Phosphorylation-independent Association of CXCR2 with the Protein Phosphatase 2A Core Enzyme*

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Protein phosphatase 2A (PP2A) is postulated to be involved in the dephosphorylation of G protein-coupled receptors. In the present study, we demonstrate that the carboxyl terminus of CXCR2 physically interacts with the PP2A core enzyme, a dimer formed by PP2Ac and PR65, but not with the PP2Ac monomer, suggesting direct interaction of the receptor with PR65. The integrity of a sequence motif in the C terminus of CXCR2, KFRHGL, which is conserved in all CC and CXC chemokine receptors, is required for the receptor binding to the PP2A core enzyme. CXCR2 co-immunoprecipitates with the PP2A core enzyme in HEK293 cells and in human neutrophils. Overexpression of dominant negative dynamin 1 (dynamin 1 K44A) in CXCR2-expressing cells blocks the receptor association with the PP2A core enzyme, and an internalization-deficient mutant form of CXCR2 (I323A, L324A) also exhibits impaired association with the PP2A core enzyme, suggesting that the receptor internalization is required for the receptor binding to PP2A. A phosphorylation-deficient mutant of CXCR2 (331T), which has previously been shown to undergo internalization in HEK293 cells, binds to an almost equal amount of the PP2A core enzyme in comparison with the wild-type CXCR2, suggesting that the interaction of the receptor with PP2A is phosphorylation-independent. The dephosphorylation of CXCR2 is reversed by treatment of the cells with okadaic acid. Moreover, pretreatment of the cells with okadaic acid increases basal phosphorylation of CXCR2 and attenuates CXCR2-mediated calcium mobilization and chemotaxis. Taken together, these data indicate that PP2A is involved in the dephosphorylation of CXCR2. We postulate that this interaction results from direct binding of the regulatory subunit A (PR65) of PP2A to the carboxyl terminus of CXCR2 after receptor sequestration and internalization.

Chemokines comprise a family of about 50 low molecular weight proteins that mediate inflammatory responses, chemotaxis, immune cell development, and leukocyte homing. These have been classified into C, CC, CXC, and CX3C chemokines, based on the presence and the position of conserved cysteine amino acid residues (1–3). The biological functions of chemokines are mediated through interaction with their cognate receptors, which are members of the G protein-coupled receptor (GPCR) superfamily. Like other members of the GPCR superfamily, the functional status of many chemokine receptors is determined largely by the phosphorylation state (4–6). Agonist treatment enhances phosphorylation of the receptors by protein kinases, presumably G protein-coupled receptor kinases and protein kinase C, which results in desensitization of the receptors (4, 7, 8). This phenomenon is common to many hormonal and neurotransmitter signaling systems (9), but the underlying mechanisms are still only partially understood, especially in the case of chemokine receptors. Based on work on several chemokine receptors, the phosphorylated receptor is then internalized via clathrin-coated pits into early endosomes (6, 10–14) and subsequently dephosphorylated by intracellular protein phosphatases (10). The dephosphorylated receptors might be either recycled through sorting endosomes back to the plasma membrane or transported to the lysosomes for degradation. The recycling and degradation rate might vary among different chemokine receptors. For example, after down-modulation by interleukin-8 (CXCL8), the expression of CXCR1 fully recovers within 1.5 h, while the recovery rate of CXCR2 expression is very slow and never reaches 40% of the control level during a 3-h culture period (15).

Several investigations have demonstrated that neutrophil chemotactic responses require chemokine receptor internalization and recycling (16). Recycling of chemokine receptors might be very important to maintain the directional migration of cells toward a chemokine concentration gradient. Moreover, dephosphorylation of the receptors appears to play a key role in the recycling and resensitization of chemokine receptors. Therefore, regulation of the receptor dephosphorylation by protein phosphatases represents an important mechanism for modulating the function of chemokine receptors.

Four major classes of serine/threonine-specific protein phosphatases (PPs) have been described. These include PP1, PP2A, PP2B (calcineurin), and PP2C. PP2B and PP2C are calcium-dependent, whereas PP1 and PP2A are not. PP1 and PP2A are widely expressed in the cytoplasm of mammalian cells and have been reported to be involved in signal transduction, proliferation, and metabolic events (17). Studies on the β2-adrenergic receptor (β2-AR) suggest that PP2A is involved in the dephosphorylation of the receptor. Pitche et al. (18) have identified a plasma and vesicular membrane-associated phosphatase that dephosphorylates the β2-AR phosphorylated by G

1 The abbreviations used are: GPCR, G protein-coupled receptor; β2-AR, β2-adrenergic receptor; FF, protein phosphatase, HEK293 cells, human embryonic kidney 293 cells; GST, glutathione S-transferase; DMEM, Dulbecco's modified Eagle's medium; OA, okadaic acid; PAGE, polyacrylamide gel electrophoresis; RIPA buffer, radioimmune precipitation buffer; PCR, polymerase chain reaction.
protein-coupled receptor kinases. This phosphatase, referred to as G protein-coupled receptor phosphatase, is a subclass of protein-coupled receptor kinases. Chemokine receptors can interact with the phosphatase but also prevent receptor dephosphorylation. In addition, PP2A has been indicated to be the chief enzyme acting on the cholecystokinin receptor dephosphorylation. In addition, PP2A has been characterized to be the chief enzyme acting on the cholecystokinin receptor dephosphorylation.

**Experimental Procedures**

**Plasmid Construction**—Wild-type and truncated mutant (331T) of CXCR2 were constructed previously (4). For the construction of PAS2/CXCR2 tail for the two hybrid screen, the CXCR2 carboxy-terminal domain was cut from PR/CMV-CXCR2 with NcoI and HindIII, blunt-ended with T4 DNA polymerase, and inserted into the PAS2 vector that had been digested with NcoI and blunted with T4 DNA polymerase. The correct orientation and in-frame fusion of the insert were determined by DNA sequencing. Constructs for glutathione S-transferase (GST) fusion proteins of the C-terminal tails of wild-type or mutant CXCR2 were generated using PCR-amplified fragments. A BamHI site was included in the 5’ primer, and a HindIII site was included in the 3’ primer. pGEX-KG and the PCR-generated fragments were cut with BamHI and HindIII, ligated, and used to transformation Escherichia coli DH5α.

**Cell Culture and Transfection**—Human embryonic kidney 293 (HEK293) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM), containing 10% fetal bovine serum and 1:100 dilution of penicillin/streptomycin (BioWhittaker), at 37 °C in a humidified atmosphere of 95% air and 5% CO2. Cells were transfected with wild-type or mutant CXCR2 using LipofectAMINE plus reagent (Life Technologies, Inc.) Stably transfected cells were selected with 560 μg/ml Genetin (G418) and evaluated for receptor expression using 125I-labeled mela-nochrome growth-stimulatory activity/growth-related protein (125I-1-CXL1) binding (PerkinElmer Life Sciences, catalog no. NEX-321).

**Yeast Two-hybrid Assay**—Yeast two-hybrid techniques were performed as described (23, 24). For Screening cDNA libraries, the bait plasmid PAS2/CXCR2 tail was transformed into yeast strain Y190 (CLONTECH) using a lithium acetate protocol (CLONTECH manual). After confirming expression of the bait protein, a human B lymphocyte library in the vector PACT2 was transformed into the strain harboring the bait plasmid. The transformants expressing both the bait and the prey proteins were selected on medium lacking leucine, tryptophan, and histidine (SD/-Leu/-Try/-His). Colonies capable of growing on the SD/-Leu/-Try/-His medium were then tested for interaction (LacZ+) using the filter lift assay. Clones that were consistently phenotypically His+ and LacZ− were further characterized. Approximately 2.6 × 106 transformants were screened, and several of them were His+ and LacZ−. A single clone was chosen for further pursuit based on its strong His+/LacZ− phenotype.

**Filter Lift Assay**—A dry nitrocellulose filter was placed on the yeast colonies. The filter was then carefully lifted, transferred (colonies facing up) into liquid nitrogen, completely submersed for 10 s, and then allowed to thaw at room temperature. The filter was then placed on a Whatman filter paper soaked in Z buffer (60 mM NaH2PO4, 40 mM Na2HPO4, 10 mM KCl, 1 mM MgSO4, pH 7.0) containing 50 mg/ml chloroform and 3-indolylgalactopyranoside, with colonies face up until the color appeared.

**In Vitro Binding Assay**—Bacteria encoding GST or GST fusion proteins were cultured overnight at 37 °C, and then isopropyl-1-thio-β-D-galactopyranoside was added, and incubation was continued for another 3 h to induce protein expression. The bacteria were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM NaF, 1 mM Na3VO4, 10 μg/ml phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin and aprotinin) and then sonicated on ice for 10 s. The supernatant of the bacterial lysate was incubated with glutathione-Sepharose at 4 °C for 30 min. After washing three times with RIPA buffer, the beads were resuspended in RIPA buffer. Purified PP2A subunits (a generous gift from Dr. Brian Wadzinski or HEK293 cell lysates were incubated with the GST or GST fusion proteins bound to glutathione-Sepharose at 4 °C for 30 min with 4 °C with rotation. Beads were pelleted by centrifugation (12,000 rpm) for 2 min and washed four times with RIPA buffer. Bound proteins were released by boiling in SDS-PAGE sample buffer for 5 min and detected by SDS-PAGE and Western blot.

**Co-immunoprecipitation and Western Blot**—Human neutrophils were isolated from fresh heparinized peripheral blood from single human donors as described previously (25). HEK293 cell stable expressing CXCR2 were serum-starved overnight in DMEM containing 5% fetal bovine serum before the experiment. The cells were treated with or without agonists, and then the cells were washed three times with ice-cold PBS and lysed in 1 ml of RIPA buffer. The cell debris was removed by centrifugation for 4 min at 13,000 rpm in an Eppendorf microcentrifuge. The supernatant was precleared for 1 h to reduce nonspecific binding by the addition of 40 μl of protein A-agarose (Pierce). After removal of the protein A-agarose by centrifugation in an Eppendorf microcentrifuge at 3000 rpm for 1 min, the cleared supernatant was collected, and 10 μl of antibody-purified anti-CXCR2 antibody (prepared in our laboratory) was added for overnight precipitation at 4 °C. 40 μl of protein A/agarose was then added and incubated at 4 °C for 2 h. The protein A/agarose-antibody-complex was then collected by washing three times with ice-cold RIPA buffer. The final pellet was resuspended in 50 μl of SDS sample buffer containing 5% β-mercaptoethanol and heated to 50 °C for 10 min. 20 μl of this preparation was electrophoresed on a 10% SDS-polyacrylamide gel, and the proteins on the gel were transferred to nitrocellulose membranes (Bio-Rad) as previously described (16). Membranes were probed with a goat polyclonal antibody (catalog no. SC6112; Santa Cruz Bio-technology, Inc., Santa Cruz, CA) and a mouse monoclonal antibody (catalog no. P4772; Transduction Laboratories, respectively).

**In Vitro Phosphorylation and Dephosphorylation**—CXCR2 was immunoprecipitated with a rabbit anti-CXCR2 antibody and protein A/agarose beads as described above. The immunoprecipitates were then washed four times with kinase buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 500 μM CaCl2. The immunoprecipitates were incubated with 100 μg/ml phosphatidylserine, 2.5 μg/ml of [32P]ATP, and 50 pmol of purified protein kinase C (Pierce) at 30 °C for 30 min in kinase assay buffer in a final volume of 20 μl. 1 unit of protein kinase C activity is defined as the amount of enzyme required to transfer 1 pmol of phosphate from ATP to histone H1 per min at 30 °C. The immunoprecipitates were then washed with phosphate assay buffer containing 5 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 0.1% 2-mercaptoethanol, and 1 mg/ml bovine serum albumin. Dephosphorylation was carried out by incubation of the immunoprecipitates with different concentrations of purified PP2A core enzyme in the phosphate assay buffer in a volume of 30 μl at 30 °C for 30 min. The reaction was terminated by adding Laemmli sample buffer and heating at 50 °C for 10 min. Samples were subjected to 10% SDS-PAGE, and phosphorylated CXCR2 was detected by autoradiography.

**In Vivo Phosphorylation and Dephosphorylation**—Receptor phosphorylation assay was performed as described previously (10). In brief, the transfected cells were replated on six-well plates 1 day after the transfection. On the following day, after incubating in serum- and phosphate-free media for 1 h, cells were stimulated for 4 h by adding phosphatase (100 μCi/ml) (PerkinElmer Life Sciences) in the same medium at 37 °C for 2 h. Cells were then stimulated with or without agonists. Dephosphorylation was performed by allowing cells to recover in fresh serum-free media at 37 °C for 1 h. The cells were then lysed in RIPA buffer. CXCR2 was immunoprecipitated as described above with a specific antibody. The immunoprecipitates were electrophoresed.
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FIG. 1. The carboxyl-terminal sequence of CC and CXC chemokine receptors and mutagenesis of the CXCR2 carboxyl tail. A, schematic of the carboxyl terminus of CXCR2 and mutations. The indicated amino acid residues were changed to alanine (underlined). B, alignment of the carboxyl-terminal amino acid sequence of CC and CXC chemokine receptors. Conserved residues potentially involved in the binding to PP2A are boxed.

RESULTS

In an attempt to isolate chemokine receptor-associated proteins, we used the yeast two-hybrid system to identify proteins that interact with the carboxyl terminus of the chemokine receptor, CXCR2 (Fig. 1A). Screening of a human B lymphocyte library fused to the GAL4 transactivation domain (generous gift from Dr. Stephen J. Elledge) yielded several potential candidate genes that were both His+ and LacZ+. The prey cDNAs were recovered from yeast and transformed into bacteria. The cDNAs were then sequenced using primers complementary to 5’ or 3’ ends of the inserts. Among them, one (clone 91) encoding PR65 was chosen for further study based on its moderately strong LacZ+. The specificity of the interaction in yeast was tested by retransforming PACT2/clone 91 along with the original bait PAS2/CXCR2 tail or PAS2 alone back into yeast strain Y190. The interaction between the receptor C terminus and PR65 specifically allowed growth on SD medium lacking leucine, tryptophan, and histidine (SD/−Leu−Trp−His) (Fig. 2A, left panel). Neither the bait protein, PAS2/CXCR2 tail, nor the prey was able to activate transcription of the reporter genes in the presence of only empty prey or bait vectors, respectively (Fig. 2A, left panel). Using the β-galactosidase assay, we found that only the yeast co-transformed with PACT2/clone 91 and the PAS2/CXCR2 tail displayed LacZ+ (Fig. 2A, right panel).

To confirm the specific biochemical interaction between the C terminus of CXCR2 and PR65, we used an in vitro binding assay to test for direct interaction. A GST fusion protein containing the C terminus of CXCR2 was made (Fig. 1A) and tested for binding to the purified PP2Ac monomer or PP2Ac/PR65 dimer. We observed that the GST-CXCR2 tail fusion protein specifically bound the purified PP2Ac/PR65 dimer but not the PP2Ac monomer (Fig. 2B), suggesting that the C terminus of CXCR2 only binds to PR65. Several GST fusion proteins show a consistent pattern of lower molecular weight bands in the purified sample. These are believed to be either unstable degradation products or incompletely translated products.

through a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad). The phosphorylated receptors were then detected by autoradiography. The amount of receptor immunoprecipitated was determined by blotting the membrane with a monoclonal antibody against CXCR2 to evaluate relative receptor phosphorylation.

Chemotaxis Assay—A 96-well chemotaxis chamber (Neuroprobe Inc.) was used for chemotaxis assays, and the lower compartment of the chamber was loaded with 400-μl aliquots of 1 mg/ml ovalbumin/DMEM (chemotaxis buffer) or CXCL8 diluted in the chemotaxis buffer (1–200 ng/ml). Polycarbonate membranes (10-μm pore size) were coated on both sides with 20 μg/ml human collagen type IV, incubated for 2 h at 37 °C, and then stored at 4 °C overnight. To prepare cells for chemotaxis assay, they were removed by trypsinization, washed, fixed, and stained with a Diff-Quik kit. Cell chemotaxis was quantified by counting the number of migrating cells present in 10 microscope fields (× 20 objective).

Calcium Fluorimetry—HEK293 cells stably expressing CXCR2 were grown until confluent. Cells were released by shaking, collected by centrifugation at 300 × g for 6 min, and washed with Hanks’ buffer containing 5 mM HEPES. Cells were resuspended at 2 × 106 cells/ml and incubated with 2.5 μM Fluo-3 (Molecular Probes, Inc., Eugene, OR) for 30 min at 37 °C. After incubation, the cells were washed once with Hanks’ buffer containing 5 mM HEPES and 2 mM Ca2+. The cells were finally adjusted to 2 × 106 cells/ml. Ca2+ mobilization experiments were performed as described previously (26).
We next identified the region of CXCR2 involved in the PP2A binding by using GST fusion proteins encoding various fragments of the C terminus of CXCR2 (Fig. 1A). We found that the GST-CXCR2-(311–330) bound to an equal amount of the PP2A core enzyme as compared with GST-CXCR2 tail, whereas the GST-CXCR2-(331–355) did not bind to the PP2A core enzyme, implying that the minimal CXCR2 binding region resides in residues 311–330 of the C-terminal domain of CXCR2 (Fig. 3A). Interestingly, this domain is immediately upstream of the phosphorylation sites, suggesting that PP2A does not bind to the C-terminal phosphorylation sites of the receptor.

To identify which residues are required for the binding of the PP2A core enzyme, in vitro binding assays using GST fusion proteins with group or single mutations in the C terminus of CXCR2 were performed (Fig. 1A). We found that the GST-CXCR2 carboxyl-terminal mutants GQK313–315A, FRH316–318A, and GLL319–321A bound less PP2A core enzyme than the wild-type GST-CXCR2 carboxyl terminus fusion protein (Fig. 3B). A GST pull-down assay using single mutants revealed that residues Lys315, Arg317, His318, and Leu320 were important for the receptor binding to the PP2A core enzyme (Fig. 3C). These data indicate that the integrity of the sequence motif, KFRHGL, is required for the interaction of CXCR2 with PP2A. This motif is conserved in all CC and CXC chemokine receptors (Fig. 1B) and in some other GPCRs (not shown).

We next examined whether a functional complex consisting of CXCR2 and the PR65/PP2Ac dimer could be detected in HEK293 cells overexpressing the receptors using an immunoprecipitation assay. Immunoprecipitation of CXCR2 from HEK293 cells revealed a weak basal association of the receptors with the PP2A core enzyme, and CXCL1 (100 ng/ml) treatment time-dependently increased the association between the immunoreactive PP2A core enzyme and the receptor, which peaked at 10 min (Fig. 4, A and B). We also tested the interaction of CXCR2 with PP2A in human neutrophils. Treatment of the cells with CXCL8 (100 ng/ml) for 10 min significantly increased the association of CXCR2 with the PP2A core enzyme (Fig. 4C).

Our previous study has shown that inhibition of CXCR2 internalization blocks the receptor dephosphorylation (10), suggesting that receptor internalization is required for the association of the receptors with PP2A. The internalization of CXCR2 and other chemokine receptors can be blocked by mutation of the carboxyl-terminal dileucine motifs (26) or cotransfection of dominant negative dynamin 1 (dynamin 1 K44A) (10, 27). As shown in Fig. 5, A and B, mutation of Ile323Leu324 greatly impaired the receptor binding to the PP2A core enzyme. Overexpression of dynamin 1 K44A in HEK293 cells stably transfected with CXCR2 also significantly decreased the interaction of the receptors with the PP2A core enzyme (Fig. 5, C and D). These data indicate that the receptor internalization is required for the agonist-dependent association of the receptors with PP2A.

To test whether agonist-induced phosphorylation is required for the association of the receptors with the PP2A core enzyme, the carboxyl-terminal truncated mutant of CXCR2 (331T) was used to co-immunoprecipitate with the PP2A core enzyme. Because 331T, which no longer undergoes agonist-induced phosphorylation, still transduces downstream signaling and undergoes agonist-induced internalization in HEK293 cells (26), it can be used as a model to investigate the potential role of phosphorylation in the association of the receptor with PP2A. Interestingly, compared with wild-type CXCR2, 331T coimmunoprecipitated an almost equal amount of the PP2A core enzyme in response to agonist treatment (Fig. 6, A and B). These data support the hypothesis that agonist-induced phosphorylation of the receptor is not required for the receptor binding to PP2A.

To assess the potential involvement of PP2A in the dephosphorylation of CXCR2, we used okadaic acid (OA), a potent cell-permeant inhibitor of PP1 and PP2A (28, 29). As shown in Fig. 7A, exposure of HEK293 cells expressing CXCR2 to CXCL8 resulted in a robust phosphorylation of the receptors (Fig. 7A, lane 1). The phosphorylation was reversed after withdrawal of the agonist followed by continued incubation for 1 h at 37°C (Fig. 7A, lane 2). Treatment of the cells with concentrations of OA ranging from 0.1 nM to 1 μM inhibited the dephosphorylation of the receptors in a concentration-dependent fashion (Fig. 7A, lanes 3–7). A maximal effect was obtained for concentrations of OA equal to or higher than 100 nM. Quantification of the phosphorylation of CXCR2 indicated that the IC50 for OA to inhibit the dephosphorylation of CXCR2 was about 10 nM (Fig. 7B). In addition, OA was also found to increase the basal level of phosphorylation in the absence of CXCL8 stimulation (Fig. 7C, compare lane 2 with lane 1). Thus, the results indicate that a protein phosphatase(s) sensitive to OA, presumably PP2A and/or PP1, is involved in regulating the phosphorylation state of CXCR2. Although the inhibition of PP2A has been reported to occur in vitro at subnanomolar concentrations of OA, whereas that of PP1 requires 100-fold higher concentration (30), the pharmacological sensitivity of the inhibitor is likely to be quite different in intact cells. The relatively high concentra-
tion of OA (equal to or higher than 100 nM) that is required for a maximal effect might reflect the abundance of PP2A present in our experimental conditions, i.e. at high cell density, rather than a preference for PP1, since treatment of the cells with the predominant PP1 inhibitor, tautomycin (500 nM), did not affect the receptor dephosphorylation (data not shown).

To confirm that CXCR2 is dephosphorylated by PP2A, an in vitro phosphorylation and dephosphorylation experiment was performed. HEK293 cells stably expressing CXCR2 were immunoprecipitated (IP) with a rabbit polyclonal anti-CXCR2 antibody. A preimmune rabbit serum (mock) was used in a parallel experiment to confirm the specificity of the immunoprecipitation. Proteins were separated using 10% SDS-PAGE and transferred to a nitrocellulose membrane. Co-precipitated PP2Ac and PR65 were blotted using specific anti-PP2Ac and anti-PR65. The membrane was stripped and reblotted with a mouse monoclonal anti-CXCR2 antibody (E2; Santa Cruz Biotechnology) to confirm equal loading.

Fig. 4. CXCR2 associates with the PP2A core enzyme in HEK293 cells and in neutrophils. A, HEK293 cells stably expressing CXCR2 were exposed to CXCL1 (100 ng/ml) for the indicated times, and the cell lysates were immunoprecipitated (IP) with a rabbit polyclonal anti-CXCR2 antibody. A preimmune rabbit serum (mock) was used in a parallel experiment to confirm the specificity of the immunoprecipitation. Proteins were separated using 10% SDS-PAGE and transferred to a nitrocellulose membrane. Co-precipitated PP2Ac and PR65 were blotted using specific anti-PP2Ac and anti-PR65. The membrane was stripped and reblotted with a mouse monoclonal anti-CXCR2 antibody (E2; Santa Cruz Biotechnology) to confirm equal loading.

Fig. 3. Identification of the PP2A binding domain in the carboxyl terminus of CXCR2. A, the indicated GST-CXCR2 carboxyl tail, GST-CXCR2-(311–330), and GST-CXCR2-(331–355) fusion proteins were incubated with HEK293 cell lysate. GST pull-down assays were performed as described in the legend to Fig. 2B. A, a series of the indicated GST fusion proteins of the wild-type or group mutants of the carboxyl-terminal domain of CXCR2 (shown schematically in Fig. 1A) were incubated with HEK293 cell lysate. The pull-down assays were performed as described in the legend to Fig. 2B. B, a series of the indicated GST fusion proteins of the wild-type or single mutant carboxyl-terminal domain of CXCR2 shown schematically (Fig. 1A) were incubated with HEK293 cell lysate. The pull-down assay was performed as described in the legend to Fig. 2B. Data shown are representative of three independent experiments.
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Fig. 5. Role of internalization in the association of CXCR2 with the PP2A core enzyme in HEK293 cells. A, cells stably expressing wild-type (lanes 1 and 2) or I323A,L324A (IL/AA) mutant (lanes 3 and 4) CXCR2 were treated without (lanes 1 and 3) or with (lanes 2 and 4) CXCL1 (100 ng/ml) for 10 min, and the cell lysates were immunoprecipitated (IP) with a rabbit polyclonal anti-CXCR2 antibody. Proteins were separated using 10% SDS-PAGE and transferred to a nitrocellulose membrane. Co-precipitated PP2Ac and PR65 were blotted using specific anti-PP2Ac and anti-PR65. The membrane was stripped and rebotted with a mouse monoclonal anti-CXCR2 antibody to confirm equal loading. Data shown represent one of three independent experiments. B, quantification of the density of bands (mean ± S.E.) representing PR65 was determined by densitometric scanning without CXCL1 treatment (black bars) or with CXCL1 treatment (striped bars). The data were analyzed using Student’s t test. *, *p < 0.05, compared with the control cells transfected with wild-type CXCR2. C, cells stably expression CXCR2 were transiently transfected with vector (lanes 1 and 2) or dynamin 1 K44A (lanes 3 and 4) and then treated without (lanes 1 and 3) or with (lanes 2 and 4) CXCL1 (100 ng/ml) for 10 min. The cell lysates were immunoprecipitated with a rabbit polyclonal anti-CXCR2 antibody. Proteins were separated using 10% SDS-PAGE and transferred to a nitrocellulose membrane. Co-precipitated PP2Ac and PR65 were blotted using specific anti-PP2Ac and anti-PR65. The membrane was stripped and rebotted with a mouse monoclonal anti-CXCR2 antibody to confirm equal loading. Data shown represent one of three independent experiments. D, quantification of the density of bands (mean ± S.E.) representing PR65 was determined by densitometric scanning without CXCL1 treatment (black bars) or with CXCL1 treatment (striped bars). *, *p < 0.05, for CXCL1-treated as compared with the control cells transfected with vector.

To assess the functional role of PP2A in the signaling of CXCR2, OA was used to pretreat HEK293 cells stably expressing CXCR2, and CXCL8-induced calcium mobilization and chemotaxis were observed. Pretreatment of the cells with OA (100 nM) for 1 h significantly attenuated CXCR2-mediated calcium mobilization (Fig. 8A) and chemotaxis (Fig. 8B).

DISCUSSION

One of the most important functions of chemokine receptors is to mediate chemotaxis of neutrophils and lymphocytes. The receptor desensitization and resensitization processes are postulated to provide an on-off mechanism for the receptor-mediated chemotaxis (10). Compared with the well-established mechanisms for the receptor desensitization (5, 31–34), the mechanisms underlying the receptor resensitization are poorly understood. Dephosphorylation appears to play a key role in the recycling and the subsequent resensitization of the receptors (10). However, little is known about the mechanisms underlying the receptor dephosphorylation. We present evidence in this study that PP2A is involved in the dephosphorylation of the chemokine receptor, CXCR2, by physically interacting with the receptors. It is surprising that CXCR2 only binds to the purified PP2A core enzyme, a dimer composed of the regulatory subunit A (PR65) and the catalytic subunit (PP2Ac), but not the PP2Ac monomer, suggesting that the receptor interacts exclusively with PR65. More interestingly, a binding domain in the C terminus of CXCR2, which is localized upstream of the potential phosphorylation sites and is conserved in all CC and CXC chemokine receptors as well as many other kinds of GPCRs, was identified. Several charged residues in this domain are required for the receptor binding to PP2A. We were unable to test the significance of this binding domain in an in vivo study, since mutation of any residue in this domain seriously impaired the receptor localization on the cell membrane (data not shown). In future experiments, it will be of interest to investigate the binding domain in the sequence of PR65 that is involved in the interaction between PP2A and CXCR2 or other types of GPCRs.

An in vivo interaction of CXCR2 with PP2A was demonstrated in this study. Agonist treatment induced an association of CXCR2 with the PP2A core enzyme in a time-dependent manner. The association peaked at about 10 min and lasted for at least 30 min, whereas, within the same time frame, most portions of CXCR2 and other GPCRs become sequestered into endosomes (26, 27, 31, 35, 36). We previously demonstrated that inhibition of CXCR2 internalization impairs the receptor dephosphorylation (10). In addition, studies of β2-ARs indicate that the receptor is dephosphorylated in endosomal vesicles. It has been shown that β2-ARs in vesicular fractions are in a less phosphorylated state than are receptors in the plasma membrane (37). Suppressing the receptor internalization by internalization-blocking reagents or mutation of the internalization
motifs impairs the receptor dephosphorylation and the subsequent resensitization (38, 39). Moreover, an increase in endosomal pH suppresses the receptor dephosphorylation and co-immunoprecipitation with PP2A (19). Based on these data, we sought to investigate the potential requirement of the internalization of CXCR2 for its association with PP2A.

CXCR2 internalization is regulated differentially in a variety of cell types. The carboxyl-terminal domain of CXCR2, which includes the phosphorylation domain appears to be required for the receptor internalization in 3ASubE cells but is not required for internalization in HEK293 cells (4, 26). Although the underlying mechanisms are not yet fully understood, it is postulated that different adaptor proteins present in these cell lines might be responsible for the difference in CXCR2 internalization (26). Previous studies have demonstrated that CXCR2 binds to AP-2 and β-arrestins, two adaptor proteins involved in the internalization of CXCR2, through its C-terminal dileucine motifs and the downstream phosphorylation sites, respectively (26). It has been suggested that in HEK293 cells, the internalization of the truncated mutant CXCR2 (331T), which loses all of the C-terminal phosphorylation sites and fails to bind to β-arrestins, is mediated, at least in part, by interaction with AP-2 (26). We postulate that the level of AP-2 in 3ASubE cells is not sufficient to mediate the internalization of 331T in the absence of β-arrestin association with the carboxyl-terminal domain of the receptor. To investigate the potential role of CXCR2 internalization in the interaction of the receptor with PP2A, the receptor internalization was blocked by overexpression of a dominant negative mutant (K44A) of dynamin 1, a major component of clathrin-coated pits (10), or by mutation of the dileucine motifs in the receptor C terminus (26). The results demonstrated that blocking the receptor internalization impaired the receptor binding to the PP2A core enzyme. These data strongly support the hypothesis that the internalized CXCR2 associates with PP2A in the endosomes.

We propose that PP2A binding to the receptors is to reverse the phosphorylated state of the receptors and that agonist-induced phosphorylation of the receptors facilitates the receptor association with PP2A. CXCR2 provides an ideal model to investigate the potential role of phosphorylation in the interaction between GPCRs and PP2A, because truncation of the C-terminal domain containing all of the potential phosphorylation sites totally abolishes agonist-induced phosphorylation of the receptor yet does not affect the receptor/G protein coupling and internalization in HEK293 cells (4, 26, 31). Interestingly, the present data clearly demonstrate that the agonist-induced phosphorylation of CXCR2 is not required for the receptor binding to the PP2A core enzyme, since the C-terminal truncated mutant (331T) of CXCR2 associated with an almost equal amount of PP2A, as compared with the wild-type CXCR2. These data, taken together with the evidence from the GST pull-down assay, indicating that truncation of the C-terminal domain containing the serine/threonine residues did not affect the receptor C terminus binding to the PP2A core enzyme,
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Inhibition of PP2A attenuates CXCR2-mediated signaling. A, HEK293 cells stably expressing CXCR2 were preincubated with OA (100 nM) in serum-free DMEM at 37 °C for 1 h. The cells were then loaded with Fluo-3, stimulated with CXCL8 (100 ng/ml), and analyzed for ability to mobilize intracellular free Ca\(^{2+}\). The data shown are representative of at least three independent experiments. B, HEK293 cells stably expressing CXCR2 were preincubated at 37 °C with OA (100 nM) for 1 h. Chemotaxis in response to CXCL8 (1–200 ng/ml) stimulation was performed as described under “Experimental Procedures.” Values represent the mean ± S.E. of three different experiments. The data were analyzed using Student’s paired t test. Statistical significance is indicated (*, p < 0.05).

In conclusion, the present study demonstrates for the first time that the G protein-coupled chemokine receptor, CXCR2, interacts with the PP2A core enzyme, probably through its C-terminal association with the regulatory subunit A (PR65) of PP2A. A conserved sequence motif in the carboxyl-terminal domain, upstream of the serine/threonine residues, is the potential PP2A binding domain. Agonist-induced increase in the interaction of the receptors with the PP2A core enzyme is phosphorylation-independent but internalization-dependent. PP2A is involved in the dephosphorylation of CXCR2 and plays an important role in regulating the receptor signaling.

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