
Structural basis for the coordinated regulation of transglutaminase 3 by guanine nucleotides and calcium/magnesium.

Bijan Ahvazi, Karen M. Boeshans, William Idler, Ulrich Baxa, Peter M. Steinert, and Fraydoon Rastinejad

This paper has been withdrawn.

VOLUME 280 (2005) PAGES 27195–27204

Methionine sulfoximine treatment and carbon starvation elicit Snf1-independent phosphorylation of the transcription activator Gln3 in Saccharomyces cerevisiae.

Jennifer J. Tate, Rajendra Rai, and Terrance G. Cooper

PAGES 27196 –27197:
Under “Materials and Methods,” subheading “Phosphatase Treatment,” the first sentence should read: “Crude protein extracts were prepared as described above with the following extraction buffer: 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and a commercial mixture of protease inhibitors (Roche Applied Science).”

VOLUME 281 (2006) PAGES 9400 –9406

Formation, structure, and dissociation of the ribonuclease S three-dimensional domain-swapped dimer.

Jorge P. López-Alonso, Marta Bruix, Josep Font, Marc Ribó, Maria Vilanova, Manuel Rico, Giovanni Gotte, Massimo Libonati, Carlos González, and Douglas V. Laurens

PAGE 9400:
Add the following to the grant support footnote: This work was also supported by Spanish “Ministerio de Educacion y Ciencia” Grant GEN2003-20642-C09-03.

VOLUME 281 (2006) PAGES 23445–23455

Human DNA polymerase N (POLN) is a low fidelity enzyme capable of error-free bypass of 5S-thymine glycol.

Kei-ichi Takata, Tatsuhiko Shimizu, Shigenori Iwai, and Richard D. Wood

PAGE 23447:
In the left column, lines 6 –8 (under “Experimental Procedures”), the sentence reading “The oligonucleotide containing an AP site or 5R-thymine glycol were purchased from Glen Research” should instead read: “The phosphoramidite precursors for an AP site analog and a 5R-thymine glycol were purchased from Glen Research (Sterling, VA), incorporated into 30-mers by the University of Pittsburgh oligonucleotide synthesis facility, and gel-purified.”

PAGE 23449:
In the legend for Fig. 4, the second sentence currently reading “The first template base denoted by X was changed from A (lanes 1–4) to C (lanes 5–8), G (lanes 9–12), and T (lanes 13–16)” should instead read: “The first template base denoted by X was changed from G (lanes 1–4) to T (lanes 5–8), A (lanes 9–12), and C (lanes 13–16).”

PAGE 23449:
In Table 1, the last entry in the left column, currently reading “Insertion opposite 5S-Tg” should instead read: “Insertion opposite 5R-Tg.”

VOLUME 281 (2006) PAGES 37302–37310

Antiangiogenic antithrombin blocks the heparan sulfate-dependent binding of proangiogenic growth factors to their endothelial cell receptors. EVIDENCE FOR DIFFERENTIAL BINDING OF ANTIANGIOGENIC AND ANTICOAGULANT FORMS OF ANTITHROMBIN TO PROANGIOGENIC HEPARAN SULFATE DOMAINS.

Weiqing Zhang, Richard Swanson, Yan Xiong, Benjamin Richard, and Steven T. Olson

PAGE 37302:
Benjamin Richard was supported by a fellowship from the Fondation pour la Recherche Médicale.
Chemokine CXCL12 induces binding of ferritin heavy chain to the chemokine receptor CXCR4, alters CXCR4 signaling, and induces phosphorylation and nuclear translocation of ferritin heavy chain.

Runsheng Li, Cherry Luo, Marjelo Mines, Jingwu Zhang, and Guo-Huang Fan

PAGE 37616:

The first line of the support footnote should read as follows (with mention of Veterans Affairs Merit Award and SNRP grant deleted): “This work was supported by Science and Technology Commission of Shanghai Municipality Project Grant 04DZ14902 and Research Center for Minority Institute Grant RR03032-19.”

ZHX proteins regulate podocyte gene expression during the development of nephrotic syndrome.

Gang Liu, Lionel C. Clement, Yashpal S. Kanwar, Carmen Avila-Casado, and Sumant S. Chugh

PAGE 39689:

The first two lines of the legend for Fig. 7 should read as follows: “FIGURE 7. Effect of ZHX mRNA knockdown and overexpression on protein distribution. Knockdown studies (panels A–D) were performed in cultured mouse GECs, whereas overexpression studies (panels E–L) to specifically identify tagged ZHX proteins were performed in HEK 293 cells.”
Chemokine CXCL12 Induces Binding of Ferritin Heavy Chain to the Chemokine Receptor CXCR4, Alters CXCR4 Signaling, and Induces Phosphorylation and Nuclear Translocation of Ferritin Heavy Chain

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Runsheng Li, Cherry Luo, Marjelo Mines, Jingwu Zhang, and Guo-Huang Fan

From the Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and Shanghai Jiaotong University School of Medicine, Shanghai 200025, People’s Republic of China, the Department of Veterans Affairs, Nashville, Tennessee 37212, and the Department of Biomedical Sciences, Meharry Medical College, Nashville, Tennessee 37208

Chemokine receptor-initiated signaling plays critical roles in cell differentiation, proliferation, and migration. However, the regulation of chemokine receptor signaling under physiological and pathological conditions is not fully understood. In the present study, we demonstrate that the CXC chemokine receptor 4 (CXCR4) formed a complex with ferritin heavy chain (FHC) in a ligand-dependent manner. Our in vitro binding assays revealed that purified FHC associated with both the glutathione S-transferase-conjugated N-terminal and C-terminal domains of CXCR4, thereby suggesting the presence of more than one FHC binding site in the protein sequence of CXCR4. Using confocal microscopy, we observed that stimulation with CXCL12, the receptor ligand, induced colocalization of the internalized CXCR4 with FHC into internal vesicles. Furthermore, after CXCL12 treatment, FHC underwent time-dependent nuclear translocation and phosphorylation at serine residues. By contrast, a mutant form of FHC in which serine 178 was replaced by alanine (S178A) failed to undergo phosphorylation, suggesting that serine 178 is the major phosphorylation site. Compared with the wild type FHC, the FHC-S178A mutant exhibited reduced association with CXCR4 and constitutive nuclear translocation. We also found that CXCR4-mediated extracellular signal-regulated kinase 1/2 (ERK1/2) activation and chemotaxis were inhibited by overexpression of wild type FHC but not FHC-S178A mutant, and were prolonged by FHC knockdown. In addition to CXCR4, other chemokine receptor-initiated signaling appeared to be similarly regulated by FHC, because CXCR2-mediated ERK1/2 activation was also inhibited by FHC overexpression and prolonged by FHC knockdown. Altogether, our data provide strong evidence for an important role of FHC in chemokine receptor signaling and receptor-mediated cell migration.

Chemokines comprise a large family of low molecular mass (8–10 kDa) proteins with chemotactic and proactivatory effects on different leukocyte lineages. Approximately 40 chemokines have been identified and several studies have established their central role in a number of physiological situations, including T helper cell responses, hematopoiesis, homeostasis, and angiogenesis (1). Chemokines mediate their biological effects by binding to specific seven-transmembrane domain G protein-coupled receptor subtypes, designated CXCR1 through CXCR6, CCR1 through CCR11, XCR1, and CX3CR1, based on their specific preference for certain chemokines (2, 3). Among these chemokine receptors, CXCR4 has received a great deal of attention because this receptor subtype plays a role not only in leukocyte homing but also in human immunodeficiency virus infection, development of the immune and central nervous systems, and cancer metastasis (4–9). Its ligand, CXCL12, which also binds to RDC1 that is proposed to rename as CXCR7 (10), has been found to induce migration of peripheral blood lymphocytes (11), CD34+ progenitor cells (4), and pre- and pro-B cell lines (12). Mice embryos lacking either the CXCL12 protein or its receptor CXCR4 display multiple lethal defects, including abnormalities in B cell lymphopoiesis and bone marrow myelopoiesis, lack of blood vessel formation in the gut, severe ventricular septal defects, and altered cerebellar neuron migration (5, 11, 13).

Stimulation of CXCR4 by its ligand triggers various intracellular signaling cascades (14–18), including activation of extracellular signal-regulated kinase (ERK1/2), a member of the mitogen-activated protein kinase (MAPK) family that plays an important role in cell proliferation, differentiation, and migration (18–20). However, the other two MAPK family members, c-Jun N-terminal kinase (JNK) and p38 MAPK, are not activated by stimulation of CXCR4 (18–20). Several important diseases, such as cardiovascular disease, allergic inflammatory disease, transplantation, neuroinflammation, cancer, and human immunodeficiency virus-associated disease, have been linked to inappropriate activation of the chemokine network (21). Whereas the mechanisms by which alterations of chemokine

These abbreviations were used: ERK1/2, extracellular signal-regulated kinase 1/2; FHC, ferritin heavy chain; TNF, tumor necrosis factor; CXCL12, CXC chemokine 12; GST, glutathione S-transferase; HEK293 cells, human embryonic kidney cells; MAPK, mitogen-activated protein kinase; HA, hemagglutinin; siRNA, small interfering RNA; FITC, fluorescein isothiocyanate; FACS, fluorescence cell sorter; MFI, mean fluorescence intensity; JNK, c-Jun N-terminal kinase.

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† To whom correspondence should be addressed. Tel.: 615-327-6363; Fax: 615-327-6757; E-mail: gfan@mmc.edu.
Ferritin Regulates CXCR4 Signaling

EXPERIMENTAL PROCEDURES

Plasmids—Plasmids encoding Myc-CXCR4, HA-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). For the construction of CXCR4 into pRC/CMV vector, the cDNA of CXCR4 was amplified by PCR from HA-CXCR4 and the amplified fragment was inserted into the HindIII site of the pRC/CMV vector. For the construction of FHC in pcDNA3.1, the cDNA of FHC was amplified by PCR from the cDNA library of HEK293 cells. The amplified product was inserted into BamHI and XbaI sites of pcDNA3.1. The GST-conjugated CXCR4 N terminus was constructed by amplifying the receptor N-terminal fragment (1–32 amino acid) using PCR and insertion of this fragment into the BamHI and XbaI sites of the pGEX/KG vector. Two specific FHC siRNAs, FHC siRNA-1 and siRNA-2, were used for this study. FHC siRNA-1 targeting control siRNA (sc-37007) consisting of a scrambled sequence that will not lead to the specific degradation of any cellular message, were purchased from Santa Cruz Biotechnology, Inc. Mutagenesis of specific FHC serine 178 to alanine (S178A) was conducted by PCR-directed mutagenesis.

Cell Culture and Transfection—Human embryonic kidney (HEK293) cells and HeLa cells were grown in Dulbecco’s modified Eagle’s medium, containing 10% fetal bovine serum and a 1:100 dilution of penicillin/streptomycin, at 37 °C in a humidified atmosphere of 95% air and 5% CO2. Cells were transfected with plasmids encoding CXCR2, CXCR4, or FHC using Lipofectamine Plus reagent (Invitrogen). HEK293 cells stably expressing CXCR4 or CXCR2 were selected with 560 μg/ml Geneticin and evaluated for receptor expression by radioligand binding assay using 125I-CXCL12 or 125I-CXCL8, respectively. Jurkat T cells were grown in RPMI medium containing 10% fetal bovine serum and a 1:100 dilution of penicillin/streptomycin, at 37 °C in a humidified atmosphere of 95% air and 5% CO2. For transient transfection of FHC constructs, 20 × 10^6 cells were electroporated with 20 μg of DNA, in 0.4 ml of preheated (37 °C) RPMI 1640 containing 10 mM dextrose and 0.1 mM dithiothreitol, using Gene Pulser (Bio-Rad), and a setting of 234 V and 1000 microfarads, with a 0.4-cm preheated cuvette (Bio-Rad). Transfected cells were resuspended in growth medium and used for experiments 16–24 h after transfection.

In Vitro Binding Assay—The GST or GST-CXCR4 C terminus fusion protein or GST-CXCR4 N terminus fusion protein 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 Na3VO4, 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 FHC fusion proteins were purified from bacteria strain DH5α transformed with plasmids encoding His-FHC. Briefly, E. coli were cultured at 37 °C for 12 h, then isopropyl β-D-thiogalactopyranoside was added and incubation was continued for another 3 h to induce protein expression. The bacteria were resuspended in TMP 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-nitriotriacetic acid-agarose by gravity flow, and His-FHC was eluted with elution buffer containing 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-FHC 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-mercaptoproline in the above pathological conditions remain largely unknown.

Ferritin is a ubiquitous and highly conserved iron-binding protein. Cytosolic ferritin is composed of two types of subunits, termed heavy chain (FHC) and light chain (FLC) that assemble together to form a 24-subunit protein. FLC contributes to the nucleation of the iron core, whereas FHC possesses a ferroxidase activity that catalyzes the oxidation of iron into a ferrihydrite mineral core in the center of the protein (22). Perturbations in cellular FHC are emerging as an important element in the pathogenesis of disease. These changes in FHC are important not only in the classic diseases of iron acquisition, transport, and storage, but also in diseases characterized by inflammation, infection, injury, and repair. Among these are most common diseases such as Parkinson disease (23), Alzheimer disease (24), rheumatoid arthritis (25), pulmonary inflammation (26), atherosclerosis (27), and cancers (28). In cellular models, FHC is induced by inflammatory cytokines (29, 30), reactive oxygen species (31), growth factors (32), and secondary messengers (33). The transcription factor, nuclear factor-κB, plays a critical role in the transcriptional regulation of FHC (33). A recent study demonstrated that overexpression of FHC inhibited tumor necrosis factor α (TNFα)-induced sustained JNK activation and, thereby, apoptosis triggered by TNFα (33). This suggested an involvement of FHC in the signal transduction initiated by TNFα.

Although FHC is involved in a number of diseases in which chemokines and their receptors are dysregulated, the potential role of this protein in chemokine receptor signaling has not been addressed. In the present study, we demonstrate that FHC stimulates CXCR4 expressing cells induced FHC phosphorylation and, thereby, apoptosis triggered by TNFα/H9251. This suggested an involvement of FHC in the signal transduction initiated by TNFα.

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**FIGURE 1. In vivo and in vitro association of FHC with CXCR4.** A. HEK293 cells stably expressing Myc-CXCR4 were exposed to CXCL12 (10 nM) for the indicated time intervals, and CXCR4 was immunoprecipitated (IP) from the cell lysate using an anti-Myc antibody. In a parallel experiment, parental HEK293 cells were treated with CXCL12 for 10 min, and immunoprecipitation was performed as described above (mock). Co-precipitated proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose membrane. Potentially associated FHC proteins were detected by Western blotting using a specific FHC antibody. B. After the immunostaining, the cells were counterstained with Hoechst 33342 to visualize nuclei. 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.

**Co-immunoprecipitation and Western Blot**—HEK293 cells stably expressing Myc-CXCR4 were treated with carrier buffer (control) or CXCL12 (10 nM) for the indicated time intervals, washed three times 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-Myc antibody (Santa Cruz Biotechnology, Inc.) for overnight incubation at 4 °C. Protein A/G-antibody-antigen 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 12% SDS-polyacrylamide gel buffer and incubated with an antibody mixture containing an anti-Myc antibody (Santa Cruz Biotechnology, Inc.) and a rabbit polyclonal anti-FHC 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, Eugene, OR) and a FITC-conjugated anti-rabbit antibody (Molecular Probes) for 30 min. The gels were exposed to X-ray film.

**FIGURE 2. FHC colocalized with CXCR4.** HeLa cells transiently expressing Myc-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-Myc monoclonal antibody and a rabbit polyclonal anti-FHC antibody, followed by a secondary antibody mixture containing a Cy3-conjugated anti-mouse antibody and a FITC-conjugated anti-rabbit antibody. After immunostaining, the cells were counterstained with Hoechst 33342 to visualize nuclei. Confocal images demonstrating the subcellular localization of Myc-CXCR4 (red), FHC (green), and colocalization of these two proteins (yellow) are shown. Small arrows indicate colocalization between CXCR4 and FHC. Large arrows indicate nuclear translocation of FHC.
and the proteins on the gel were transferred to nitrocellulose membranes (Bio-Rad). Co-precipitated proteins were detected by Western blotting using specific antibodies.

**MAPK Assay**—HEK293 cells stably expressing CXCR4 or CXCR2 were transiently transfected with vector (control), FHC expressing vector, control siRNA, or FHC-specific siRNA. Cells were treated with CXCL12 (10 nM) or CXCL8 (10 nM) for different time intervals. Cells were lysed in RIPA buffer. Lysates containing equal amounts of protein 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 ERK2 antibody to confirm equal loading.

**FHC 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. FHC proteins were immunoprecipitated from the cell lysates. Equal amounts of immunoprecipitates were subjected to SDS-PAGE. Phosphorylated FHC proteins were detected by Western blot analysis with an anti-phosphoserine antibody (BD Transduction Laboratories). The same blots were stripped and reprobed with a FHC antibody to confirm equal loading.

**Densitometry Analysis of Western Blots**—The relative amount of the Western blot bands was measured by densitometry analysis using NIH Image software. 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.

**Chemotaxis Assay**—The migration of HEK293 cells stably expressing CXCR4 and transiently transfected with the FHC construct or FHC-specific siRNA was evaluated using the chemotaxis assay. Briefly, polycarbonate filters (10-μm pore size) coated with 2 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 × 10⁵/100 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 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.

**Fluorescence-activated Cell Sorting (FACS) Analysis**—HEK293 cells stably expressing Myc-CXCR4 were transfected with vector (control), plasmids encoding FHC or FHC-S178A, control siRNA, or FHC-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 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).

**RESULTS**

In a recent effort to identify CXCR4-interacting proteins, we performed proteomic studies by treating HEK293 cells stably expressing Myc-CXCR4 with or without CXCL12, immunoprecipitating CXCR4 from the cell lysates, and analyzing the co-immunoprecipitated proteins by mass spectrometry. We have identified a number of proteins that potentially interact with CXCR4, and have characterized some of them (35). One of the proteins potentially associated with CXCR4 is FHC (data not shown). To confirm the association of FHC with CXCR4, we repeated the co-immunoprecipitation experiment after treating HEK293 cells stably expressing Myc-CXCR4 with CXCL12 (10 nM) for different time intervals and detected the possible association of FHC with CXCR4 by Western blot analysis using a specific anti-FHC antibody. As shown in Fig. 1, A and B, immunoprecipitation of CXCR4 from HEK293 cells revealed a weak basal association of the receptor with FHC prior to ligand treatment. CXCL12 (10 nM) treatment resulted in a time-dependent increase in the association of FHC with CXCR4, which peaked at 30 min and returned to near basal level after 120 min of incubation. By comparing the density of
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FIGURE 4. Phosphorylation-deficient mutant of FHC underwent reduced association with CXCR4 but constitutive nuclear translocation. A, HEK293 cells stably expressing Myc-CXCR4 were transiently transfected with plasmids encoding HA-FHC or HA-FHC-S178A. Cells were exposed to CXCL12 (10 nM) for 30 min. CXCR4 was immunoprecipitated (IP) from the cell lysate using an anti-Myc antibody and co-precipitated HA-tagged FHC proteins were detected by Western blotting (IB). The blots were stripped and reprobed with a specific anti-Myc antibody to confirm equal loading. B, quantification of the density of bands representing co-immunoprecipitated HA-FHC was assessed by densitometric scanning. Data are mean ± S.E. from three independent experiments. C, HeLa cells transiently transfected with plasmids encoding HA-FHC or HA-FHC-S178A were treated with or without CXCL12 (10 nM) for 30 min before being fixed in methanol. Cells were incubated with a rabbit anti-HA antibody for 30 min followed by a FITC-conjugated anti-rabbit antibody for 30 min. After immunostaining, the cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Confocal microscopy images demonstrating the subcellular localization of HA-tagged FHC or FHC-S178A mutant are shown. Arrows indicate nuclear translocation of FHC. Bars, 10 μm.

CXCL12-induced CXCR4 internalization (data not shown). These data suggest that CXCR4 internalization is required for its association with FHC.

The C terminus of chemokine receptors has been known to interact with various intracellular proteins (35, 37). To test whether the CXCR4 C terminus is involved in receptor association with FHC, we incubated purified GST (control) or GST-CXCR4 C terminus fusion proteins with HEK293 cell lysates and determined co-precipitated FHC by immunoblotting. To assess the possible interaction of the receptor N terminus with FHC, we also incubated purified GST-CXCR4 N terminus fusion proteins with HEK293 cell lysates and detected possible co-precipitated FHC by immunoblotting. As shown in Fig. 1D, the GST-CXCR4 C terminus fusion proteins but not the GST alone (control) associated with FHC. Unexpectedly, we also observed a robust association of the GST-CXCR4 N terminus fusion proteins with FHC, suggesting that both the C terminus and N terminus contain FHC binding sites.

The in vitro and in vivo associations of FHC with CXCR4 suggest that these two proteins are co-distributed. To visualize the subcellular localization of CXCR4 and FHC, we treated HeLa cells transiently expressing Myc-CXCR4 with CXCL12 (10 nM) for different time intervals (0, 15, and 30 min), immunostained both Myc-CXCR4 and FHC, and performed confocal microscopy. As shown in Fig. 2, prior to ligand stimulation, CXCR4 proteins were exclusively expressed on the cell surface, whereas FHC was predominantly expressed in the cytoplasm. In response to ligand treatment for 15 min, a small proportion of Myc-CXCR4 receptors were internalized and partially colocalized with FHC in the cytoplasm (small arrows indicated). Ligand treatment for 30 min induced more pronounced CXCR4 internalization and colocalization with FHC (small arrows indicated). Interestingly, we observed significant nuclear translocation of FHC after CXCL12 treatment for 15 min (large arrows indicated) and maximal nuclear translocation was observed after 30 min of ligand stimulation (large arrows indicated).

FHC is enriched with serine and threonine residues in the protein sequence. This encouraged us to assess whether FHC undergoes phosphorylation after CXCL12 treatment. We treated HEK293 cells stably expressing CXCR4 with CXCL12 (10 nM) for different time intervals (0, 5, 10, 30, and 60 min), immunoprecipitated FHC from the cell lysates with anti-FHC antibody (Santa Cruz Biotechnology, Inc.), and detected FHC phosphorylation. We have previously shown that CXCR4 undergoes internalization in response to CXCL12 stimulation in a time-dependent manner and maximal receptor internalization occurs after exposure to CXCL12 for 30 min (35). The corresponding peak CXCR4 internalization time and peak FHC-CXCR4 association time (30 min) suggests that only the internalized CXCR4 associates with FHC. To test this hypothesis, the internalization of Myc-CXCR4 in HEK293 cells was inhibited by pretreatment of the cells with 0.4 mM sucrose for 1 h at 37 °C in medium without fetal bovine serum (36) and then the co-immunoprecipitation assay was performed to examine the association of CXCR4 with FHC after CXCL12 (10 nM) treatment for 30 min. As shown in Fig. 1C, CXCL12 treatment resulted in a significant increase in the association of CXCR4 with FHC in the control cells. In contrast, the ligand-induced association was remarkably reduced in the sucrose-pretreated cells. In a parallel experiment, we observed that sucrose pretreatment inhibited


the immunoblots for FHC co-precipitated with CXCR4 from 1 ml of cell lysate and the immunoblots of FHC in 40 μl of cell lysate, we estimated that 20% of the FHC proteins were co-immunoprecipitated with CXCR4. The CXCR4–FHC interaction is obviously specific because using the anti-Myc antibody failed to pull down any FHC from the cell lysate of parental HEK293 cells, which do not express CXCR4 endogenously.

We have previously shown that CXCR4 undergoes internalization in response to CXCL12 stimulation in a time-dependent manner and maximal receptor internalization occurs after exposure to CXCL12 for 30 min (35). The corresponding peak CXCR4 internalization time and peak FHC–CXCR4 association time (30 min) suggests that only the internalized CXCR4 associates with FHC. To test this hypothesis, the internalization of Myc–CXCR4 in HEK293 cells was inhibited by pretreatment of the cells with 0.4 mM sucrose for 1 h at 37 °C in medium without fetal bovine serum (36) and then the co-immunoprecipitation assay was performed to examine the association of CXCR4 with FHC after CXCL12 (10 nM) treatment for 30 min. As shown in Fig. 1C, CXCL12 treatment resulted in a significant increase in the association of CXCR4 with FHC in the control cells. In contrast, the ligand-induced association was remarkably reduced in the sucrose-pretreated cells. In a parallel experiment, we observed that sucrose pretreatment inhibited
proteins did not undergo phosphorylation in response to ligand treatment, suggesting that serine 178 is likely the phosphorylation site. 

Among the several serine residues in the protein sequence of FHC, serine 178 was predicted to be a phosphorylation site by PROSITE. To assess possible phosphorylation of FHC at this residue, we generated a mutant FHC in which serine 178 was replaced with alanine (S178A). The HA-tagged wild type FHC or FHC-S178A mutant was overexpressed in HEK293 cells stably expressing Myc-CXCR4. Cells were treated with CXCL12 (10 nM) or FHC-S178A mutant was overexpressed in HEK293 cells stably expressing CXCR4. Cells were treated with CXCL12 (10 nM) for the indicated time intervals and ERK1/2 phosphorylation was determined by Western blot analysis using an anti-phospho-ERK1/2 antibody. The blots were stripped and reprobed with an ERK2 antibody to confirm equal loading. In a parallel experiment, Western blot analysis of FHC expression was performed to determine FHC expression levels in these transfected cells. B, quantification of the density of bands representing ERK1/2 phosphorylation in cells transfected with vector, wild-type FHC construct, and FHC-S178A construct was assessed by densitometric scanning. Data are mean ± S.E. of three independent experiments. C, quantification of the density of bands representing ERK1/2 phosphorylation in cells transfected with control siRNA, FHC siRNA-1 and FHC siRNA-2 were assessed by densitometric scanning. Data are mean ± S.E. of three independent experiments. *, p < 0.05 compared with control cells with the same treatment. RNAi, RNA interference.

FIGURE 5. FHC was involved in CXCR4-mediated ERK1/2 activation in HeLa cells. A, cells were transiently transfected with vector (control), wild-type FHC construct, FHC-S178A construct, control siRNA, or FHC-specific siRNA-1 and siRNA-2. Cells were treated with CXCL12 (10 nM) for the indicated time intervals and ERK1/2 phosphorylation was determined by Western blot analysis using an anti-phospho-ERK1/2 antibody. The blots were stripped and reprobed with an ERK2 antibody to confirm equal loading. In a parallel experiment, Western blot analysis of FHC expression was performed to determine FHC expression levels in these transfected cells. B, quantification of the density of bands representing ERK1/2 phosphorylation in cells transfected with vector, wild-type FHC construct, and FHC-S178A construct was assessed by densitometric scanning. Data are mean ± S.E. of three independent experiments. C, quantification of the density of bands representing ERK1/2 phosphorylation in cells transfected with control siRNA, FHC siRNA-1 and FHC siRNA-2 were assessed by densitometric scanning. Data are mean ± S.E. of three independent experiments. *, p < 0.05 compared with control cells with the same treatment. RNAi, RNA interference.

To evaluate the role of FHC phosphorylation in its association with CXCR4, HEK293 cells stably expressing Myc-CXCR4 were transiently transfected with plasmids encoding HA-tagged FHC or FHC-S178A. After treatment of the cells with or without CXCL12 for 30 min, CXCR4 was immunoprecipitated from the cell lysate and associated HA-tagged FHC proteins were detected by Western blot analysis using an anti-HA antibody. As shown in Fig. 4, A and B, CXCL12 treatment for 30 min resulted in a significant increase in the association of FHC with CXCR4 over the basal level. However, the ligand-dependent association of FHC-S178A with CXCR4 was remarkably reduced, suggesting requirement of FHC phosphorylation in its association with CXCR4.

To examine the role of FHC phosphorylation in its nuclear translocation, we treated HeLa cells transiently expressing HA-tagged FHC or FHC-S178A with or without CXCL12 (10 nM) for 30 min, and immunostained the overexpressed wild type or mutant FHC with an anti-HA antibody. As shown in Fig. 4C, in cells overexpressing HA-FHC, little HA-FHC was observed in the nucleus prior to CXCL12 treatment, but CXCL12 treatment for 30 min induced a remarkable nuclear translocation (arrows indicated), consistent with that of the endogenous FHC shown in Fig. 2. Strikingly, in the cells overexpressing HA-FHC-S178A, a proportion of HA-FHC-S178A proteins were observed in the nucleus prior to CXCL12 treatment and ligand treatment did not result in more FHC-S178A in the nucleus. These suggest constitutive nuclear translocation of the FHC-S178A mutant.

CXCR4-mediated activation of ERK1/2 signaling cascades plays an important role in cell proliferation and survival (38). To investigate the potential role of FHC overexpression in receptor signaling, we transfected HeLa cells with vector (control), wild type FHC construct, or FHC-S178A construct and treated the cells with CXCL12 (10 nM) for different time intervals (0, 2, 5, 10, 30, and 60 min) before phosphorylation of ERK1/2 was assessed by Western blot analysis using an anti-phospho-ERK1/2 antibody (Santa Cruz Biotechnology, Inc.). The same blots were reprobed with an anti-ERK2 antibody (Santa Cruz Biotechnology, Inc.) to confirm equal loading. As shown in Fig. 5, A and B, ligand stimulation induced a typical bell-shape time-dependent ERK1/2 phosphorylation in control transfected cells, which peaked at 5–10 min and returned to basal level after 30 min of ligand treatment. In contrast, in the cells overexpressing FHC, CXCL12-induced ERK1/2 phosphorylation was significantly inhibited. Overexpression of the FHC-S178A mutant did not result in inhibition of CXCL12-
induced ERK1/2 activation. Western blot of FHC showed that the expression level of FHC or FHC-S178A was significantly elevated in the overexpressing cells compared with that in the control cells. These data indicate that overexpression of FHC inhibits CXCR4-mediated ERK1/2 activation and that phosphorylation of FHC is required for this function. Based on these results, we proposed that knockdown of FHC may exert an opposite effect. To test this hypothesis, HeLa cells transiently transfected with control siRNA or two FHC-specific siRNAs (siRNA-1 and siRNA-2) were treated with CXCL12 for different time intervals and phosphorylation of ERK1/2 was assessed. As shown in Fig. 5, A and C, cells transfected with the control siRNA exhibited similar bell-shape time-dependent ERK1/2 phosphorylation as that of the control cells (transfected with empty vector). However, in the cells transfected with FHC-specific siRNA-1 or siRNA-2, CXCL12 stimulation resulted in sustained ERK1/2 phosphorylation, which peaked at 5–10 min but never returned to basal level over the entire experiment period (60 min). Western blot of FHC showed that the expression level of FHC was robustly reduced in both the specific siRNA-1 and siRNA-2, but not in expressing the empty vector, with a peak migration occurring at a concentration around 1 nM CXCL12. Also, the cellular migration response followed a typical bell-shaped curve in which the chemotaxis was inhibited at higher concentrations of CXCL12 (Fig. 7, A and B). Strikingly, cells overexpressing FHC exhibited marked attenuation of CXCR4-mediated chemotaxis. Cells overexpressing FHC-S178A mutant did not exhibit a reduced chemotactic response to CXCL12 gradients (Fig. 7, A and B). These data indicate that overexpression of FHC inhibits CXCR4-mediated chemotaxis and that phosphorylation of FHC is required for this function. Based on these findings, we proposed that knockdown of FHC might result in opposite chemotactic responses. To test this hypothesis, we transfected HEK293 cells stably expressing CXCR4 with control siRNA, FHC siRNA-1, or FHC siRNA-2 and examined chemotactic migration toward CXCL12 gradients. We observed a similar bell-shaped chemotactic in the control siRNA-transfected cells. However, cells transfected with FHC-specific siRNA-1 or siRNA-2 expressing the empty vector, with a peak migration occurring at a concentration around 1 nM CXCL12. Also, the cellular migration response followed a typical bell-shaped curve in which the chemotaxis was inhibited at higher concentrations of CXCL12 (Fig. 7, A and B). Strikingly, cells overexpressing FHC exhibited marked attenuation of CXCR4-mediated chemotaxis. Cells overexpressing FHC-S178A mutant did not exhibit a reduced chemotactic response to CXCL12 gradients (Fig. 7, A and B). These data indicate that overexpression of FHC inhibits CXCR4-mediated chemotaxis and that phosphorylation of FHC is required for this function. Based on these findings, we proposed that knockdown of FHC might result in opposite chemotactic responses. To test this hypothesis, we transfected HEK293 cells stably expressing CXCR4 with control siRNA, FHC siRNA-1, or FHC siRNA-2 and examined chemotactic migration toward CXCL12 gradients. We observed a similar bell-shaped chemotactic in the control siRNA-transfected cells. However, cells transfected with FHC-specific siRNA-1 or siRNA-2...
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FHC was involved in CXCR4-mediated chemotaxis. A, HEK293 cells stably expressing CXCR4 were transiently transfected with empty vector, FHC expression vector, or FHC−S178A expressing vector. After transfection, chemotactic migration of the cells toward CXCL12 gradients was assessed. Values represent the mean ± S.E. of three independent experiments. B, representative images showing chemotaxis of the above transfected cells in response to 1 nM CXCL12 treatment. C, HEK293 cells stably expressing CXCR4 were transiently transfected with control siRNA, FHC siRNA-1, or FHC siRNA-2. After transfection, chemotactic migration of the cells toward CXCL12 gradients was assessed. D, representative images showing chemotaxis of the above transfected cells in response to 1 nM CXCL12 treatment. Data were analyzed using Student’s paired t test. *, p < 0.05, compared with control.

The above findings indicate that FHC negatively regulates CXCR4 signaling, but the underlying mechanisms remain unclear. One possible mechanism is that cell surface expression or ligand-induced down-regulation of CXCR4 is affected by FHC overexpression or knockdown. To test this hypothesis, we transfected HEK293 cells stably expressing CXCR4 with empty vector (control), FHC expression vector, FHC−S178A expressing vector, control siRNA, FHC siRNA-1, or FHC siRNA-2 and treated the cells with or without CXCL12 (10 nM) for 30 min before FACS analysis was performed to determine cell surface CXCR4. As shown in Fig. 8, comparable levels of cell surface CXCR4 expression were observed among these transfected cells prior to CXCL12 treatment (Fig. 8, A and B). After CXCL12 treatment for 30 min, a significant CXCR4 internalization was observed in the control cells (Fig. 8A) with ~35% reduction of the mean fluorescence intensity (MFI) of CXCR4 (Fig. 8B). Cells overexpressing FHC or FHC−S178A exhibited similar CXCR4 internalization in response to CXCL12 treatment (Fig. 8A), with comparable reduction of CXCR4 MFI (~39 and ~37% for cells overexpressing FHC and FHC−S178A, respectively). However, cells transfected with the FHC-specific siRNA-1 and siRNA-2 exhibited remarkable inhibition of CXCR4 internalization (Fig. 8A), with significantly less reduction of CXCR4 MFI (~13 and ~15% for cells transfected with siRNA-1 and siRNA-2, respectively) than that of the control siRNA-transfected cells (MFI reduction of ~36%).

It should be noted that the above inhibitory roles of FHC in CXCR4 signaling were observed in non-leukocyte cell lines (HEK293 and HeLa cells). To examine whether similar roles of FHC in CXCR4 signaling could be observed in leukocyte cell lines, we used Jurkat T cells transfected with vector (control), FHC expression vector, control siRNA, or FHC-specific siRNA-1, to evaluate CXCR4-mediated ERK1/2 activation. As shown in Fig. 9, A and B, in the control cells, CXCL12 (10 nM) treatment for 10 min induced a significant increase in ERK1/2 phosphorylation, which returned to basal level after 60 min of incubation. However, cells overexpressing FHC exhibited a slightly reduced basal ERK1/2 phosphorylation and significantly reduced ERK1/2 activation in response to CXCL12 treatment. As expected, cells transfected with the FHC-specific siRNA-1 exhibited sustained ERK1/2 activation (Fig. 9, A and B), consistent with that in HeLa cells (Fig. 5).

DISCUSSION

In recent years it has been established that immune cell migration to and function at inflammatory sites is controlled by the coordinated action of the cytokine-chemokine network (39−41). Ferritin is modulated by cytokines, such as TNFα (33) and interferon γ (42), during inflammation. However, potential roles of ferritin in chemokine receptor signaling have not been addressed until the present study. Here, we demonstrate for the first time that FHC interacts with CXCR4. Several lines of evidence support this conclusion. First, FHC was co-immunoprecipitated with CXCR4 in a ligand-dependent manner. Second, the GST pull-down assay verified that the purified FHC interacted with both the C- and N-terminal domains of CXCR4. Third, confocal assay further confirmed the interaction between CXCR4 and FHC in transfected cells. Moreover, we provided evidence for the requirement of phosphorylation for FHC association with CXCR4. The CXCR4-FHC interaction is of functional significance, because CXCR4-mediated ERK1/2 activation and chemotaxis were inhibited by overexpression of FHC and were prolonged by FHC knockdown.

One of the interesting findings in this study is the physical interaction between CXCR4 and FHC. Using GST pull-down, we found that FHC interacted with both the C terminus and N terminus of CXCR4. The CXCR4 C terminus resides in the
cytoplasm and is known to associate with a number of proteins such as heat shock protein 73 (35), so it is not surprising that it interacts with FHC. However, the finding that the CXCR4 N terminus associated with FHC is unexpected, because besides its specific ligand, CXCL12, little has been found to bind to the N terminus of CXCR4 thus far. Because of the significant differences in the protein sequences of the N and C terminus, we do not predict a precise FHC binding structure. Co-immunoprecipitation of CXCR4 with FHC confirmed the interaction in intact cells. It remains unclear whether both the C and N terminus of CXCR4 are involved in receptor interaction with FHC in vivo. Moreover, in our co-immunoprecipitation experiments association of CXCR4 with FHC was increased upon CXCL12 stimulation, indicating that the interaction was inducible. The FHC-CXCR4 complex formation in response to CXCL12 stimulation may occur via several mechanisms. One possibility is that ligand stimulation results in CXCR4 internalization, which favors the association of the CXCR4 with FHC. This is supported by the confocal images showing only the internalized CXCR4 receptors colocalized with FHC, and by the co-immunoprecipitation results showing that inhibition of CXCR4 internalization by sucrose reduced FHC association with CXCR4. Another possibility is that activation of CXCR4 results in modification of FHC, such as phosphorylation, which facilitates its association with CXCR4. This is supported by the result showing that mutation of the FHC phosphorylation site inhibited its association with CXCR4. Detailed mechanisms, however, remain to be investigated.

Another interesting finding of the present study is CXCR4-mediated FHC nuclear translocation. We observed that FHC proteins were predominantly localized in the cytoplasm prior to CXCL12 treatment. In response to CXCL12 stimulation, a proportion of FHC proteins were translocated into nuclei. Although previous studies have demonstrated nuclear translocation of FHC in response to treatment with inflammatory cytokines, such as TNFα and IL-1 (43), to our knowledge, this is the first report of chemokine-induced FHC nuclear translocation. However, the mechanism by which FHC enters the nucleus and the function of nuclear ferritin remain unclear. A nuclear localization signal, or any other sequence that promotes nuclear translocation, has yet to be detected on ferritin. Analyses with deletion constructs for ferritin failed to detect any discrete region that might function as a non-consensus nuclear localization signal (44). Transfection analyses using Myc-tagged ferritin deletion constructs in corneal epithelial cells showed that, for nuclear transport in corneal epithelial cells, more than 83% of the FHC must be intact. Thus, there seems to be no specific region that serves as an...
nuclear localization signal. A ferritin chaperone, referred to as a “ferrotoid,” that may mediate nuclear translocation has been described, but it appears to be specific to avian corneal epithelial cells and, thus, may not account for the increasing number of cells in which ferritin nuclear translocation has been observed (45). In the present study, we observed a similar time frame between the phosphorylation and nuclear translocation of FHC, therefore, it would be predicted that inhibition of FHC phosphorylation may block its nuclear translocation. However, unexpectedly, we found that a phosphorylation-deficient mutant form of FHC (S178A) underwent constitutively nuclear translocation. Thus, the precise role of FHC phosphorylation in its nuclear translocation remains a question. Nuclear ferritin has been shown to protect DNA from UV- or iron-dependent DNA damage (43, 46) and to inhibit TNFα-induced apoptosis by suppressing reactive species (33). Based on the previous observations that ferritin is present in rapidly growing tumors (43), that it can be chemically cross-linked to DNA in live astrocytoma cells (43), and that it binds DNA in vitro (43, 47–49), we propose that CXCR4-mediated ferritin nuclear translocation may play a role in the receptor-mediated tumor growth. CXCR4 is expressed in a variety of cancer cell lines and tissues and plays a role in tumorigenesis (7). It would be of interest to investigate if CXCR4-mediated FHC nuclear translocation correlates with its tumorigenic activity. Also, ferritin has been observed in the nucleus of rodent neurons during development (50) and the critical role of CXCR4 in the development of the central nervous system has been addressed (13), although the underlying mechanisms remain unclear. It remains an open question whether CXCR4-mediated ferritin nuclear translocation is involved in the role of ferritin in neurodevelopment.

The most intriguing finding of the present study is the inhibitory role of FHC in CXCR4 signaling. We demonstrated that CXCR4-mediated ERK1/2 activation was reversed by overexpression of wild type but not the phosphorylation-deficient mutant of FHC, suggesting a critical role of FHC phosphorylation in its inhibitory effect on CXCR4 signaling. The inhibitory effect of FHC on CXCR4 signaling was confirmed by a parallel experiment showing that knockdown of FHC with RNA interference prolonged CXCR4-mediated ERK1/2 activation. It is likely that other chemokine receptor signaling is similarly regulated by FHC based on the inhibitory effect of FHC on CXCR2 signaling. However, mechanisms by which FHC negatively regulate chemokine receptor signaling remain to be investigated. The possibility that FHC inhibits CXCR4 expression has been excluded. Although knockdown of FHC with RNA interference inhibited CXCR4 internalization, overexpression of FHC did not result in enhancement of CXCR4 internalization. Thus, it is likely that the effect of FHC on CXCR4 internalization is not related to its inhibitory role in CXCR4 signaling. This is supported by previous studies showing little correlation between chemokine receptor internalization and receptor-mediated ERK1/2 activation (51, 52). The inhibitory effect of FHC on CXCR4 signaling is also not likely due to the inhibition of receptor-G protein coupling, because the chemokine receptor-initiated immediate signaling, such as calcium mobilization, was not affected by FHC overexpression or knockdown.3 Interestingly, recent studies demonstrated that JNK, another MAPK family member activated by inflammatory cytokines such as TNFα, was also inhibited by FHC overexpression (33). It is therefore likely that a signaling molecule commonly employed by both ERK1/2 and JNK signaling pathways is inhibited by the up-regulated FHC. Investigation of detailed mechanisms underlying the inhibitory role of FHC in chemokine-initiated ERK1/2 activation is underway. Functionally, stimulation of chemokine receptors predominantly activates the ERK1/2 pathway, which plays an important role in cell survival (53, 54), whereas stimulation by TNFα or several other cytokines predominantly activates the JNK pathway, which is involved in cell apoptosis (55). This suggests that ferritin plays a critical role in controlling the balance between cell survival and death. Under the situation of FHC up-regulation, such as inflammation and embryonic development, whether cells undergo survival or apoptosis likely depends largely on the predominance of either ERK1/2 or JNK signaling pathways.

Finally, we provide evidence for a role of FHC in chemokine receptor-mediated cell migration. We observed that overexpression of wild type but not the phosphorylation-deficient mutant of FHC attenuated CXCR4-mediated cell migration, whereas FHC knockdown enhanced the receptor-mediated cell migration. Although the underlying mechanisms remain largely unclear, the chemokine receptor-initiated signaling pathways necessary for chemotaxis might be involved. Previous
studies have demonstrated that several signaling pathways initiated by CXCR4 and other chemokine receptors are involved in chemotactic migration. These include MEK-ERK1/2 and phosphatidylinositol 3-kinase-Akt signaling cascades (19, 20, 56–58). However, we only observed that FHC overexpression inhibited CXCR4- or CXCR2-mediated ERK1/2 activation, without affecting the receptor-mediated Akt activation (data not shown), thereby suggesting that the inhibitory effect of overexpressed FHC on chemotactic migration likely results from its inhibition of CXCR4-mediated ERK1/2 activation. However, other mechanisms may not be excluded. Nevertheless, because chemokine receptor-mediated cell migration plays a crucial role in inflammation and cancer metastasis (7, 9, 59, 60), these findings may provide significant insight into the pathogenesis of these diseases.

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