated peptides derived from enzymatic digestion of purified CXCR4

pS denotes phosphorylated residues as predicted by LC/MS/MS. The brackets denote that one of the highlighted residues is phosphorylated as predicted by LC/MS/MS.

Peptide	Predicted *m/z* ratio	Observed *m/z* ratio
Lys-C digestion		
^{311}TSAQHALTSVSRGSSLK^{326}	1730	1730
^{311}TSAQHALTSVSRGS**pS**LK^{326}	1730	1810
^{311}TSAQHALTSVSRG**pSpS**LK^{326}	1730	1890
^{311}TSAQHALTSV**pS**RGSSLK^{326}	1730	1810
^{311}TSAQHALTSV**pS**RGS**p**SLK^{326}	1730	1890
^{334}RGGHSSVSTESESSSFHSS^{352}	1952	1951
^{334}RGGHSSVSTESE**SSS**FH[**SS**]^{352}	1952	2031
^{334}RGGHSSVSTESE[**SSS**]FHSS^{352}	1952	2031
^{334}RGGH[**SS**V**S**]TESESSSFHSS^{352}	1952	2031
Chymotrypsin digestion		
^{316}ALTSVSRGSSLKIL^{328}	1432	1432
^{316}ALTSVSRGS**pS**LKIL^{328}	1432	1511
^{316}ALTSVSRG**pSpS**LKIL^{328}	1432	1591
^{316}ALTSVSRGSSL^{326}	1077	1077
^{316}ALTSVSRGS**pS**L^{326}	1077	1157
^{318}TSVSRGSSLKIL^{328}	1248	1248
^{318}TSVSRG**pSpS**LKIL^{328}	1248	1408
^{310}KTSAQHALTSVSRGSSLKIL^{328}	2084	Not observed
^{310}KTSAQHALTSVSRG**pSpS**LKIL^{328}	2084	2244

has two phosphates attached (M-$(H_3PO_4)_2$). The loss of phosphates upon fragmentation was apparent when Ser-324 and Ser-325 were present in the fragment (see peaks b9-$(H_3PO_4)_2$ and [b10-$(H_3PO_4)_2$]$^{2+}$) but not in a peptide containing Thr-318, Ser-319, and Ser-321 (peak b4), demonstrating that both Ser-324 and Ser-325 are phosphorylated. These results were confirmed from two separate experiments as well as with peptides derived from Lys-C digestion (Table 1). Because peptides containing Ser-325 phosphorylation alone were also observed, we speculate that Ser-325 is phosphorylated before Ser-324, although we were unable to generate a pS325-specific antibody to directly test this. Overall, these studies identified six sites of phosphorylation after a 10-min stimulation with CXCL12: Ser-321, Ser-324, Ser-325, one residue from Ser-338-Ser-341, one residue from Ser-346-Ser-348, and either Ser-351 or Ser-352 (Fig. 2*D*). The specific sites of phosphorylation that occur from Ser-338 to Ser-352 were not able to be specifically determined by LC/MS/MS due to the serine-rich nature of this region.

Protein Kinase C Is Primarily Responsible for Phosphorylation of Ser-324/5—Previous mutagenesis studies have identified Ser-324/5 as critical for CXCL12-induced internalization (22) and degradation (23) as well as phorbol 12-myristate 13-acetate-promoted internalization (22, 29). To further characterize Ser-324/5 phosphorylation, we generated a phospho-specific antibody to evaluate the kinetics of phosphorylation and kinase specificity for these residues. A 10-min stimulation with CXCL12 resulted in robust phosphorylation of Ser-324/5 that was blocked by preincubation with the immunizing phosphopeptide but not with vehicle or unphosphorylated peptide (Fig. 3*A*). FLAG CXCR4 cells were then stimulated for various times with CXCL12, and cell lysates were electrophoresed and blotted with anti-Ser(P)-324/5 to assess the kinetics of phosphorylation. Phosphorylation of Ser-324/5 rapidly increased peaking at ~4-fold over basal within 5–10 min and returned to near basal levels within 60 min (Fig. 3*B*). Furthermore, the increase in Ser(P)-324/5 immunoreactivity paralleled the observed reduction in electrophoretic mobility of CXCR4 after stimulation with CXCL12 (Figs. 1*D* and 3*B*).

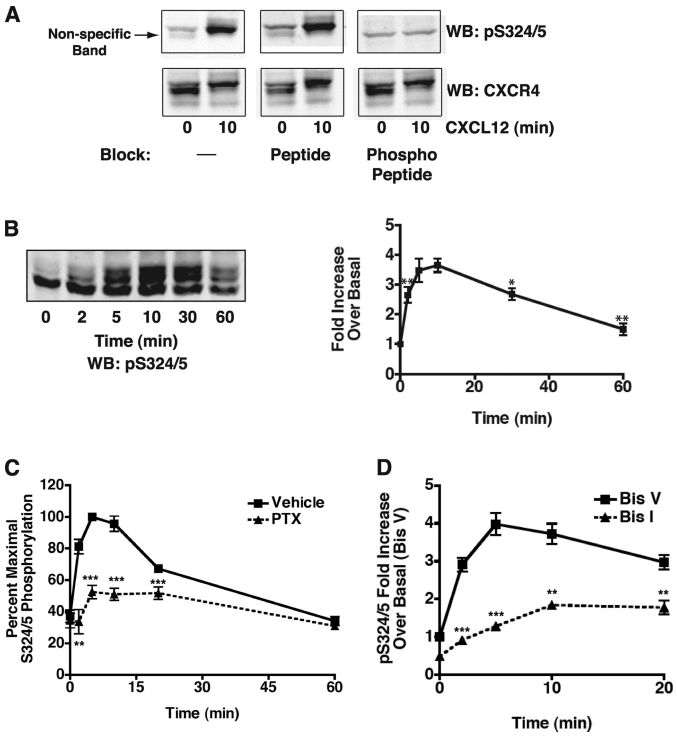

FIGURE 3. **Characterization of anti-Ser(P)-324/5 (*pS324/5*).** *A*, shown is a representative Western blot (*WB*) demonstrating the specificity of the Ser(P)-324/5 antibody. Ten μg of purified antibody was incubated for 10 min with vehicle (PBS), 10 μg of peptide (C-Ahx-RGSSLKIL), or 10 μg of phosphopeptide (C-Ahx-RG(pS)(pS)LKIL) before overnight incubation with the nitrocellulose blots. *B*, cells stably expressing FLAG CXCR4 were stimulated at the time points indicated with 100 nM CXCL12. Lysates were processed and separated to visualize the agonist promoted gel shift of CXCR4. Blots were incubated overnight with a 1:1000 dilution of crude Ser(P)-324/5 antibody. Ser(P)-324/5 was normalized to total CXCR4, and data are presented as the -fold increase over basal (±S.E., $n = 4$). The -fold increase at 10 min is significantly different from 2, 30, and 60 min ($p = 0.004$, 0.05, and 0.009, respectively) but not 5 min. *C*, cells stably expressing FLAG CXCR4 were treated overnight with vehicle (*PBS*) or pertussis toxin (*PTX*, 100 ng/ml) before stimulation with 100 nM CXCL12. Ser(P)-324/5 was normalized to total CXCR4, and data are presented as the percent maximum of vehicle-treated cells (± S.E., $n = 3$). *D*, cells stably expressing FLAG CXCR4 were serum-starved for 6 h. 30 min before CXCL12 stimulation cells were pretreated with 2.5 μM Bis I or Bis V. Ser(P)-324/5 was normalized to total CXCR4, and data are presented as the -fold increase over basal (*Bis V*) (±S.E., $n = 4$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

CXCR4, like other chemokine receptors, primarily couples to the G_i family of heterotrimeric G proteins (1). To determine whether G protein activation was involved in receptor phosphorylation, cells were pretreated with pertussis toxin before CXCL12 stimulation. Pertussis toxin pretreatment significantly attenuated both the rate and extent of Ser-324/5 phosphorylation (Fig. 3*C*). This suggests the possibility that activation of second messenger-dependent kinases such as PKC may contribute to phosphorylation of these residues. Indeed, the primary amino acid sequence of CXCR4 reveals that both Ser-324 and Ser-325 fall within a PKC consensus motif (RGSSLK). To initially address the potential role of PKC in agonist-promoted phosphorylation of Ser-324/5, cells were pretreated with the broad-spectrum PKC inhibitor bisindolylmaleimide I (Bis I) or the negative control bisindolylmaleimide V (Bis V) (36) before CXCL12 stimulation. Bis I treatment led to a significant reduc-

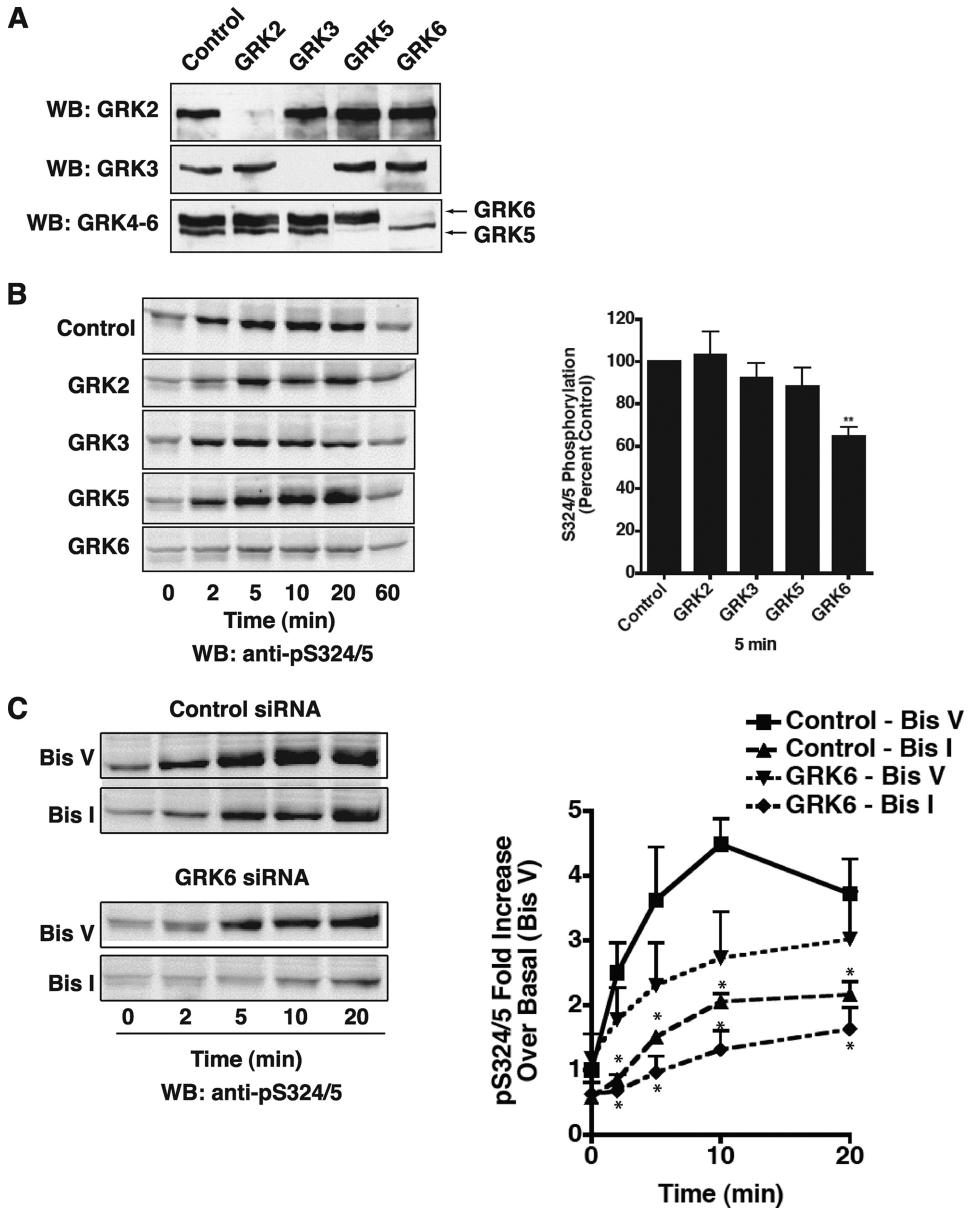

FIGURE 4. **GRK6 contributes to Ser-324/5 phosphorylation after CXCL12 stimulation.** *A*, shown is a representative Western blot (*WB*) demonstrating specific and efficient knockdown of GRKs endogenously expressed in HEK293 cells 72 h post-transfection. *B*, knockdown of GRK6, but not GRK2, GRK3, or GRK5 led to a significant reduction in phosphorylation of Ser-324/5. *Left panel*, shown is a representative Western blot using purified anti-Ser(P)-324/5. *Right panel*, shown is a comparison of Ser(P)-324/5 phosphorylation after a 5-min stimulation with CXCL12. Ser(P)-324/5 was normalized to total CXCR4, and data are presented as the percent of control at 5 min (±S.E., $n = 4$). *C*, GRK6 knockdown and PKC inhibition almost completely abolished phosphorylation of Ser-324/5. Cells transfected with GRK6 siRNA were pretreated with 2.5 μM Bis I or Bis V 30 min before stimulation with CXCL12. *Left panel*, shown is a representative Western blot using purified anti-Ser(P)-324/5. *Right panel*, Ser(P)-324/5 was normalized to total CXCR4, and data are presented as -fold increase over basal in control/Bis V-treated cells (±S.E., $n = 3$; *. $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

tion in basal phosphorylation as well as CXCL12-promoted phosphorylation of Ser-324/5 in HEK293 cells (Fig. 3*D*).

Because Bis I inhibits conventional and novel PKC isoforms, we attempted to better define the PKC subtype(s) involved in Ser-324/5 phosphorylation by pretreating cells with either Gö 6976, which inhibits the conventional PKC isoforms (α, β_I, and β_{II}) (37), or rottlerin, which is reported to inhibit PKCδ (38). Treatment with rottlerin, but not with Gö 6976, led to a significant reduction of Ser-324/5 phosphorylation (supplemental Fig. S1*A*). However, because rottlerin can have off-target effects (39), we attempted to confirm the role of PKCδ in CXCR4 phosphorylation using siRNA treatment. PKCδ levels could be effectively reduced in FLAG CXCR4 cells by siRNA treatment, and this resulted in a partial decrease in Ser-324/5 phosphorylation, although the effect was not statistically significant (supplemental Fig. S1*B*). In contrast, knockdown of PKCα had no effect on Ser-324/5 phosphorylation (supplemental Fig. S1*B*). Taken together, these data demonstrate that PKC plays a major role in phosphorylating Ser-324/5 after CXCL12 stimulation. The significant effect of Bis I pretreatment coupled with the fact that knockdown of PKCδ only partially decreased phosphorylation suggests that multiple PKC isoforms likely mediate CXCR4 phosphorylation.

GRK6 Contributes to Ser-324/5 Phosphorylation—Because some phosphorylation of Ser-324/5 was still evident after pertussis toxin treatment or PKC inhibition, we hypothesized that GRKs also contribute to phosphorylation of these residues. The GRKs consist of seven members, four of which (GRK2, -3, -5, and -6) are expressed in HEK293 cells (31, 40). Because there are no specific GRK inhibitors available, we assessed the effect of individual GRK knockdown on Ser-324/5 phosphorylation. Although efficient and specific knockdown of each of the individual GRKs expressed in HEK293 cells was achieved (Fig. 4*A*), only GRK6 knockdown had a significant effect on Ser-324/5 phosphorylation (Fig. 4*B*). Because our results suggest that both GRK6 and PKC contribute to Ser-324/5 phosphorylation, we also evaluated the effect of PKC inhibition and GRK6 knockdown. Although PKC inhibition or GRK6 knockdown alone resulted in an ~50 and ~40% reduction in phosphorylation, respectively, the combination resulted in an almost complete loss of Ser-324/5 phosphorylation (Fig. 4*C*). These data demonstrate that both PKC and GRK6 are needed for maximal agonist-promoted phosphorylation of Ser-324/5 after CXCL12 stimulation.

Ser-330 and Ser-339 Are Phosphorylated by GRK6—Previous studies have demonstrated a prominent role of Ser-330 in regulating CXCL12-promoted degradation (23), whereas Ser-339 contributes to receptor internalization (21, 22). Although a

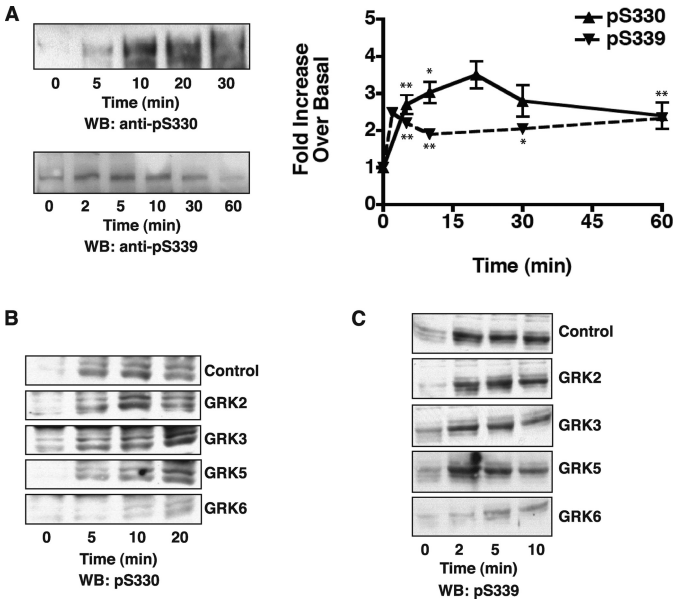

FIGURE 5. **Ser-330 and Ser-339 in CXCR4 are phosphorylated by GRK6.** *A*, cells expressing FLAG CXCR4 were serum-starved for 6 h before stimulation with 100 nM CXCL12 for the times indicated. An equal volume of lysate was separated by SDS-PAGE and blotted with purified anti-Ser(P)-330 (*top left panel*) or anti-Ser(P)-339 (*bottom left panel*). Ser(P)-330 blots were processed to visualize the gel shift of CXCR4. *Right panel*, Ser(P)-330 or Ser(P)-339 blotting was normalized to total CXCR4, and data are presented as -fold increase over basal (±S.E., $n = 4$). For Ser-330, the -fold increase at 20 min was significantly different from 5, 10, and 60 min ($p = 0.01$, 0.02, and 0.004, respectively) but not 30 min. For Ser-339, the -fold increase at 2 min is significantly different from 5, 10, and 30 min ($p = 0.01$, 0.01, and 0.02, respectively). Seventy-two hours post-transfection, an equal volume of cell lysate was separated to visualize the gel shift of CXCR4 and blotted using purified anti-Ser(P)-330 (*B*) or anti-Ser(P)-339 (*C*). Shown are representative Western blots (*WB*) of four separate experiments.

peptide containing Ser-330 was not observed in our mass spectrometry analysis, we generated and characterized a phospho-specific antibody to this site. In addition, a phospho-specific antibody has been generated against Ser-339 (41), and phosphorylation within this region was also detected by mass spectrometry (Fig. 2*D*). As shown in Fig. 5*A*, both Ser-330 and Ser-339 undergo agonist-promoted phosphorylation, albeit with different kinetics. Phosphorylation of Ser-330 was relatively slow, peaking at ~20 min, whereas phosphorylation of Ser-339 was very rapid, peaking at ~2 min (Fig. 5*A*). Interestingly, despite the kinetic differences, we found that both Ser-330 and Ser-339 were primarily phosphorylated by GRK6 (Figs. 5, *B* and *C*).

Overall, we found that GRK6 phosphorylates multiple sites including Ser-324/5, Ser-330, and Ser-339, whereas GRK2, -3, and -5 do not contribute to CXCR4 phosphorylation at these sites. Additional studies demonstrate that PKC inhibition had no effect on CXCL12-promoted phosphorylation of Ser-330 and Ser-339 in HEK293 cells (data not shown), demonstrating that phosphorylation of these residues is completely GRK6-dependent in response to CXCL12.

Protein kinase C Mediates Ser-324/5 Phosphorylation in Human Astrocytes—To further validate Ser-324/5 and Ser-330 as phosphorylation sites, we also examined human astroglia, normal primary cells that express CXCR4 endogenously (30). Ser-324/5 was found to be rapidly phosphorylated after CXCL12 treatment, and PKC inhibition effectively attenuated phosphorylation (supplemental Fig. S2*A*). Ser-330 was also phosphorylated in astroglia, albeit more slowly. Interestingly, PKC inhibition significantly enhanced the basal phosphorylation of Ser-330 and eliminated agonist-promoted phosphorylation (supplemental Fig. S2*B*). These results suggest that PKC may negatively regulate the basal phosphorylation of Ser-330, possibly by regulating the activity of another protein kinase or a phosphatase. Taken together, these studies demonstrate that the kinetics of CXCR4 phosphorylation in HEK293 and astroglia are comparable and that PKC plays a major role in the agonist-dependent phosphorylation of Ser-324/5.

GRKs Differentially Regulate CXCR4 Signaling in HEK293 Cells—Phosphorylation of GPCRs is one of the earliest mechanisms of regulation, initiating the process of desensitization (9). Recent evidence suggests that differential phosphorylation of GPCRs can have specific but disparate effects on receptor regulation (30, 40, 42, 43). Because CXCR4 activation in HEK293 cells leads to calcium mobilization (Fig. 1*A*) and activation of ERK1/2 (Fig. 1*B*), we next evaluated the functional role of GRKs in regulating CXCR4-mediated signaling. Knockdown of GRK2 or GRK6, but not GRK3 or GRK5, led to a significant increase in the peak calcium transient observed after CXCL12 stimulation of endogenous CXCR4 in HEK293 cells (Fig. 6*A*). Interestingly, knockdown of GRK2 led to an ~30% increase in ERK1/2 activation, whereas knockdown of GRK3 or GRK6 led to an ~40% reduction in ERK1/2 activation (Figs. 6, *B* and *C*). In contrast, knockdown of GRK5 (Fig. 6*C*) or inhibition of PKC by Bis I (data not shown) had no effect on activation of ERK1/2 after CXCL12 activation.

We also evaluated whether the stable overexpression of CXCR4 altered GRK-mediated regulation. Similar to endogenous CXCR4, knockdown of either GRK2 or GRK6 in the FLAG CXCR4 cells enhanced calcium mobilization, although GRK6 had a larger effect (supplemental Fig. S3*A*). Similarly, knockdown of GRK2 enhanced, whereas knockdown of GRK3 or GRK6 decreased activation of ERK1/2 (supplemental Fig. S3, *B* and *C*). Thus, stable overexpression of CXCR4 in HEK293 cells did not alter the signaling or regulation of CXCR4. Our data suggest that phosphorylation of CXCR4 by GRK6 and possibly GRK2 uncouples the receptor from activation of G_i, decreasing calcium mobilization. In contrast, GRK3- and GRK6-mediated phosphorylation of CXCR4 positively regulates activation of ERK1/2.

Arrestins Differentially Regulate Signaling after CXCR4 Activation—Agonist-dependent phosphorylation of many GPCRs leads to the recruitment of the non-visual arrestins, arrestin2 and arrestin3 (also termed β-arrestin1 and β-arrestin2, respectively). This effectively uncouples the receptor from heterotrimeric G proteins, targets the receptor for internalization, and nucleates G protein-independent signaling events (12, 13). Therefore, we next examined the effect of siRNA-mediated knockdown of arrestin2 and -3 on CXCR4 signaling in HEK293 cells. Knockdown of arrestin3 led to a significant increase, whereas arrestin2 had only a modest effect in the peak calcium transient observed after CXCL12 stimulation of endogenous (Fig. 7*A*) or overexpressed (Fig. 7*B*) CXCR4. Conversely, knockdown of arrestin2 led to a significant reduction in ERK1/2 activation after CXCL12 stimulation of endogenous (Fig. 7*C*) or overexpressed (Fig. 7*D*) CXCR4, whereas knockdown of arres-

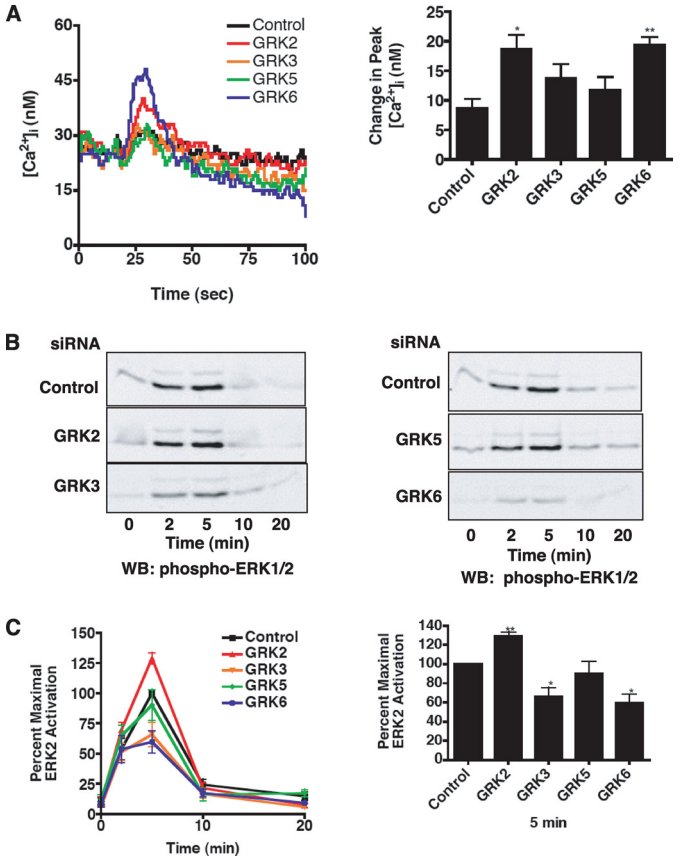

FIGURE 6. **GRKs differentially regulate signaling after activation of endogenous CXCR4 in HEK293 cells.** *A*, HEK293 cells were loaded with the ratiometric calcium indicator Fura-2A/M 72 h after siRNA transfection. Cells were stimulated with 100 nM CXCL12, and changes in intracellular calcium were calculated from changes in fluorescence. *Left panel*, shown is a representative trace from six separate experiments. *Right panel*, shown is the mean ± S.E. increase in peak calcium transient calculated from six separate experiments. *B*, shown is the effect of GRK knockdown on CXCL12-mediated activation of ERK1/2. Seventy-two hours post-transfection cells were serum-starved for 6 h before stimulation with CXCL12 (100 nM). Shown is a representative Western blot (*WB*) from five independent experiments. *C*, *left panel*, pERK2 was normalized to total ERK2, and data are presented as the percent maximal ERK2 activation as compared with control (±S.E., $n = 4$). *Right panel*, comparison of maximal ERK2 activation (5 min) after stimulation with CXCL12 (100 nM) (±S.E., $n = 4$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

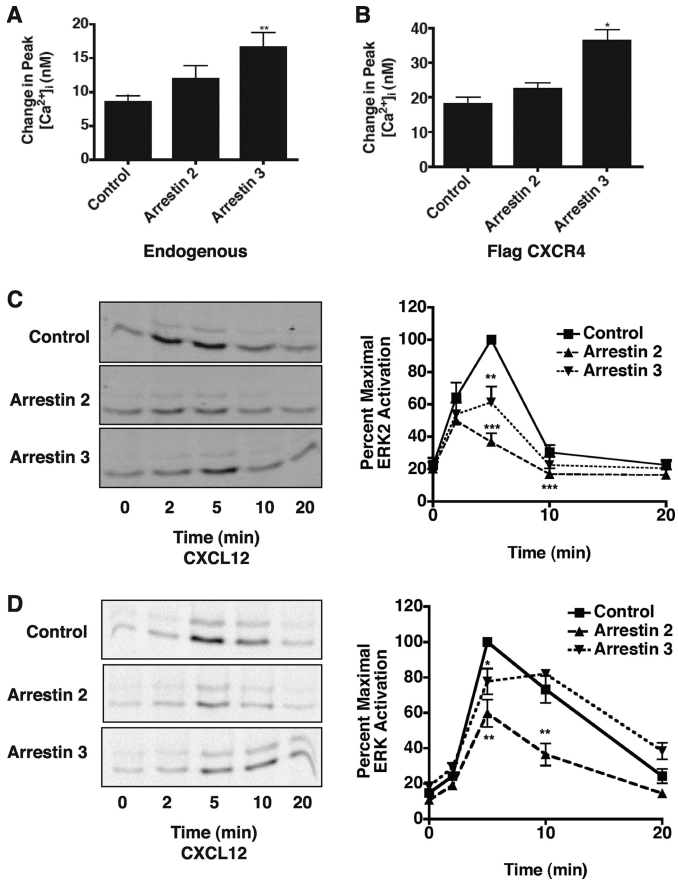

FIGURE 7. **Non-visual arrestins differentially regulate CXCR4-mediated signaling.** *A*, mean (±S.E.) increase in peak calcium transient after stimulation of endogenous CXCR4 calculated from seven separate experiments is shown. *B*, mean (±S.E.) increase in peak calcium transient after stimulation of FLAG CXCR4 calculated from three separate experiments is shown. *C* and *D*, shown is the effect of arrestin knockdown on ERK1/2 activation after activation of HEK293 (*C*) or FLAG CXCR4 (*D*) cells. Seventy-two hours post-transfection, cells were serum-starved for 6 h before stimulation with CXCL12 (100 nM). *Left panels*, shown are representative Western blots from seven (*C*) and four (*D*) separate experiments. *Right panels*, pERK2 was normalized to total ERK2, and data are presented as the percent maximal ERK2 activation as compared with control (± S.E.; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

tin3 had lesser effects. These results reveal that both arrestins contribute to regulating CXCR4 signaling, although arrestin3 appears to play the primary role in desensitization of calcium mobilization, whereas arrestin2 plays the primary role in activation of ERK1/2 signaling.

Site-specific Phosphorylation of CXCR4 Differentially Regulates Arrestin Recruitment—The observed decrease in ERK activation after GRK3 or GRK6 knockdown could arise from either the inability to form a stable CXCR4-arrestin2 complex (44, 45) or an altered conformation of arrestin2 (40, 42) that does not allow for full activation of ERK1/2. To distinguish between these possibilities and further delineate the functional role of site-specific phosphorylation of CXCR4 in arrestin binding, we performed bioluminescence resonance energy transfer studies between luciferase-tagged CXCR4 and GFP-tagged arrestin2 and -3.

CXCL12 stimulation resulted in a time-dependent increase in the BRET ratio observed between CXCR4 and arrestin2 (Fig. 8*A*) or arrestin3 (Fig. 8*B*). Interestingly, slightly different kinetics were observed for the two arrestins, arrestin3 being recruited more rapidly than arrestin2. Mutation of the last five serine residues in CXCR4 (5A Tail) to mimic loss of GRK2/3-mediated phosphorylation nearly abolished the recruitment of arrestin2 (Fig. 8*C*) and arrestin3 (Fig. 8*D*), suggesting that phosphorylation of the extreme C terminus of the receptor is needed for arrestin recruitment. In striking contrast, mutation of the GRK6 phosphorylation sites in CXCR4 (Ser-324/5, Ser-330, and Ser-339; GRK6SA) resulted in an apparent increase in the BRET ratio observed between CXCR4 and arrestin2 or 3 (Figs. 8, *C* and *D*). This suggests that phosphorylation of the GRK6 sites either inhibits arrestin binding to CXCR4 or results in a receptor/arrestin complex that adopts a conformation that is distinct from that promoted by phosphorylation of the extreme C terminus. Taken together, these results reveal that site-specific phosphorylation of CXCR4 has distinct effects on not only the recruitment of arrestins but also the conformation that these proteins adopt. These processes

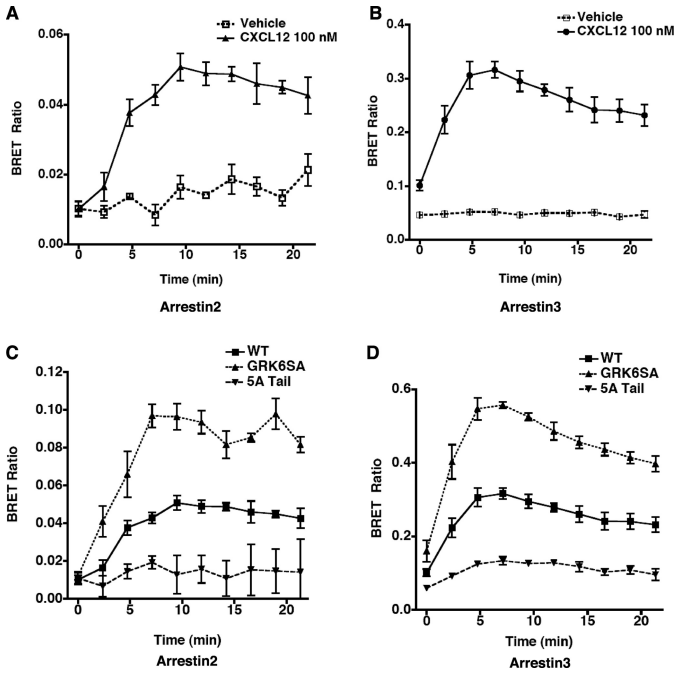

FIGURE 8. **Phosphorylation of CXCR4 differentially regulates the recruitment and conformation of the non-visual arrestins.** A BRET assay was performed to determine whether CXCR4 was able to recruit the non-visual arrestins after activation. HEK-293T cells were transiently transfected with wild type (*WT*) *R*LucII-tagged CXCR4 and either GFP-tagged arrestin2 or arrestin3. CXCL12 stimulation resulted in the recruitment of both arrestin2 (*A*) and arrestin3 (*B*). Interestingly, alanine substitution of the proposed GRK2/3 sites (5A Tail) eliminated arrestin2 recruitment (*C*) and significantly decreased arrestin3 recruitment (*D*). In contrast, alanine substitution of the identified GRK6 sites (GRK6SA) resulted in an increase in the BRET ratio observed, suggesting an altered conformation of the arrestins (*C* and *D*).

work together to dynamically modulate CXCR4-mediated signaling.

DISCUSSION

Phosphorylation and arrestin recruitment have long been appreciated as critical steps in the process of GPCR desensitization (9, 10). More recently, a newly appreciated role in initiating G protein-independent signaling pathways has emerged for both the GRKs and arrestins (46). Studies using the vasopressin and angiotensin receptors have enabled the elucidation of a model where GRK2/3 negatively regulates arrestin-dependent signaling, whereas GRK5/6 plays a positive role in signaling (40, 42). Because of these initial reports, similar phenomena have been observed for a number of GPCRs, including the β_2-adrenergic receptor and the follicle-stimulating hormone receptor (47, 48), suggesting that this model applies to many GPCRs.

Taking into consideration the large diversity of the GPCR superfamily, characterizing the mechanisms involved in receptor regulation under "normal" physiological conditions will add significantly to our understanding of receptor signaling as well as receptor dysregulation in disease. CXCR4 has emerged as a prominent GPCR due to its reported role in cancer progression and metastasis (4), and recent studies have provided evidence for the dysregulation of CXCR4 in cancer cells (49, 50). Given the wealth of knowledge on the role of CXCR4-mediated signaling in cancer (51), it is surprising that the regulation of CXCR4 function is not better understood. In this report, we characterize site-specific phosphorylation of CXCR4 and provide evidence that kinase-specific phosphorylation has distinct effects on CXCR4 signaling.

With few exceptions, the specific sites and functional role of GPCR phosphorylation are largely unknown. To determine the functional role of site-specific phosphorylation, the sites phosphorylated, and the kinases involved need to be identified. Because mutagenesis and metabolic labeling studies have suggested that multiple regions of the C-terminal tail of CXCR4 may be phosphorylated (22), we used mass spectrometry and phospho-specific antibodies to define specific sites of phosphorylation. Using these approaches we found that CXCL12 promotes the phosphorylation of seven serines in the C-terminal tail (Fig. 2*D*), although we cannot rule out the presence of additional sites. We confirmed previous studies suggesting that Ser-324 and Ser-325 are phosphorylated (21, 22) as well as one study showing that Ser-339 is phosphorylated (41) (Fig. 2, *C* and *D*). In addition, we found that Ser-321 is phosphorylated, a site that has not been previously implicated in CXCR4 regulation. Finally, we demonstrated that Ser-330 is also phosphorylated in response to CXCL12 (Fig. 5*A*).

Phospho-specific antibodies have provided a powerful tool to enable characterization of phosphorylation kinetics for a number of GPCRs (52–54). Here, we successfully used phospho-specific antibodies against Ser-324/5, Ser-330, and Ser-339 and found that phosphorylation at these sites occurs with disparate kinetics, peaking at 5–10, ~20, and ~2 min after CXCL12 stimulation, respectively. Based on these results, we hypothesize that phosphorylation at these sites would have distinct effects on desensitization, signaling, and/or trafficking after CXCR4 activation. In this regard previous studies have suggested that Ser-324/5 and Ser-330 play an important role in CXCR4 degradation (23), whereas CXCR4 recycles to the plasma membrane with varying efficiencies in different cell lines (55–58). These findings suggest cell type differences in Ser-324/5 or Ser-330 phosphorylation may regulate differential CXCR4 sorting. These phospho-specific antibodies should enable a more in-depth analysis of CXCR4 phosphorylation in various tissues and cells.

To address if site-specific phosphorylation can differentially dictate the regulation of CXCR4 signaling, we evaluated the role of site- and kinase-specific phosphorylation in CXCR4-mediated signaling. We provide novel evidence for PKC in selectively phosphorylating Ser-324/5 after CXCL12 stimulation in HEK293 cells and human astroglia (Fig. 3 and supplemental Fig. S2*A*) with no effect on Ser-330 or Ser-339 in HEK293 cells (data not shown). Previously, PKC has been thought to be primarily involved in heterologous desensitization of CXCR4, downstream of a number of receptors (1). Interestingly, Ser-324 and Ser-325 have been shown to play a prominent role in PKC-mediated internalization of CXCR4 (21, 22), although there have been conflicting reports for the role of these residues in CXCL12-mediated internalization. For example, mutation of Ser-324/5 to alanine had no effect on internalization in Mv-1-Lu cells (21) but effectively attenuated internalization in HEK293 cells (22). Because Ser-324/5 was robustly phosphorylated after CXCL12 stimulation in HEK293 cells, cell

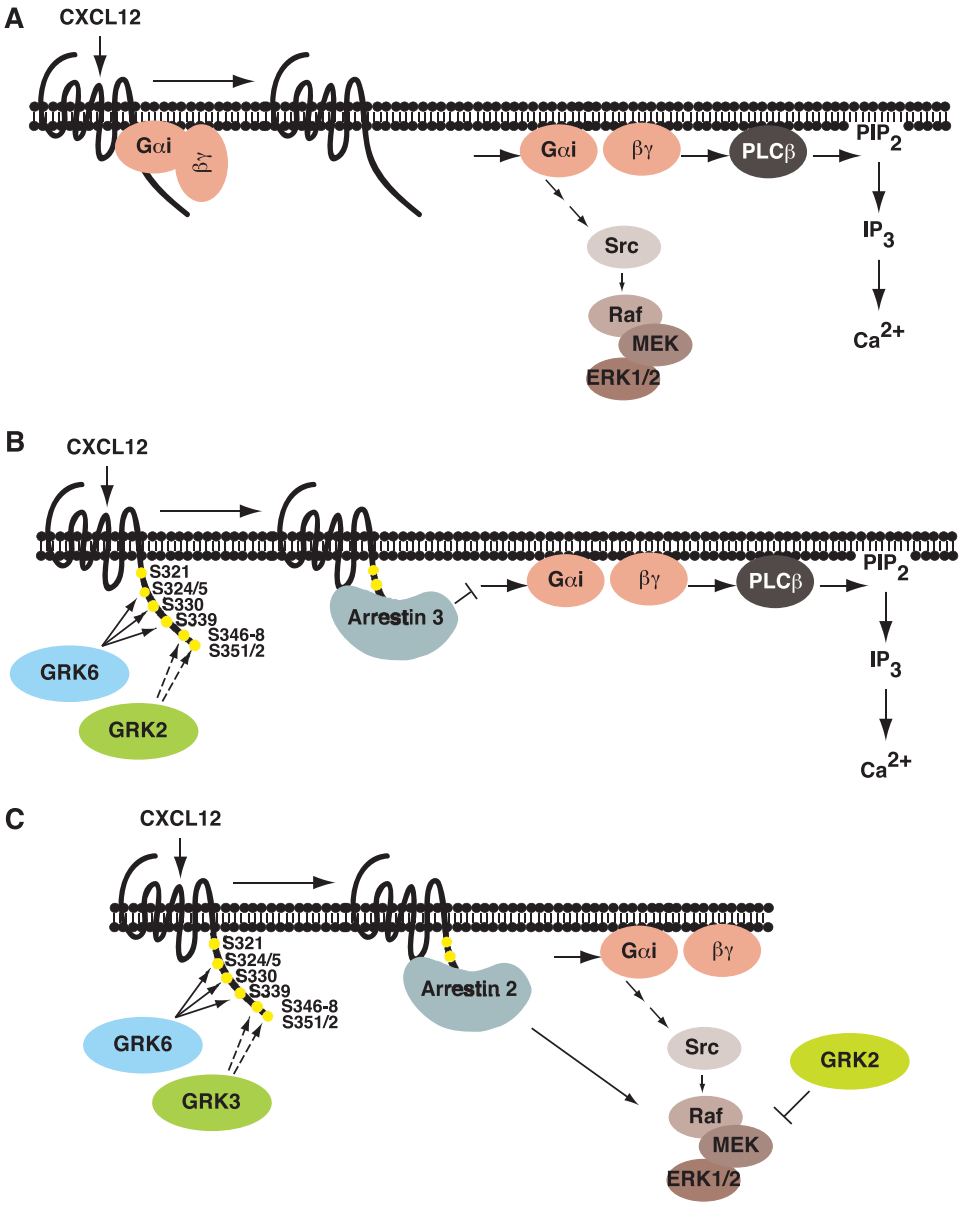

FIGURE 9. **Regulation of CXCR4 activity and signaling.** *A*, upon ligand binding, CXCR4 activates a number of signaling cascades resulting in calcium release from intracellular stores and phosphorylation of ERK1/2. *B*, phosphorylation of CXCR4 by GRK6 (Ser-324/5, Ser-330, and Ser-339) and GRK2 (residues between Ser-346 and Ser-352) resulted in the recruitment of arrestin3 to CXCR4, thereby attenuating G protein activation and calcium release. *PLCβ*, phospholipase C*β*; PIP_2, phosphatidylinositol disphosphate; IP_3, inositol trisphosphate. *C*, phosphorylation of CXCR4 by GRK6 (Ser-324/5, Ser-330, and Ser-339) and GRK3 (residues between Ser-346 and Ser-352) resulted in a conformation of arrestin2 that allows for full activation of ERK1/2. In contrast, GRK2 inhibits ERK1/2 activation most likely by regulating the activity of MEK (25).

type-dependent differences in trafficking may be attributed to differences in phosphorylation or in subsequent protein/protein interactions. Despite this robust phosphorylation, PKC inhibition had no effect on CXCR4 activation of ERK1/2 (data not shown). Based on the critical role of Ser-324/5 in receptor degradation (23), it is reasonable to hypothesize that PKC-mediated phosphorylation drives receptor degradation. In fact, phosphorylation of these residues is required for the interaction of AIP4 with CXCR4 and subsequent ubiquitination (59).

We have found that GRK6 accounts for the majority of CXCR4 phosphorylation, contributing to Ser-324/5 phosphorylation and being principally responsible for Ser-330 and Ser-339 phosphorylation (Figs. 4 and 5). Although no GRK-specific phosphorylation motifs have been identified, GRK2 and -3 prefer acidic residues N-terminal to the phosphorylation site, whereas GRK5 and -6 prefer basic residues (10). Consistent with our GRK6 results, there are basic residues located N-terminal to Ser-324/5, Ser-330, and Ser-339 (Fig. 2*D*). Previous studies have shown that the deletion of GRK6 in a mouse had marked effects on the activity of CXCR4, leading to enhanced function and a lack of desensitization (27, 28). Surprisingly, loss of GRK6 had different effects on CXCL12-mediated chemotaxis in neutrophils and T cells, suggesting that the regulation of CXCR4 function by phosphorylation may be cell type-dependent (27, 28). Although these previous studies did not evaluate signaling, our work has shown that loss of GRK6 significantly increased calcium mobilization while reducing ERK1/2 activation. Finally, mutation of the identified GRK6 phosphorylation sites to alanine resulted in an apparent increase in arrestin recruitment (Fig. 8, *C* and *D*). However, as BRET is dependent on the proximity of the donor and acceptor tags (60) and there was no change in the timing of arrestin recruitment (Fig. 8), we propose that loss of GRK6 phosphorylation results in arrestin2 and 3 adopting a conformation that is unable to effectively serve as a platform for ERK activation.

Two residues at the extreme C terminus of CXCR4 (one between Ser-346–348 and either Ser-351 or Ser-352) are also phosphorylated in response to CXCL12 stimulation (Fig. 2*D*). Based on the acidotropic nature of these serines, we postulate that these residues are phosphorylated by GRK2 and/or GRK3 (Fig. 2*D*). Although we were unable to directly test this in our system, recent studies suggest that GRK3 phosphorylates the far C-terminal region of CXCR4 (26). Using fibroblasts or leukocytes derived from patients with WHIM syndrome, Balabanian *et al.* (26) showed that only overexpression of GRK3 enhanced internalization of wild type CXCR4 but not a C-terminal-truncated CXCR4 lacking the last 15 amino acids (which contains 10 serines and threonines). By comparison, McCormick *et al.* (61) recently showed that a CXCR4 mutant lacking the last 19 amino acids is defective in GRK6 and

arrestin3 recruitment compared with WT CXCR4, whereas there was no affect on GRK3 or arrestin2 association. However, knockdown of GRK3 or GRK6 affected the trafficking of WT and mutant CXCR4 to a similar extent (61), suggesting that both kinases regulate CXCR4 function. Because previous work has demonstrated that Ser-338 is not phosphorylated in response to CXCL12 (41) and we have shown that Ser-339 is phosphorylated by GRK6 (Fig. 5*C*), the effects observed by Balabanian *et al.* (26) and McCormick *et al.* (61) could be explained by GRK3-mediated phosphorylation of the far C-terminal residues in response to CXCL12 stimulation, although none of these studies exclude a potential role for GRK2. Interestingly, knockdown of GRK3 had no effect on calcium mobilization but significantly decreased ERK1/2 activation (Fig. 6 and supplemental Fig. S3). Mutation of the last five serine residues to alanine decreased, but did not eliminate arrestin3 recruitment to CXCR4, suggesting this was sufficient for receptor desensitization. However, GRK3-mediated phosphorylation of C-terminal residues may act in concert with GRK6-mediated phosphorylation to participate in ERK1/2 activation through altering the conformation of activated arrestin (Fig. 8).

Interestingly, we found that GRK2 negatively regulates CXCR4-mediated activation of both calcium flux and ERK1/2 (Fig. 6 and supplemental Fig. S3). Previous overexpression studies showed that GRK2 enhances CXCR4 internalization (22, 24) and negatively regulates CXCL12-mediated ERK activation downstream of the receptor in HEK293 cells, possibly through interaction with MEK (25). Thus, we envision that GRK2-mediated phosphorylation likely promotes arrestin3 binding to CXCR4 and inhibits calcium signaling, whereas GRK2 inhibition of ERK may occur via interaction with MEK as previously shown (25).

The positive role of GRK3 and GRK6 on ERK1/2 activation prompted us to evaluate the role of arrestins in CXCR4 signaling. Previous studies have suggested that arrestin3 is involved in desensitization, internalization, and activation of p38 and ERK1/2 (22, 24, 27, 62), whereas arrestin2 is involved in sorting CXCR4 to the lysosomes for degradation (63). Similar to studies on the angiotensin and vasopressin receptors (37, 64), we found that arrestin2 and -3 have different effects on CXCR4 signaling. Our results suggest that arrestin3 plays a primary role in desensitizing G protein activation (*i.e.* inhibiting calcium mobilization), a pathway that appears partially dependent on GRK2 and GRK6 phosphorylation of CXCR4. Conversely, arrestin2 plays a positive role in ERK1/2 activation and appears to require phosphorylation of the receptor by both GRK3 and GRK6. This pattern of arrestin-mediated activation of ERK1/2 is somewhat unique as most GPCRs either require both arrestins (12) or arrestin3 (40, 64) for ERK activation, although there is one report that PAR1 selectively utilizes arrestin2 (65). This is the first demonstration that GRK2/3 and GRK5/6 cooperate in terms of signaling.

In summary, our results support a model where GRK2 and GRK6-mediated phosphorylation leads to recruitment of arrestin3 (Fig. 9*B*). This serves to uncouple CXCR4 from activation of G_i thereby regulating calcium release. In addition, GRK3- and GRK6-mediated phosphorylation of CXCR4 promotes interaction with arrestin2 and results in full activation of ERK1/2 (Fig. 9*C*). These results provide a foundation to better understand how CXCR4 is regulated and identify distinct regulatory molecules that can be targeted to modulate CXCR4 signaling in disease.

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