Cyclophilin A Is Required for CXCR4-mediated Nuclear Export of Heterogeneous Nuclear Ribonucleoprotein A2, Activation and Nuclear Translocation of ERK1/2, and Chemotactic Cell Migration*

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The chemokine receptor CXCR4-mediated signaling cascades play an important role in cell proliferation and migration, but the underlying mechanisms by which the receptor signaling is regulated remain incompletely understood. Here, we demonstrate that CXCR4 was co-immunoprecipitated with cyclophilin A (CyPA) from the lysate of HEK293 cells stably expressing CXCR4. Although both the glutathione S-transferase-CXCR4 N- and C-terminal fusion proteins were associated with the purified CyPA, truncation of the C-terminal domain of CXCR4 robustly inhibited the receptor co-immunoprecipitation with CyPA in intact cells, thereby suggesting a critical role of the receptor C terminus in this interaction. Ligand stimulation of CXCR4 induced CyPA phosphorylation and nuclear translocation, both of which were inhibited by truncation of the C-terminal domain of CXCR4. CyPA was associated with transportin 1, and knockdown of transportin 1 by RNA interference (RNAi) blocked CXCL12-induced nuclear translocation of CyPA, thereby suggesting a transportin 1-mediated nuclear import of CyPA. CyPA formed a complex with heterogeneous nuclear ribonucleoprotein (hnRNP) A2, which underwent nuclear export in response to activation of CXCR4. Interestingly, the CXCR4-mediated nuclear export of hnRNP A2 was blocked by RNAi of CyPA. Moreover, CXCR4-evoked activation of extracellular signal-regulated kinase 1/2 (ERK1/2) was attenuated by CyPA RNAi, by overexpression of a PPIase-deficient mutant of CyPA (CyPA-R55A), and by pretreatment of the immunosuppressive drugs, cyclosporine A and sanglifehrin A. Finally, CXCL12-induced chemotaxis of HEK293 cells stably expressing CXCR4 or Jurkat T cells was inhibited by CyPA RNAi or CsA treatment.

Chemokines comprise a large family of low molecular mass (8–10 kDa) proteins with chemoattractant properties whose main function is leukocyte recruitment in inflammatory sites. Chemokines mediate their biological effects by binding to specific seven-transmembrane domain G protein-coupled receptors, designated CXCR1 through CXCR6, CCR1 through CCR11, CX3CR1, and XCR1, based on their specific preference for certain chemokines. Among these chemokines and chemokine receptors, the stromal cell-derived factor 1/CXCL12 and its cognate receptor CXCR4 have attracted much attention because this receptor/ligand pair play important roles in human immunodeficiency virus infection, development of immune and central nervous systems, angiogenesis, tumorigenesis, and metastasis of many cancer types (1–8). CXCR4-mediated signal transduction pathways leading to cell proliferation and migration are critical for these functions.

Stimulation of CXCR4 by its ligand triggers various intracellular signaling cascades (9–13). A major CXCR4-initiated signaling pathway that controls cell proliferation and differentiation is the activation of Ras on the plasma membrane, followed by activation of c-Raf, mitogen-activated protein kinase kinase (MAPKK or MEK1/2),2 and ultimately extracellular signal-regulated kinase (ERK1/2). The activated ERK1/2 enters the nucleus (14, 15), where it activates several transcription factors, including Elk and early growth response factor-1 (16–18). However, the other two MAPK family members, c-Jun N-terminal kinase and p38 MAPK, are not activated by CXCR4 (19, 20). Several important diseases, including human immunodeficiency virus infection, cardiovascular disease, allergic inflammatory disease, neuroinflammation, and cancer, have been linked to inappropriate activation of the chemokine network (21). However, mechanisms for the alterations of chemokine receptor signaling in the above pathological conditions remain largely unclear. In our recent proteomic studies, we identified a
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number of CXCR4-interacting proteins, and some of them play a role in the signaling of CXCR4 (22, 23). Thus, characterizing the functions of the CXCR4-interacting proteins would be very helpful for uncovering the mechanisms that regulates CXCR4 signaling under physiological and pathological conditions.

One of the identified CXCR4-interacting proteins is cyclophilin A (CyPA), a prototypical member of the cyclophilin family that is expressed in the cytoplasm and nucleus of all cell types. Like other cyclophilin family members, CyPA has peptidyl-prolyl cis-trans isomerase (PPIase) activity, which regulates protein-protein interactions and the function of mature proteins, contributing to conformational stability (24–27). CyPA was subsequently shown to direct the immunosuppressive effect of cyclosporin A (CsA) (28). The CyPA–CsA complex forms a composite surface that binds to and inhibits calcineurin (29), a serine-threonine phosphatase required for cytokine induction in response to stimulation of T cells (30, 31). Recently, the CyPA–binding molecule sanglifehrin (SfA) was shown to inhibit macrophage colony stimulatory factor-induced activation of ERK1/2 and cell proliferation (32), and CsA was shown to inhibit chemokine-induced dendritic cell chemotaxis (33), thereby suggesting involvement of CyPA in the MAPK signaling pathway and in chemotaxis. Here, we provide evidence that CyPA interacts with the chemokine receptor CXCR4 after ligand-induced internalization of the receptor in endosomal compartments. Ligand stimulation of CXCR4 induced CyPA phosphorylation and translocation to nucleus, where it formed a complex with heterogeneous nuclear ribonucleoprotein (hnRNP) A2. CyPA plays a role in CXCR4-mediated nuclear export of hnRNP A2, activation and nuclear translocation of ERK1/2, and chemotactic cell migration. These data indicate critical role of CyPA in CXCR4 signaling involved in cell proliferation and migration.

EXPERIMENTAL PROCEDURES

Plasmids and siRNAs—Plasmids encoding HA-CXCR4, HA-CXCR4 (Δ34), Myc-CXCR4, and glutathione S-transferase (GST)-conjugated CXCR4 C terminus were obtained from Dr. Gang Pei (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences). The GST-conjugated CXCR4 N terminus was constructed previously (22). For the construction of CyPA in pcDNA3.1, the cDNA of CyPA was amplified by PCR from the cDNA library of HEK293 cells, and the amplified fragment was inserted into BamHI and XbaI sites of pcDNA3.1. The CyPA siRNA, which is a pool of three target specific 20–25 nucleotide siRNAs (number 5478), and a scramble siRNA that does not lead to the specific degradation of any cellular message

Purification of GST-CXCR4 Tail and His-CyPA Fusion Proteins and in Vitro Protein Binding Assay—The GST, GST-CXCR4 C terminus fusion proteins, or GST-CXCR4 N terminus fusion proteins were purified from the bacteria strain DH5α transformed with plasmids encoding the above proteins. Briefly, after incubation of the Escherichia coli at 37 °C for 12 h, isopropyl β-D-thiogalactopyranoside was added and incubation was continued for another 3 h to induce protein expression. The bacteria were lysed in RIPA buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 1 mM NaVO₃, 1 mM phenylmethylsulfonyl fluoride, and 10 μg each of 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 purified GST or GST fusion protein-bound beads were resuspended in RIPA buffer. The His-tagged CyPA fusion proteins were purified from bacteria strain DH5α transformed with plasmids encoding His-CyPA. Briefly, the E. coli were cultured at 37 °C for 12 h, then isopropyl β-D-thiogalactopyranoside was added and incubation continued for another 3 h to induce protein expression. The bacteria were resuspended in TEM buffer containing 50 mM Tris-HCl, pH 8.0, 20 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and then sonicated. The supernatant of the bacterial lysate was applied to equilibrated nickel-nitriilotriacetic acid–agarose by gravity flow, and His-CyPA was eluted with elution buffer containing TMP
and 100 mM NaCl, 100 mM imidazole, and 10% glycerol. For the in vitro binding assay, aliquots of the glutathione-Sepharose bound GST or GST fusion proteins were incubated with equal amounts of His-CyPA fusion proteins at 4 °C for 2 h with rotation. Beads were pelleted by centrifugation (15,000 × g, 2 min), and washed four times with RIPA buffer. Bound proteins were released by boiling in loading buffer containing 5% β-mercaptoethanol for 5 min and detected by SDS-PAGE and Western blot.

**Immunofluorescence and Confocal Microscopy**—For the co-immunostaining of CXCR4 and CyPA, HEK293 cells stably expressing HA-CXCR4 were treated with carrier buffer or CXCL12 for various time intervals and fixed with methanol. Cells were washed with phosphate-buffered saline and incubated with an antibody mixture containing a mouse monoclonal anti-HA antibody (Santa Cruz Biotechnology, Inc.) and a rabbit polyclonal anti-CyPA antibody (Santa Cruz Biotechnology, Inc.) for 30 min. Cells were washed and incubated with a secondary antibody mixture containing a CY3-conjugated anti-mouse antibody (Molecular Probes) and a FITC-conjugated anti-rabbit antibody (Molecular Probes) for 30 min. For co-localization between CyPA and EGFP-Rab5, HeLa cells transiently transfected with EGFP-Rab5 plasmids were treated with or without CXCL12 for different time intervals and immunostaining of CyPA was performed as described above. After the immunostaining of CyPA, the cells were counterstained with DAPI for 5 min. For the co-immunostaining of CyPA and ERK1/2, HeLa cells treated with CXCL12 for different time intervals were incubated with an antibody mixture containing a mouse monoclonal anti-ERK1/2 antibody (Santa Cruz Biotechnology, Inc.) and a rabbit polyclonal anti-CyPA antibody (Santa Cruz Biotechnology, Inc.) for 30 min, followed by a secondary antibody mixture containing a CY3-conjugated anti-mouse antibody and a FITC-conjugated anti-rabbit antibody for 30 min. After the immunostaining of CyPA, the cells were counterstained with DAPI for 5 min. Confocal microscopy was performed on a LSM-510 laser scanning microscope (Carl Zeiss, Thornwood, NY) with a 63 × 1.3 numerical aperture oil immersion lens using dual excitation (488 nm for FITC, 568 nm for Cy3) and emission (515–540 nm for FITC, 590–610 nm for Cy3) filter sets. All digital images were captured at the same settings to allow direct quantitative comparison of staining patterns. Final images were processed using Adobe Photoshop software. The percentage of CyPA co-localized with EGFP-Rab5 was analyzed by calculating the colocalized CyPA fluorescence from the total EGFP-Rab5 fluorescence.

**Immunoprecipitation**—HEK293 cells stably expressing HA-CXCR4 or HeLa cells were washed with ice-cold phosphate-buffered saline and lysed in 1 ml of RIPA buffer. The cell debris was removed by centrifugation (15,000 × g, 15 min). The supernatant was pre-cleared by incubation with 40 µl of protein A/G-agarose (Pierce) for 1 h at 4 °C to reduce nonspecific binding. After removal of the protein A/G-agarose by centrifugation (15,000 × g, 1 min), the cleared supernatant was collected and 10 µl of affinity purified anti-HA antibody (Santa Cruz Biotechnology, Inc.) or anti-CyPA antibody (Santa Cruz Biotechnology, Inc.) was added for overnight incubation at 4 °C. Protein A/G beads were then added and incubated at 4 °C for 2 h. The protein A/G-antibody-antigen complex was collected by washing three times with ice-cold RIPA buffer. The final pellet was re-suspended 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 12% SDS-polyacrylamide gel. Proteins on the gel were either stained with Coomassie Blue or transferred to nitrocellulose membranes (Bio-Rad) for Western blot analysis.

**CyPA Phosphorylation Assay**—HEK293 cells stably expressing CXCR4 were grown in 6-well plates. Cells were treated with CXCL12 (10 nM) for different time intervals before being lysed in RIPA buffer. CyPA proteins were immunoprecipitated from the cell lysates. Equal amounts of immunoprecipitates were subjected to SDS-PAGE. Phosphorylated CyPA proteins were detected by Western blot analysis with an anti-phosphoserine/threonine antibody (BD Transduction Laboratories). The same blots were stripped and reprobed with a CyPA antibody to confirm equal loading.

**MAPK Assay**—HEK293 cells stably expressing CXCR4 were treated with CXCL12 (10 nM) for different time intervals before being lysed in RIPA buffer. Equal amounts of proteins were subjected to SDS-PAGE. Phosphorylated ERK1/2 was detected by Western blot analysis using a phosphospecific ERK1/2 antibody (Santa Cruz Biotechnology, Inc.). The blots were stripped and reprobed with an ERK2 antibody to confirm equal loading.

**Densitometric Analysis of Western Blots**—The relative amount of the Western blot bands was measured by densitometric analysis using NIH Image software (rsb.info.nih.gov/nih-image). The relative density of the protein bands was calculated in the area encompassing the immunoreactive protein band and subtracting the background of an adjacent nonreactive area in the same lane of the protein of interest.

**Fluorescence-activated Cell Sorting (FACS) Analysis**—HEK293 cells stably expressing Myc-CXCR4 were transfected with control siRNA or CyPA-specific siRNA. Cells were incubated in Hepes (20 mM)-buffered Dulbecco’s modified Eagle’s medium at 37 °C for 30 min in the presence or absence of CXCL12 (10 nM) for 30 min. Cells were washed in ice-cold medium followed by continued incubation in ligand-free medium at 37 °C for 60 min. Cells were incubated with a mouse monoclonal anti-Myc antibody at 4 °C for 60 min, followed by FITC-conjugated anti-mouse secondary antibody at 4 °C for 60 min. Cells were washed and fixed in 2% formaldehyde in phosphate-buffered saline and analyzed by flow cytometry equipped with CellQuest software (BD Biosciences).

**Chemotactic Cell Migration (Chemotaxis) Assay**—The migration of HEK293 cells stably expressing CXCR4 was evaluated using the Boyden chamber chemotaxis assay. Briefly, polycarbonate filters (10-µm pore size) coated with 20 µg/ml human collagen type IV were placed between the upper and lower compartments of the Boyden chambers (Neuroprobe, Gaithersburg, MD). The lower compartment of the chamber was loaded with 400-µl aliquots of 1 mg/ml ovalbumin/Dulbecco’s modified Eagle’s medium (chemotaxis buffer) or CXCL12 diluted in the chemotaxis buffer (0.001–10 nM). Cells (5 × 10^5/100 µl) were loaded into the upper compartments and incubated for 240 min at 37 °C in a 5% CO₂ atmosphere. Cells migrated toward media or CXCL12 gradients were counted...
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under the microscope (×20 objective) after being stained with a Diff-Quik kit. The migration index was calculated based on the ratio of the number of cells crossing the filter toward CXCL12 gradients to the number of cells migrating toward media (control). Each experiment was performed at least three times.

RESULTS

In a recent proteomic study by immunoprecipitating CXCR4 and analyzing the co-immunoprecipitated proteins by mass spectrometry, we identified a number of CXCR4-interacting proteins and characterized some of them (22, 34). One of these proteins that attracted our attention was CyPA. To replicate the co-immunoprecipitation of CyPA with CXCR4, we stimulated HEK293 cells stably expressing HA-CXCR4 with CXCL12 (10 nM) for different time intervals (0, 10, 30, 60, and 120 min), immunoprecipitated CXCR4 from the cell lysate, and detected the co-precipitated CyPA by Western blotting. The immunoprecipitation revealed a weak basal association of CyPA with CXCR4 prior to ligand treatment. CXCL12 (10 nM) stimulation resulted in a time-dependent association of CyPA with CXCR4, which peaked at 10–30 min and returned to near basal level after 120 min of incubation (Fig. 1A). The association of CyPA with CXCR4 appears to be specific because a pre-immune serum did not pull down any CyPA proteins (Fig. 1A).

CyPA binds to target proteins containing X-Pro motifs (where X is any amino acid) (35). Because both the C- and N-terminal domains of CXCR4 contain the X-Pro motifs, we examined the possible direct binding of CyPA to the C- and/or N-terminal domains of CXCR4. Purified GST (control), GST-CXCR4 N terminus fusion proteins, or GST-CXCR4 C terminus fusion proteins were incubated with the purified CyPA and the co-precipitated CyPA was detected by Western blot analysis. We observed that the purified CyPA was co-precipitated with both the GST-conjugated C- and N-terminal domains of CXCR4 but not with the GST alone (Fig. 1B), thereby suggesting a physical interaction between CyPA and CXCR4.

Previous studies have shown that CXCR4 undergoes rapid internalization in response to ligand stimulation (23, 34). The same time frame for the receptor internalization and its association with CyPA raises an interesting question regarding the role of CXCR4 internalization in its interaction with CyPA. To address this question, we evaluated the association of CyPA with a C-terminal 34-amino acid deletion mutant of CXCR4 (Δ34), which has exhibited reduced internalization (11). As shown in Fig. 1C, CXCL12 (10 nM) stimulation for 10 min
induced a robust increase in the association of CyPA with the full-length CXCR4. By contrast, the ligand-induced association of CyPA with CXCR4 (Δ34) was significantly reduced. These data suggest that the intact C-terminal domain of CXCR4 is critical for the receptor association with CyPA in intact cells.

We also examined the effect of pertussis toxin (PTX) on CXCR4 interaction with CyPA. HEK293 cells stably expressing HA-CXCR4 were pretreated with or without PTX (200 ng/ml) for 24 h before being treated with or without CXCL12 (10 nM) for 10 min. The HA-CXCR4 proteins were immunoprecipitated from the cell lysate using an anti-HA antibody, and co-precipitated CyPA was detected by Western blot analysis. As shown in Fig. 1D, PTX pretreatment did not affect the association of CyPA with CXCR4, thereby suggesting that activation of G proteins is not involved in this interaction.

To visualize the association between CyPA and CXCR4 in intact cells, we treated HEK293 cells stably expressing HA-CXCR4 with or without CXCL12 (10 nM) for 30 min, immunostained the cells with both HA-CXCR4 and CyPA antibodies, and visualized the subcellular localization of both CXCR4 and CyPA by confocal microscopy. As shown in Fig. 1E, prior to ligand stimulation, CXCR4 proteins were predominantly localized on the cell surface, whereas CyPA proteins were predominantly in the cytoplasm. CXCL12 stimulation for 30 min resulted in a marked internalization of CXCR4, and strikingly, the internalized CXCR4 receptors were co-localized with CyPA in the internal vesicles (small arrows indicated).

Previous studies have shown the endosomal localization of the internalized CXCR4 (37). The co-localization between CyPA and the internalized CXCR4 suggests possible interaction of these two proteins in endosomal compartments. To test this hypothesis, we examined whether both CXCR4 and CyPA are co-localized with Rab5, an early endosomal marker (37). To visualize the subcellular localization of CXCR4 and Rab5 by confocal microscopy, HeLa cells transfected with plasmids encoding EGFP-CXCR4 were treated with or without CXCL12 (10 nM) before being immunostained with a Rab5 antibody. Fig. 2A shows that prior to ligand stimulation, the EGFP-CXCR4 receptors were localized exclusively on the cell surface, whereas the endogenous Rab5 proteins were predominantly in the cytoplasm. After CXCL12 (10 nM) treatment for 30 min, a large proportion of CXCR4 receptors were internalized and co-localized with Rab5, thereby suggesting early endosomal localization of the internalized CXCR4, consistent with the previous report in stem cells (11). To visualize the subcellular distribution of CyPA and Rab5, HeLa cells, which endogenously express CXCR4 (38), were transfected with plasmids of EGFP-Rab5. Cells were treated with CXCL12 (10 nM) for different time intervals (0, 30, and 60 min) before being immunostained with a CyPA antibody and counterstained with DAPI, a nuclear marker. As shown in Fig. 2B, prior to ligand stimulation, CyPA proteins were predominantly localized in the cytoplasm and co-localized with the EGFP-Rab5 proteins (small arrows indicated), whereas a small proportion of CyPA proteins were localized in the nucleus. CXCL12 stimulation resulted in a gradual decrease of the amount of CyPA proteins in the EGFP-Rab5-positive early endosomes and a gradual increase of the amount of CyPA proteins in the nucleus (Fig. 2B, large arrows indicated). These data indicate that CyPA is predominantly localized in the early endosomal compartments in quiescent cells, and that CyPA undergoes nuclear translocation, most likely from early endosomes, in response to ligand stimulation of CXCR4. Taken together, the results that both CyPA and CXCR4 were co-localized with Rab5 suggest an interaction of these two proteins in early endosomes.

How does activation of CXCR4 induce CyPA nuclear translocation? One possible mechanism is that CyPA undergoes a covalent modification that promotes its association with the nuclear transport machinery. CyPA is enriched in Ser and Thr residues, which favor its phosphorylation by Ser/Thr protein kinases. To test this possibility, CyPA was immunoprecipitated from the cell lysate of HeLa cells treated with CXCL12 (10 nM) for different time intervals (0, 10, 30, and 60 min), and Western blot analysis was performed using an anti-Ser/Thr antibody. As shown in Fig. 3A, CyPA underwent a time-dependent phosphorylation in response to ligand stimulation, which peaked at 10 min and lasted for at least 60 min. In contrast to the ligand-

**FIGURE 1. In vivo and in vitro interaction between CyPA and CXCR4.** A, co-immunoprecipitation of CyPA with CXCR4. HEK293 cells stably expressing HA-CXCR4 were exposed to CXCL12 (10 nM) for the indicated time intervals, and CXCR4 was immunoprecipitated (IP) from the cell lysate using an anti-HA antibody. In a parallel experiment, cells were treated with CXCL12 for 10 min and immunoprecipitation was performed using a preimmune serum (mock). Co-precipitated proteins were separated by 12% SDS-PAGE, and blots were stripped and reprobed with a specific anti-CyPA antibody. Bar graphs underneath represent -fold changes of CyPA proteins detected in the immunoprecipitated complex with HA-CXCR4 after agonist stimulation versus no agonist (the latter defined as control, or 1.0-fold). *p < 0.05, compared to the control without CXCL12 treatment. B, co-immunoblot. C, in vitro association of purified CyPA with the GST-conjugated N- or C-terminal domain of CXCR4. Glutathione-Sepharose bound GST (lane 1), GST-CXCR4 N-terminal fusion proteins (lane 2), or GST-CXCR4 C-terminal fusion proteins (lane 3) were incubated with an equal amount of purified CyPA proteins. Co-precipitated proteins were subjected to 12% SDS-PAGE, and CyPA proteins were detected by Western blot analysis using a specific CyPA antibody. D, co-immunoprecipitation of CyPA with full-length CXCR4 or CXCR4 (Δ34). HEK293 cells stably expressing HA-CXCR4 or HA-CXCR4 (Δ34) were exposed to CXCL12 (10 nM) for 30 min. The full-length HA-CXCR4 or HA-CXCR4 (Δ34) proteins were immunoprecipitated from the cell lysate using an anti-HA antibody, and co-precipitated CyPA was detected by Western blot analysis as described above. Bar graphs on the right represent -fold changes of CyPA proteins detected in the immunoprecipitated complex with HA-CXCR4 after agonist stimulation versus no agonist (the latter defined as control, or 1.0-fold). E, co-localization between CyPA and CXCR4 in cells. HEK293 cells stably expressing HA-CXCR4 were treated with CXCL12 (10 nM) for different time intervals (0, 15, and 30 min). The treated cells were fixed in methanol and incubated with an antibody mixture containing a mouse anti-HA monoclonal antibody and a rabbit polyclonal anti-CyPA antibody, followed by a secondary antibody mixture containing a FITC-conjugated anti-mouse antibody and a Cy3-conjugated anti-rabbit antibody. Confocal images demonstrating the subcellular localization of HA-CXCR4 (green), CyPA (red), and co-localization of these two proteins (yellow) are shown. Small arrows indicate co-localization between CXCR4 and CyPA. Large arrows indicate nuclear translocation of CyPA.
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![Image](https://example.com/image1.png)

**FIGURE 2. Endosomal co-distribution between CXCR4 and CyPA and CXCR4-mediated nuclear translocation of CyPA.** A, co-localization between Rab5 and CyPA or internalized CXCR4. HeLa cells transfected with plasmids encoding EGFP-CXCR4 were treated with or without CXCL12 (10 nM) for 30 min. Cells were incubated with a mouse monoclonal Rab5 antibody for 30 min followed by a Cy3-conjugated anti-mouse secondary antibody for 30 min before being immunostained with a Rab5 antibody. Confocal images demonstrating the subcellular localization of EGFP-CXCR4 (green), Rab5 (red), and co-localization of these two proteins (yellow) are shown. Arrows indicate co-localization between EGFP-CXCR4 and Rab5. Data are representative of three independent experiments with similar results. B, HeLa cells transiently transfected with the EGFP-Rab5 construct were stimulated with CXCL12 (10 nM) for different time intervals (0, 30, and 60 min) before being fixed in methanol. Cells were incubated with a rabbit polyclonal anti-CyPA antibody followed by a Cy3-conjugated anti-rabbit secondary antibody and counterstained with DAPI. Confocal images demonstrating the subcellular localization of EGFP-CXCR4 (green), Rab5 (red), and co-localization of these two proteins (yellow) are shown. Small arrows indicate co-localization between EGFP-Rab5 and CyPA. Large arrows indicate nuclear translocation of CyPA. Bar graphs are quantitative results from three independent experiments showing a time-dependent decrease of CyPA co-localized with Rab5 and a time-dependent increase of CyPA entered nucleus after CXCL12 treatment. *, p < 0.05, comparing the percentage of CyPA co-localized with EGFP-Rab5 or the -fold increase of CyPA in the nucleus after agonist stimulation versus no agonist (the latter defined as control, or 100%).

...induced robust phosphorylation of CyPA in the full-length CXCR4 expressing cells, CXCL12-induced phosphorylation of CyPA in the CXCR4 (Δ34) expressing HEK293 cells was significantly reduced (Fig. 3B), thereby suggesting a critical role of the receptor C terminus in CXCR4-mediated CyPA phosphorylation.

It raises an interesting question regarding the role of the C-terminal domain of CXCR4 in the nuclear translocation of CyPA. To address this question, HEK293 cells stably expressing full-length CXCR4 or CXCR4 (Δ34) were stimulated with or without CXCL12 (10 nM) for 60 min before cytoplasmic and nuclear samples were isolated using a Nuclear Extraction Kit (Chemicon) and Western blot for CyPA was performed. Fig. 3C shows that ligand-induced nuclear translocation of CyPA was significantly reduced in the CXCR4 (Δ34) expressing cells relatively to that in the full-length CXCR4 expressing cells.

Because CyPA does not possess a classical nuclear localization signal (39), until the present study, it is elusive regarding the mechanism by which CyPA undergoes nuclear translocation. Here, we identified a M9-like motif embedded in the glycine-rich domain (amino acids 41–125) on CyPA by comparing the protein sequences between CyPA and hnRNP A1 (Fig. 4A), which is known to contain a M9 motif that is recognized by transportin 1, an importin family member that specifically directs the nuclear import of hnRNP A1 (40). This suggests that transportin 1 may interact with CyPA and direct its nuclear import. To test this hypothesis, we first immunoprecipitated CyPA from the cell lysate of HEK293 cells stably expressing full-length CXCR4 or CXCR4 (Δ34) and detected the potentially associated transportin 1 by Western blot analysis. Fig. 4B shows that transportin 1 was associated with CyPA in a ligand-dependent manner in the full-length CXCR4 expressing cells, and in contrast, a significantly reduced association was observed in the CXCR4 (Δ34)-expressing cells. We then visualized CyPA nuclear translocation in HeLa cells with or without transportin 1 knockdown. As shown in Fig. 4C, in the scramble siRNA-transfected cells, CXCL12 (10 nM) stimulation induced a remarkable nuclear translocation of CyPA, which appear to co-localize with transportin 1 in the nucleus. By contrast, in the cells trans-
**FIGURE 3.** Requirement of the C-terminal domain of CXCR4 for the receptor-mediated phosphorylation and nuclear translocation of CyPA. A, time course of CXCL12-induced CyPA phosphorylation. HeLa cells were treated with CXCL12 (10 nM) for different time intervals as indicated before being lysed in RIPA buffer. CyPA was immunoprecipitated (IP) from the cell lysate. Proteins samples were subjected to SDS-PAGE and CyPA phosphorylation was detected by Western blot analysis using an anti-phospho-Ser/Thr antibody. The blots were stripped and reprobed with a CyPA antibody to confirm equal loading. The bar graph on the right is the quantitative result of the density of bands representing CyPA phosphorylation that was normalized with the density of the total CyPA bands. Data are mean ± S.E. from three independent experiments. B, role of the C-terminal domain of CXCR4 in the receptor-mediated CyPA phosphorylation. HEK293 cells stably expressing full-length CXCR4 or CXCR4 (Δ34) were treated with or without CXCL12 (10 nM) for 10 min. Phosphorylation of CyPA was determined as described above. The bar graph on the right is the quantitative result of the density of bands representing CyPA phosphorylation that was normalized with the density of the total CyPA bands. Data are mean ± S.E. from three independent experiments. *, p < 0.05 compared with the control cells with the same treatment. C, role of the C-terminal domain of CXCR4 in the receptor-mediated CyPA nuclear translocation. HEK293 cells stably expressing full-length CXCR4 or CXCR4 (Δ34) were treated with or without CXCL12 (10 nM) for 60 min. The cytoplasmic and nuclear samples were isolated using a Nuclear Extraction Kit (Chemicon) and Western blot analyses for CyPA, tubulin, and P84 were performed. The bar graphs on the right are the quantitative results of the density of bands representing the cytoplasmic or nuclear CyPA that was normalized with the density of the either tubulin or P84 bands. Data are mean ± S.E. from three independent experiments. *, p < 0.05 compared with the control cells with the same treatment.
were treated with or without CXCL12 (10 nM) for 30 min. CyPA was immunoprecipitated and co-precipitated between CyPA and transportin 1. HEK293 cells stably expressing HA-tagged full-length CXCR4 or CXCR4 (proteins are highlighted.

Requirement of transportin 1 in CXCR4-mediated CyPA nuclear translocation.

**FIGURE 4.** Requirement of transportin 1 in CXCR4-mediated CyPA nuclear translocation. A, sequence analysis between the M9 motif of hnRNP A1 (amino acids 258–305) and CyPA. Residues identical in these two proteins are highlighted. B, role on the C-terminal domain of CXCR4 in the receptor-mediated association between CyPA and transportin 1. HEK293 cells stably expressing HA-tagged full-length CXCR4 or CXCR4 (Δ34) were treated with or without CXCL12 (10 nM) for 30 min. CyPA was immunoprecipitated and co-precipitated transportin 1 was detected by Western blot analysis. In a parallel experiment, an equal amount of the cell lysate was subjected to SDS-PAGE and Western blot analysis using an anti-HA antibody was performed to confirm equal loading of the samples. The bar graph on the right is the quantitative result of the density of bands representing transportin 1 co-immunoprecipitated with the wild-type or mutant CXCR4 that was normalized with the density of the total CyPA bands. Data are mean ± S.E. from three independent experiments. *, p < 0.05 comparing to the control cells with the same treatment. C, effect of transportin 1 knockdown on CXCL12-induced CyPA nuclear translocation. HeLa cells transiently transfected with scramble siRNA (control) or transportin 1-specific siRNA were treated with or without CXCL12 (10 nM) for 60 min before being fixed in methanol. Cells were incubated with an antibody mixture containing a rabbit polyclonal anti-CyPA antibody and mouse monoclonal anti-transportin 1 antibody followed by incubation of a mixture of secondary antibodies containing a FITC-conjugated anti-rabbit IgG and a Cy3-conjugated anti-mouse IgG, and counterstained with DAPI.

To identify nuclear proteins that bind to CyPA, we immunoprecipitated CyPA from HeLa cells and analyzed the co-immunoprecipitated proteins by mass spectrometry. We identified hnRNP A2 as a major nuclear protein that forms a complex with CyPA. Here we replicated the co-immunoprecipitation experiment by stimulating HeLa cells with CXCL12 (10 nM) for different time intervals, immunoprecipitating CyPA from the cell lysate, and detecting the associated hnRNP A2 by Western blot analysis. As shown in Fig. 5A, the immunoprecipitation revealed a basal association between CyPA and hnRNP A2 before CXCL12 stimulation, and CXCL12 treatment resulted in a time-dependent increase in their association.

The association of CyPA with hnRNP A2 is apparently specific because we used the same nitrocellulose membrane to probe hnRNP U or RelA but did not observe any co-immunoprecipitation of either hnRNP U or RelA with CyPA. These data provide the first evidence for CXCR4-mediated interaction between CyPA and hnRNP A2.

hnRNP A2 is a member of the hnRNP family that localizes in the nucleus but rapidly shuttles between the nucleus and cytoplasm upon stimulation. This raises the questions whether activation of CXCR4 induces hnRNP A2 nuclear export, and if yes, whether CyPA plays a role in this process? To address these questions, we transfected HeLa cells with CyPA-specific siRNA or scramble siRNA (control), treated the cells with CXCL12 (10 nM) for different time intervals (0, 10, and 30 min), extracted nuclei from the cells, and detected hnRNP A2 by Western blot analysis. As shown in Fig. 5B, in the scramble siRNA-transfected cells, CXCL12 stimulation resulted in a time-dependent decrease of hnRNP A2 and a time-dependent increase of CyPA in the nuclear fraction. In contrast, in the CyPA siRNA-transfected cells, CXCL12 stimulation did not induce a time-dependent decrease of hnRNP A2 in the nuclei (Fig. 5B), thereby suggesting requirement of CyPA in hnRNP A2 nuclear export. The comparable Western blot bands of the nuclear maker P84 indicate equal loading of the protein samples (Fig. 5B). We also visualized the subcellular localization of hnRNP A2 in HeLa cells transfected with CyPA-specific siRNA or scramble siRNA. As shown in Fig. 5C, in the cells transfected with the scramble siRNA, CyPA was predominantly localized in the cytoplasm, whereas hnRNP A2 was exclusively localized in
FIGURE 5. CyPA was associated with hnRNP A2 and was required for hnRNP A2 nuclear export. A, association of CyPA with hnRNP A2. HeLa cells were treated with CXCL12 (10 nM) for the indicated time intervals. CyPA was immunoprecipitated from the cell lysate. Aliquots of the cell lysates (left four lanes) and the immunoprecipitates (IP, right four lanes) were subjected to SDS-PAGE, and proteins were transferred to nitrocellulose membrane, followed by Western blot analysis using antibodies against hnRNP A2, hnRNP U, and RelA, respectively. Only hnRNP A2 co-immunoprecipitated with CyPA. B, effect of CyPA knockdown on CXCR4-mediated hnRNP A2 nuclear export. HeLa cells transfected with scramble siRNA or CyPA-specific siRNA were treated with CXCL12 (10 nM) for the indicated time intervals and nuclear samples were isolated as described under “Experimental Procedures.” The nuclear proteins were subjected to SDS-PAGE and subsequently transferred to nitrocellulose membrane. Western blot analysis was performed using antibodies against hnRNP A2, CyPA, and P84, respectively. Shown is a representative of three independent experiments with similar results indicating that knockdown of CyPA with specific RNAi blocked CXCL12-induced hnRNP A2 nuclear export. C, visualization of hnRNP A2 nuclear export in HeLa cells. Cells transiently transfected with scramble siRNA or CyPA-specific siRNA were treated with or without CXCL12 (10 nM) for 30 min before being fixed in methanol. Cells were incubated with an antibody mixture containing a rabbit polyclonal anti-CyPA antibody and a mouse monoclonal anti-hnRNP A2 antibody followed by incubation of a mixture of secondary antibodies containing a FITC-conjugated anti-rabbit IgG and a Cy3-conjugated anti-mouse IgG, and counterstained with DAPI. Confocal images demonstrating the subcellular localization of CyPA (green), hnRNP A2 (red), and nuclear staining (blue) are shown. The large arrows indicate agonist-induced nuclear export of hnRNP A2 in the scramble siRNA (control) but not in the CyPA siRNA-transfected cells, and the small arrows indicate the co-localization between CyPA and hnRNP A2 in the nucleus.

The nucleus under quiescent conditions. After CXCL12 stimulation for 60 min, CyPA underwent nuclear import, whereas a proportion of hnRNP A2 underwent nuclear export, and the nuclear CyPA proteins were partially co-localized with hnRNP A2 (Fig. 5C, small arrows indicated). In contrast, in the cells transfected with CyPA-specific siRNA, which resulted in robust knockdown of the CyPA protein expression level, hnRNP A2 failed to undergo nuclear export in response to CXCL12 stimulation. Taken together, these data indicate that activation of CXCR4 results in nuclear export of hnRNP A2 and CyPA plays a critical role in this process.

In addition, CyPA has recently been shown to be required for macrophage colony-stimulatory factor-induced ERK1/2 activation (32). This raises an interesting question whether CyPA is required for CXCR4-mediated ERK1/2 activation. To address this question, we transfected HeLa cells with CyPA-specific siRNA or scramble siRNA (control), treated the cells with CXCL12 (10 nM) for different time intervals (0, 5, 10, and 30 min), and detected phosphorylation of ERK1/2 by Western blot analysis. As shown in Fig. 6A, in the scramble siRNA-transfected cells, ligand stimulation induced a typical time-dependent ERK1/2 phosphorylation that peaked at 5 min and returned to basal level after 30 min. In contrast, cells transfected with the CyPA-specific siRNA exhibited a robust decrease of ERK1/2 activation in response to CXCL12 stimulation.

To examine if the PPIase activity of CyPA is required for its involvement in the CXCR4-mediated ERK1/2 activation, we attempted to generate a mutant form of CyPA that is deficient of PPIase activity. Several amino acid residues, such as His54, Trp121, Phe113, His126, Phe60, and Arg85, are crucial for the PPIase activity of CyPA (41). Whereas mutation of these individual amino acid residues differentially reduces the PPIase activity (41), Ala replacement of the Arg85 has been shown to result in maximal reduction (<1% of total) of its PPIase activity (41). Therefore, we generated the CyPA-R55A mutant by site-directed mutagenesis. We transfected the wild-type or R55A mutant form of CyPA into HeLa cells, treated the cells with CXCL12 (10 nM) for different time intervals (0, 5, 10, and 30 min), and detected ERK1/2 activation by Western blot analysis. As shown in Fig. 6B, overexpression of the wild-type CyPA did not affect CXCL12-induced ERK1/2 activation. However, overexpression of the CyPA-R55A mutant significantly attenuated the receptor-mediated ERK1/2 activation.

We then examined the effect of two CyPA inhibitors, CsA, which inhibits both the PPIase activity of CyPA and the phosphatase activity of calcineurin (29), and SfA, which inhibits the PPIase activity of CyPA without inhibiting the phosphatase activity of calcineurin (42), on CXCR4-mediated ERK1/2 activation. We treated Jurkat T cells with different concentrations of CsA (0, 0.1, 1, 10, and 100 μM) for 30 min before stimulating the cells with CXCL12 (10 nM) for 5 min. ERK1/2 phosphorylation was assessed by Western blot analysis. We observed a dose-dependent inhibition of CXCR4-evoked ERK1/2 acti-
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viation by CsA, and 10 \( \mu M \) CsA completely blocked ERK1/2 activation (Fig. 6C). Similarly, treatment of the Jurkat T cells with SFA dose-dependently inhibited CXCL12-induced ERK1/2 activation (Fig. 6D). These data indicate that CyPA is required for CXCR4-mediated ERK1/2 activation, and calcineurin appears not to be involved in this process.

Activation of ERK1/2 is necessary and sufficient for the nuclear translocation of ERK1/2 (43, 44). To examine whether CyPA is involved in CXCR4-mediated ERK1/2 nuclear translocation, we transfected HeLa cells with scramble siRNA or CyPA-specific siRNA, treated the cells with CXCL12 (10 nm) for 60 min, immunostained CyPA and ERK1/2, and visualized the subcellular localization of CyPA or ERK1/2 by confocal microscopy. As shown in Fig. 7A, in the scramble siRNA-transfected cells, CXCL12 stimulation resulted in nuclear translocation of both CyPA and ERK1/2 (arrows indicated). In contrast, in the cells transfected with the CyPA-specific siRNA, which resulted in marked knockdown of CyPA, CXCL12 stimulation failed to induce ERK1/2 nuclear translocation (arrows indicated) (Fig. 7A). Similarly, pretreatment of the HeLa cells with CsA (10 \( \mu M \)) for 30 min resulted in blockade of CXCL12-induced ERK1/2 nuclear translocation (arrows indicated) (Fig. 7B).

One of the major functions of CXCR4 is to mediate chemotaxis, a process involving multiple signal transduction pathways. The interaction between CyPA and CXCR4 and the involvement of CyPA in CXCR4 signaling encouraged us to examine the effect of CyPA on CXCR4-mediated chemotaxis. We transfected HEK293 cells stably expressing CXCR4 with CyPA-specific siRNA or scramble siRNA (control) and determined CXCL12-induced chemotaxis by Boyden chamber assay. We observed a CXCL12 concentration-dependent chemotactic response in the cells transfected in the scramble

FIGURE 6. CyPA in CXCR4-mediated MAPK activation. A, effect of CyPA knockdown on CXCR4-evoked MAPK activation. HeLa cells transiently transfected with control siRNA or CyPA-specific siRNA were treated with CXCL12 (10 nm) for the indicated time intervals and ERK1/2 phosphorylation was determined by Western blot analysis and normalized by Western blotting for total ERK2. In a parallel experiment, Western blotting of CyPA was performed to confirm knockdown of CyPA in the specific CyPA siRNA versus the scramble siRNA-transfected cells. Bar graphs represent quantitative data from three independent experiments indicating the fold change of ERK1/2 phosphorylation after agonist stimulation versus no agonist (the latter defined as control, or 1.0-fold). *, \( p < 0.05 \), comparing to the agonist-induced ERK1/2 phosphorylation in the scramble siRNA (control). B, effect of overexpression of wild-type or PPIase-deficient mutant CyPA on CXCR4-evoked ERK1/2 activation. HeLa cells transiently transfected with vector, CyPA plasmid DNA, or CyPA-R55A plasmid DNA were treated with CXCL12 (10 nm) for the indicated time intervals and ERK1/2 phosphorylation was determined by Western blot analysis and normalized by Western blotting for total ERK2. In a parallel experiment, Western blotting of CyPA was performed to confirm overexpression of CyPA or CyPA-R55A. Bar graphs represent quantitative data from three independent experiments indicating the fold change of ERK1/2 phosphorylation after agonist stimulation versus no agonist (the latter defined as control, or 1.0-fold). *, \( p < 0.05 \), comparing to the control with the same treatment. C, effect of CsA on CXCL12-induced ERK1/2 activation in Jurkat T cells. Cells preincubated with the indicated concentrations of CsA were stimulated with CXCL12 (10 nm) for 5 min. ERK1/2 phosphorylation was determined by Western blot analysis and normalized by Western blotting for total ERK2. Data from three independent experiments are summarized in the bar graph. *, \( p < 0.05 \), comparing to the control without SFA treatment.

D, effect of SFA on CXCL12-induced ERK1/2 activation in Jurkat T cells. Cells preincubated with the indicated concentrations of SFA were stimulated with CXCL12 (10 nm) for 5 min. ERK1/2 phosphorylation was determined by Western blot analysis and normalized by Western blotting for total ERK2. Data from three independent experiments are summarized in the bar graph. *, \( p < 0.05 \), comparing to the control without SFA treatment.
question regarding the role of CyPA in CXCR4 trafficking. To address this question, we transfected HEK293 cells stably expressing Myc-CXCR4 with CyPA-specific siRNA or with scramble siRNA (control), treated the cells with or without CXCL12 (10 nM) for 30 min, and analyzed the cell surface expression of CXCR4 by FACS. We observed that ligand stimulation resulted in a similar internalization of CXCR4 in both the scramble siRNA- and CyPA-specific siRNA-transfected cells, with comparable mean fluorescence intensity reduction of CXCR4 (65.6 ± 7.4 and 64.8 ± 8.3 for the control siRNA- and CyPA siRNA-transfected cells, respectively) (Fig. 8A). Western blot analysis showed marked knockdown of the CyPA proteins in the specific siRNA-transfected cells relative to the scramble siRNA-transfected cells (Fig. 9B). These data indicate that CyPA is not involved in CXCR4 endocytosis.

**DISCUSSION**

CyPA is present as a relatively abundant protein in the cytoplasm of all mammalian cells. To date, the studies of CyPA have largely focused on its structure and immunosuppressive action through association of the CyPA-CsA complex with calcineurin (45). Occasional reports have noted that CyPA associates with specific proteins, such as the YY1 suppressor of gene transcription (46), the human immunodeficiency virus type I Pr55gag polyprotein precursor (47, 48), and the dynein motor protein complex (49). However, these findings have not led to a general model for CyPA function in mammalian cells in the absence of CsA. In this study we demonstrate that CyPA forms a complex with CXCR4. Several lines of evidence support this conclusion. First, CyPA was co-immunoprecipitated with CXCR4 in a ligand-dependent manner. Second, the GST pull-down assay verified that the purified CyPA interacted with both the C- and N-terminal domains of CXCR4. Third, confocal microscopy further confirmed the interaction between CXCR4 and CyPA in endosomal compartments. Moreover, we provide evidence that ligand stimulation of CXCR4 induced phosphorylation and nuclear translocation of CyPA in a transportin-1-dependent manner. CyPA played a role in CXCR4-mediated nuclear export of hnRNP A2, in

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**FIGURE 7.** Involvement of CyPA in ERK1/2 nuclear translocation. A, effect of CyPA knockdown on CXCL12-induced ERK1/2 nuclear translocation. HeLa cells transiently transfected with control siRNA or CyPA-specific siRNA were treated with or without CXCL12 (10 nM) for 60 min before being fixed in methanol. Cells were incubated with an antibody mixture containing a rabbit polyclonal anti-CyPA antibody and a mouse monoclonal ERK1/2 antibody followed by incubation of a mixture of secondary antibodies containing a FITC-conjugated anti-rabbit IgG and a Cy3-conjugated anti-mouse IgG, and were counterstained with DAPI. Confocal images demonstrating the subcellular localization of CyPA (green), ERK1/2 (red), and nuclear staining (blue) are shown. Arrows indicate agonist-induced nuclear translocation of ERK1/2 in the scramble siRNA (control) but not the CyPA siRNA-transfected cells. B, effect of CsA on CXCL12-induced ERK1/2 nuclear translocation. HeLa cells pre-treated with vehicle or CsA (10 μM) were stimulated with or without CXCL12 (10 nM) for 60 min before being fixed in methanol. Cells were incubated with an anti-CyPA antibody for 30 min followed by a FITC-conjugated anti-rabbit IgG for 30 min before being counterstained with DAPI. Shown are representative images for the subcellular localization of CyPA. Arrows indicate agonist-induced nuclear translocation of ERK1/2 in the vehicle (control) but not CsA pre-treated cells.

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**TABLE 1.**

| siRNA      | CyPA ERK1/2 | DAPI | Overlay |
|------------|-------------|------|---------|
| Scramble   |             |      |         |
| CyPA       |             |      |         |
| Vehicle    |             |      |         |
| CsA        |             |      |         |

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**FIGURE 8.** Interaction of CyPA and CXCR4 in the early endosomes raises an interesting question regarding the role of CyPA in CXCR4 trafficking. To address this question, we transfected HEK293 cells stably expressing Myc-CXCR4 with CyPA-specific siRNA or with scramble siRNA (control), treated the cells with or without CXCL12 (10 nM) for 30 min, and analyzed the cell surface expression of CXCR4 by FACS. We observed that ligand stimulation resulted in a similar internalization of CXCR4 in both the scramble siRNA- and CyPA-specific siRNA-transfected cells, with comparable mean fluorescence intensity reduction of CXCR4 (65.6 ± 7.4 and 64.8 ± 8.3 for the control siRNA- and CyPA siRNA-transfected cells, respectively) (Fig. 8A). Western blot analysis showed marked knockdown of the CyPA proteins in the specific siRNA-transfected cells relative to the scramble siRNA-transfected cells (Fig. 9B). These data indicate that CyPA is not involved in CXCR4 endocytosis.
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FIGURE 8. CyPA was involved in CXCR4-mediated chemotaxis. A, effect of CyPA knockdown on CXCL12-induced chemotaxis. HEK293 cells stably expressing CXCR4 were transiently transfected with scramble siRNA (control) or CyPA-specific siRNA. After the transfection, chemotactic migration of the cells toward CXCL12 gradients was assessed. Values represent the mean ± S.E. of three independent experiments. *, p < 0.05, comparing to the control cells with the same concentration of CXCL12 treatment. The Western blots of CyPA indicate knockdown of CyPA in the CyPA-specific siRNA-transfected cells. The Western blots of tubulin indicate an equal cell number in both transfections. B, effect of CsA on CXCL12-induced chemotaxis in HEK203 cells stably expressing CXCR4. Cells were treated with vehicle or CsA (10 μM) before chemotactic migration of the cells toward CXCL12 gradients was assessed. Values represent the mean ± S.E. of three independent experiments. *, p < 0.05, comparing to the control cells with the same concentration of CXCL12 treatment. C, effect of CsA on CXCL12-induced chemotaxis in Jurkat T cells. Cells were treated with vehicle or CsA (10 μM) before chemotactic migration of the cells toward CXCL12 (1 nM) was assessed. Values represent the mean ± S.E. of three independent experiments. *, p < 0.05, comparing to the control cells with the same treatment.

CXCL12-induced activation and nuclear translocation of ERK1/2, and in chemotactic cell migration toward CXCL12 gradients.

A previous study has shown that CyPA is directly associated with the prolactin receptor (50), a member of the class I cytokine receptor family. However, there is no report for the association of the intracellular CyPA with any chemokine receptors until the present study showing interaction between CyPA and CXCR4 in both in vitro and in vivo systems. This interaction appears to be specific, in that a recombinant form of the highly homologous cyclophilin family member CyPB failed to interact with CXCR4 (data not shown). At present, we do not know the molecular determinants mediating recruitment of CyPA to CXCR4. However, CyPA is thought to bind to target proteins containing X-Pro motifs (35), which are embedded in both the N- and C-terminal domains of CXCR4 and other chemokine receptors, thereby suggesting that the X-Pro motif is likely the CyPA binding site on CXCR4. Although our in vitro binding results showed that the purified CyPA formed a complex with both the GST-conjugated C- and N-terminal domains of CXCR4, it appears that the C-terminal domain of CXCR4 is exclusively necessary for its interaction with CyPA in intact cells as evidenced by the result that deletion of the C-terminal domain of CXCR4 robustly inhibited the receptor association with CyPA. Whereas the in vitro interaction between CyPA and the GST-CXCR4 N-terminal domain are reminiscent of the previous report that cyclophilin-18 binds to the N-terminal domain of the chemokine receptor CCR5 and inhibits fusion and infection of T cells and macrophages by R5 viruses but not by X4 viruses (51), in our preliminary studies, we did not observe any signaling (e.g. calcium mobilization) induced by CyPA when incubating the purified CyPA with HEK293 cells stably expressing CXCR4 (data not shown), thus it remains elusive for the function of CyPA binding to the N terminus of CXCR4 in intact cells.

Until the present study, little is known about the precise subcellular localization of CyPA in the cytoplasm. In contrast, CyPB, a closely related cyclophilin, possesses a signal sequence that directs it to the endoplasmic reticulum in the cytoplasm (52). Here we demonstrate that CyPA was co-localized with Rab5 in the early endosomes where it formed a complex with the internalized CXCR4. Moreover, we observed that CyPA underwent nuclear translocation in response to CXCR4 activation. By quantitative measurement of the endosomal CyPA versus the nuclear CyPA, we clearly demonstrated that CyPA underwent nuclear translocation from the endosomal compartments in response to ligand stimulation of CXCR4. Interestingly, a recent study has shown that CyPA co-localizes with Rab24 in the nucleus (53). This data, together with our data showing the co-localization of CyPA with Rab5, indicate that CyPA is associated with different Rab GTPases in the cytoplasm and nucleus.

At this moment, it is unclear how CyPA undergoes translocation from the early endosome to the nucleus in response to CXCR4 activation. However, CyPA has been shown to bind through its PPIase domain to the cytoplasmic dynein-dynactin motor protein complex (49, 54), which links early endosomes and is involved in transport of cytoplasmic cargos to the nucleus (55, 56), thereby suggesting movement of CyPA from the early endosome to the nucleus along microtubules (55). More importantly, we demonstrated that transportin 1, a newly identified importin family member, was required for the CyPA...
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CXCR4-evoked phosphorylation of CyPA may play a role in its nuclear translocation.

The most intriguing functional interaction between CXCR4 and CyPA is the requirement of CyPA in CXCR4-mediated nuclear export of hnRNP A2. HnRNP A2, a 36-kDa protein, belongs to the hnRNP family that comprises at least 24 members that are among the most abundant nuclear proteins and play an important role in all steps of mRNA metabolism (59). Although hnRNP A2 is mainly nuclear, it shuttles rapidly between nuclear and cytoplasmic compartments. This has been implicated in trafficking of RNA containing a cis-acting element (60). The mechanism of hnRNP A2 nuclear import has been ascribed by analogy with hnRNP A1, a closely related hnRNP that has been studied extensively. Like hnRNP A1, which possesses a M9 motif that serves as a binding site for two transport receptors, transportin 1 and transportin 2b (40, 61, 62), a M9 domain (296–335) is embedded within the glycine-rich domain in the C terminus of hnRNP A2, thereby suggesting that transportin 1 and transportin 2b may mediate the nuclear import of hnRNP A2. In the present study, we demonstrate that CyPA forms a complex with hnRNP A2 and was required for CXCR4-mediated nuclear export, thereby suggesting that CyPA plays an important regulatory role in the trafficking of hnRNP A2. Interestingly, CyPA has been shown to promote the nuclear export of a zinc finger protein (41). It would be of interest to elucidate how CyPA is involved in the protein nuclear export.

Another intriguing functional interaction between CXCR4 and CyPA is the involvement of CyPA in CXCR4-evoked ERK1/2 activation and nuclear translocation. We demonstrate that CXCR4-mediated ERK1/2 activation was reversed by knockdown of CyPA, by overexpression of a PPIase-deficient mutant form of CyPA (R55A), and by two cyclophilin inhibitors, CsA, which inhibits both the PPIase activity of CyPA and calcineurin (29) and SFA, which inhibits the PPIase activity without affecting calcineurin activity (64). Our data are consistent with previous reports that SFA inhibited macrophage colony-stimulating factor-induced ERK1/2 activation in macrophages (32, 65) and that CsA inhibited ERK1/2 activation by plate-derived growth factor (19). Because SFA inhibits the phosphorylation of Raf-1 (32), it is hypothesized that the upstream signaling molecules, rather than the ERK1/2 itself are regulated by CyPA. Moreover, we showed that CXCR4-mediated ERK1/2 nuclear translocation was blocked by CyPA RNAi or CsA. Because the nuclear translocation of ERK1/2 is critical for the nuclear translocation. Transportin 1 binds to M9-motif containing proteins, such as hnRNP A1, and mediates their nuclear import (40, 57). By comparing the protein sequence between CyPA and the M9 motif of hnRNP A1, we identified a M9-like motif embedded in the glycine-rich region on CyPA. Moreover, we demonstrated that CyPA formed a complex with transportin 1 and that knockdown of transportin 1 blocked CXCR4-mediated CyPA nuclear translocation, thereby suggesting a critical role of transportin 1 in CyPA nuclear translocation.

Because CyPA does not possess a classical nuclear localization signal as does CyPB (39), it has long been a mystery for the mechanism of CyPA nuclear translocation until the present study. Now we reasonably postulate that CyPA possesses a M9 motif that directs its nuclear translocation through transportin 1, whereas CyPB possesses a classical nuclear localization signal that may direct its nuclear translocation through the importins α and β complex (58).

We demonstrated for the first time that CyPA was phosphorylated upon ligand stimulation of CXCR4, and truncation of the C-terminal domain of CXCR4 significantly inhibited the receptor-mediated CyPA phosphorylation. At this moment, we are unable to define the phosphorylation site(s) on CyPA and protein kinase(s) involved in CyPA phosphorylation. However, based on the results that truncation of the C-terminal domain of CXCR4 not only inhibited the receptor association with CyPA but also inhibited CyPA phosphorylation, its association with transportin 1, and nuclear translocation, we propose that

FIGURE 9. CyPA was not involved in the endocytosis of CXCR4. HEK293 cells stably expressing Myc-CXCR4 were transiently transfected with scramble siRNA (control) or CyPA-specific siRNA were treated with or without CXCL12 (10 nM) for 30 min before being incubated with a monoclonal anti-Myc antibody followed by a FITC-conjugated anti-mouse secondary antibody and were subjected to fluorescence-activated cell sorting analysis. The staining of cells in the absence of primary CXCR4 antibody (thick solid line) or with CXCR4 antibody in cells incubated with (thick solid line) or without (thin solid line) CXCL12 (10 nM) are shown. The Western blot results are representative of three independent experiments showing that CyPA was knocked down by transfection of the specific siRNA but not by the scramble siRNA (control).
activation of several transcription factors that regulate cell proliferation and migration (16, 17), our data suggest that CyPA plays a role in CXCR4-mediated cell proliferation.

Finally, we demonstrated that CyPA RNAi or CsA treatment attenuated CXCR4-mediated cell migration toward CXCL12 gradients. These data are consistent with the previous report (33) that CsA inhibits the migration of dendritic cells out of skin and into the secondary lymphoid organs. Although it is largely unclear why CyPA is involved in chemotaxis, several possible mechanisms are proposed. First, CyPA may play a role in CXCL12-induced chemotaxis through regulating the receptor signaling, as increasing lines of evidence have demonstrated the critical role of the MAPK signaling pathway in chemotactic cell migration (19, 20, 22, 23, 68–70). For example, CXCL12-induced chemotaxis was inhibited by the dominant negative mutant of Ras or Rac, the upstream molecules of the ERK1/2 signaling pathway, as well as by the MEK inhibitor PD98059 (36). Because both CyPA siRNA and pharmacological inhibitors (CsA and SFA) remarkably reduced the CXCR4-mediated ERK1/2 activation, it is likely that regulation of the ERK1/2 activity may be one of the mechanisms for the involvement of CyPA in chemotaxis. Second, the cytoplasm–nuclear transport activity of CyPA may play a role in chemotaxis. We showed that stimulation of CXCR4 induced translocation of CyPA into the nucleus where it formed a complex with hnRNP A2 and played a role in hnRNP A2 nuclear export. Although direct evidence for the involvement of hnRNP A2 in chemotaxis is lacking, several other hnRNP family members have been shown to be involved in cell migration. For example, hnRNP K interacts with N-WASP and plays a role in filopodia formation, cell spreading, and migration (63, 66), while hnRNP S1 forms a complex with vimentin intermediate filaments in migrating cells (67), thereby suggesting a role of these hnRNP proteins in cytoskeletal organization and cell migration. It would be of interest to investigate whether hnRNP A2 is involved in chemotaxis and whether hnRNP K and S1 are regulated by CyPA.

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