Hsc/Hsp70 interacting protein (Hip) associates with CXCR2 and regulates the receptor signaling and trafficking

Guo-Huang Fanl, Wei Yangl, Jiqing Sail, and Ann Richmondl &

From l the Department of Veterans Affairs, Nashville, TN 37212-2637, and the Department of Cancer Biology, Vanderbilt University School of Medicine, Nashville, TN 37212-2175

Running title: Chemokine receptors interact with Hip

Corresponding Author:
Ann Richmond, Ph.D.
Professor of Cell Biology
Department of Cell Biology
Vanderbilt University School of Medicine
Nashville, TN 37232

Phone: 615-343-7777
FAX: 615-343-4539
Email: ann.richmond@mcmail.vanderbilt.edu
Summary

The ligand induced trafficking of chemokine receptors plays a significant role in the regulation of inflammatory processes and HIV infection. Although many chemokine receptors have been demonstrated to internalize through clathrin-coated vesicles, a process that involves the binding of arrestins to the receptors, accumulating evidence has suggested the possible existence of other regulators. In a yeast two-hybrid screening using the C-terminal domain of CXCR2 as a bait, the Hsc70-interacting protein (Hip) was identified to interact with CXCR2. Hip binds CXCR2 through its C-terminal domain binding to the C-terminal leucine-rich domain (KILAIHGLI) of CXCR2. Hip associates with CXCR2 or CXCR4 in intact cells, and agonist stimulation increases the association. Mutation of the Ile-Leu motif in the C-terminal domain of CXCR2 blocks the agonist-dependent association of the mutant receptor with Hip. Overexpression of a tetratricopeptide repeat (TPR) deletion mutant form of Hip (ΔTPR), which is unable to bind Hsc70 (Prapapanich, et al., Mol. Endocrinol. 1996, 10: 420-431), but retains the ability to bind CXCR2, does not affect CXCR2-mediated mitogen-activated protein kinase activation. However, overexpression of ΔTPR significantly attenuates the agonist-induced internalization of CXCR2 and CXCR4, and attenuates CXCR2-mediated chemotaxis. These findings open a possibility for the regulation of chemokine receptor signaling and trafficking by protein chaperone molecules.
Chemokine receptors are a family of G protein-coupled seven transmembrane receptors (GPCRs)\(^1\) that mediate inflammatory processes, hematopoiesis, angiogenesis, and some of them (e.g. CCR5 and CXCR4) are involved in AIDS pathogenesis (1-6). Like other members of GPCR superfamily, the ability of chemokine receptors to transmit signals may be rapidly attenuated. This attenuation of signaling is tightly regulated by different mechanisms, including phosphorylation-dependent desensitization and internalization (7-9). After receptor internalization, the receptor may be dephosphorylated in the internal vesicles, recycled back to the cell surface, and resensitized to a subsequent exposure of the ligand (10, 11).

Chemokine receptor internalization and recycling have been shown to play an important role in the regulation of inflammatory processes and HIV-1 infection. Studies demonstrated that the responses of neutrophils, which consist of the major infiltrate in the course of acute inflammation, could be regulated and desensitized in the processes involving chemokine receptor internalization (12-14). The CCR5 antagonist aminooxypentane-RANTES has been shown to induce a potent inhibition of HIV-1 entry into target cells. This inhibition was proved to be a direct result of its ability to promote CCR5 internalization and to prevent its recycling back to the plasma membrane, thereby removing a key element of the fusion complex (11, 15).

Despite being extensively studied, the mechanisms underlying the trafficking of chemokine receptors are still not fully understood. Recent studies demonstrated that many chemokine receptors internalize through clathrin-coated vesicle (CCV), a process that involves the binding of arrestins to the phosphorylated receptors (9, 16-20). However, as revealed by the studies of other GPCRs, even though arrestins bind clathrin \textit{in vitro} with high affinity (21), they are not constitutively associated with clathrin-coated vesicles (22). Our previous studies...
demonstrated that truncation of the C-terminal serine-rich domain in CXCR2, which blocked arrestins binding to the mutant receptor, did not prevent the receptor internalization in certain cell types (23). Similar phenomena have been observed in the studies of other chemokine receptors, e.g. CXCR4 (8), as well as other kind of GPCRs, e.g. type A cholecystokinin receptor (24). While arrestins and dynamin appear to play an important role in phosphorylation-dependent sequestration of GPCRs, the phosphorylation-independent sequestration suggest that other potential regulators may exist in the internalization processes. Because the C-terminal domains of chemokine receptors have been demonstrated to play a key role in the receptor phosphorylation, desensitization, and internalization (7, 8, 18, 23), we established a yeast-two hybrid system to screen the intracellular proteins that interact with the C-terminal domain of CXCR2, a major chemokine receptor that mediates the migration of neutrophils to inflammatory sites in response to ELR-expressing CXC chemokines (1, 25). A number of proteins have been identified to be potentially involved in the receptor signaling and trafficking. In the present study, we demonstrate that Hsc70-interacting protein (Hip) associates with the chemokine receptors CXCR2 and CXCR4, and a tetratricopeptide repeat (TPR) deletion mutant form of Hip, which is unable to bind Hsc70 but continues to bind CXCR2, attenuates the receptor internalization and the receptor-mediated chemotaxis.

EXPERIMENTAL PROCEDURES

Plasmid construction - Wild-type CXCR2 in pRC/CMV, and the C-terminal domain of CXCR2 in PAS2 or in pGEX-KG were constructed previously (10). The wild-type and the mutant forms of Hip in pcDNA3.1 were constructed by amplifying the fragments using polymerase chain reaction (PCR) and inserted into the BamHI and XhoI sites.
Yeast-two hybrid assay - Yeast two hybrid techniques were performed as described (10). For screening cDNA libraries, the bait plasmid PAS2/CXCR2 tail was transformed into yeast strain Y190 (CLONTECH) using a lithium acetate protocol (CLONTECH Manual). After confirming expression of the bait protein, a human B lymphocyte library in PACT2 (26) was transformed into the strain harboring the bait plasmid. The transformants expressing both the bait and the prey proteins were selected on SD/-Leu/-Try/-His medium. Colonies capable of growing on the SD/-Leu/-Try/-His medium were then tested for β-galactosidase activity (LacZ⁰) using the filter lift assay. Clones that were consistently phenotypically His⁺ and LacZ⁺ were further characterized. Approximately 2.6 x 10⁶ transformants were screened, and several of them were His⁺ and LacZ⁺. One of the clones chosen for sequencing and characterization based on its strong His⁺/LacZ⁺ phenotype displayed sequence identity with the Hsc/Hsp70 interacting protein, Hip.

Filter lift assay - A dry nitrocellulose filter was placed on the yeast colonies. The filter was then carefully lifted, transferred (colonies facing up) into liquid nitrogen, completely submerged for 10 sec, and then allowed to thaw at room temperature. The filter was then placed on a Whatman filter paper soaked in Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, pH 7.0) containing 50 mg/ml 5-bromo-4-chloro-3-indolyl-galactopyranoside, with colonies face up until blue color appeared.

Cell culture and transfection - Human embryonic kidney (HEK293) cells and rat basophilic leukemia (RBL-2H3) cells were grown in Dulbecco’s modified eagle’s medium (DMEM), 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. HEK293 cells were transfected with plasmids encoding CXCR2, CXCR4, or Hip forms using LipofectAMINE plus reagent (Life Technologies, Inc.). RBL-2H3 cells were transfected with plasmids encoding CXCR4 and Hip
forms by electroporation. Stably transfected HEK293 cells were selected with 560 µg/ml Geneticin (G418), and evaluated for receptor expression using ¹²⁵I-CXCL1 binding assay (Dupond NEN, #NEX-321).

In vitro binding Assay - Bacteria transformed with plasmids encoding GST or GST fusion proteins were cultured overnight at 37 °C, then 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 (25mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10mM NaF, 1mM Na₃VO₄, 1mM phenylmethylsulfonyl fluoride, and 10 µg each leupeptin and aprotinin) and then sonicated on ice for 10 sec. 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. Aliquots of the purified GST or GST fusion proteins were incubated with HEK293 cell lysate at 4 °C for 2 h with rotation. Beads were pelleted by centrifugation (15,000 × g, 2 min), and washed 4 times with RIPA buffer. Bound proteins were released by boiling in SDS-PAGE sample buffer containing 5% β-mercaptoethanol for 5 min, and detected by SDS-PAGE and Western blot.

Co-immunoprecipitation and Western blot – The following cell types were used in these experiments: 1) Human neutrophils which endogenously express CXCR2 were isolated from fresh heparinized peripheral blood from single human donors as described previously (10); 2) RBL-2H3 cells stably expressing CXCR2 were transiently transfected with plasmids encoding His-tagged wild-type Hip or ΔTPR; 3) HEK293 cells stably expressing wild-type or the internalization-deficient mutant (IL323,324AA) of CXCR2 were transiently transfected with plasmids encoding His-tagged wild-type Hip or ΔTPR, 4) HEK293 cells transiently expressing myc-CXCR4 and wild-type Hip or ΔTPR. Cells were treated with carrier buffer (control) or
agonists (100 ng/ml CXCL8 for CXCR2, and 100 nM CXCL12 for CXCR4) for 5 min unless indicated, washed 3 times with ice-cold PBS, and lysed in 1ml 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 affinity-purified anti-CXCR2 antibody (26) or anti-myc antibody (9E10, Santa Cruz Biotechnology) was added for overnight precipitation at 4°C. 40 µl protein A/G was then added and incubated at 4 °C for 2 h. The protein A/G-antibody-antigen complex was then collected by washing 3 times with ice-cold RIPA buffer. The final pellet was re-suspended in 50µl of SDS sample buffer containing 5% β-mercaptoethanol and heated to 50 °C for 10 min. 20 µl of this preparation was electrophoresed on a 10% SDS-polyacrylamide gel, and the proteins on the gel were transferred to nitrocellulose membranes (Bio-Rad). Co-precipitated proteins were detected by Western blotting using specific antibodies.

Confocal Microscopy – Confocal microscopy was performed on a Zeiss LSM-510 laser scanning microscope using a Zeiss 63 × 1.3 NA oil immersion lens. HEK293 cells stably expressing CXCR2 and transiently expressing His-tagged Hip were grown on coverslips. Cells were treated with carrier buffer or CXCL8 (100 ng/ml) for 5 min, and fixed with methanol. Cells were washed with PBS and incubated with a mixture of a mouse monoclonal anti-CXCR2 antibody and a rabbit polyclonal anti-His antibody for 30 min. Cells were washed and incubated with a mixture of a fluorescein isothiocyanate (FITC)-conjugated anti-mouse and a rhodamine-conjugated anti-rabbit antibody for 30 min. Colocalization studies of FITC-labeled CXCR2 and rhodamine-labeled His-tagged Hip were performed using dual excitation (488, 568 nm) and
emission (515-540 nm, FITC; 590-610 nm rhodamine) filter sets. Specificity of labeling and absence of signal crossover were established by examination of single-labeled samples.

**MAP kinase assay** – HEK293 cells stably expressing CXCR2 were transiently transfected with plasmids encoding His-tagged wild-type Hip or ΔTPR. Cells were treated with CXCL8 at 37 °C for 10 min, then lysed in RIPA buffer. Lysates containing equal amounts of protein were subjected to SDS-PAGE (10 %). Proteins were transferred to nitrocellulose membrane and phosphorylated MAP kinase (ERK1/ERK2) was detected by Western blotting using a phosphospecific MAP kinase antibody (SC-7383, Santa Cruz Biotechnology).

**Chemotaxis assay** – A 96-well chemotaxis chamber (Neuroprobe Inc) was used for chemotaxis assays, and the lower compartment of the chamber was loaded with 400 µl aliquots of 1 mg/ml ovalbumin/DMEM (chemotaxis buffer) or CXCL1 diluted in the chemotaxis buffer (1-200 ng/ml). Polycarbonate membranes (10 µm pore size) were coated on both sides with 20 µg/µl human collagen type IV, incubated for 2 h at 37 °C, then stored at 4 °C overnight. To prepare HEK293 cells for chemotaxis assay, cells were removed from the culture dish by trypsinization, washed with Hank’s solution, and incubated in 10% FBS/DMEM for 2 h at 37 °C to allow time for restoration of receptors. The cells were washed with chemotaxis buffer and then loaded into the upper chamber in the chemotaxis buffer. The chamber was incubated for 4 h at 37 °C in humidified air with 5% CO₂, then the membrane was removed, washed, fixed, and stained with a Diff-Quik kit. Cell chemotaxis was quantified by counting the number of migrating cells present in 10 microscope fields (20 × objective).

**Ligand-receptor complex internalization assay** - The acid-wash technique (23) was used to determine the kinetics of CXCL8-induced internalization of CXCR2. HEK293 cells expressing CXCR2 were grown to confluence on 24-well plates which were pre-coated with 0.1
mg/ml poly-L-lysine (Sigma, MW 30,000-70,000) for 1 h and washed once with distilled water before use. Cells were incubated at 4 °C in 0.5 ml serum-free DMEM containing 75 nCi/ml \( ^{125}\text{I}\)-CXCL8 for 1 h. The medium was subsequently removed and 1 ml ice-cold serum-free DMEM was added carefully into each well, aspirated, then another 1ml aliquot of ice-cold serum-free DMEM was added prior to incubation at 37 °C for the indicated time. The medium was removed and the cells were incubated with 1 ml ice-cold 0.2 M acetic acid with 0.5 M NaCl for 6 min. After the incubation, the cells were washed once with 1ml ice-cold serum-free DMEM, and lysed with 1 ml 1% SDS with 0.1 N NaOH (lysis solution). The radioactive cell lysate was then counted on a \( \gamma \)-counter (Gamma 5500, Beckman). Total cell surface receptor binding was measured after incubation with \( ^{125}\text{I}\)-CXCL8 medium followed by washing the cells with ice-cold serum-free DMEM. Nonspecific binding was measured by adding ice-cold 0.2 M acetic acid with 0.5 M NaCl after incubation with \( ^{125}\text{I}\)-CXCL8 in binding medium. Calculation of the percentage of internalized receptor was performed as described before (23).

FACS analysis – RBL-2H3 cells were transiently co-transfected with CXCR4 and pcDNA3.1, Hip, or ΔTPR. Cells (5 × 10^5/sample) were incubated in HEPES (20 mM)-buffered DMEM at 37 °C for 30 min in the presence or absence of CXCL12 (100 nM). Cells were then washed in ice-cold medium and incubated with a monoclonal CXCR4 antibody at 4 °C for 1 h. Cells were washed with ice-cold medium followed by incubation with fluorescein isothiocyanate-labeled goat anti-mouse IgG at 4 °C for 30 min. Cells were washed and fixed in 2% formaldehyde in phosphate-buffered saline and analyzed in FACSscan equipped with CellQuest software (Becton Dickinson).
RESULTS

In an attempt to isolate chemokine receptor-associated proteins, we used the yeast two-hybrid system to identify proteins that interact with the carboxyl-terminus (amino acids 311-355) of CXCR2 (Fig. 1A). Screening of a human B lymphocyte library fused to the GAL4 transactivation domain (27) yielded several potential candidate genes that were both His$^+$ and LacZ$^+$. The prey cDNAs were recovered from yeast and transformed into bacteria. The cDNAs were then sequenced using primers complimentary to 5’ or 3’ ends of the inserts. Among them, one encoding Hip was chosen for further study based on its moderately strong LacZ$^+$. The specificity of the interaction in yeast was tested by re-transforming PACT2/Hip along with the original bait PAS2/CXCR2 C-terminus, or PAS2 alone back into yeast strain Y190. The interaction between the receptor C-terminus and Hip specifically allowed growth of the yeast on SD medium lacking leucine, tryptophan, and histidine (SD/-Leu/-Try/-His) (Fig. 1A, left panel). Deletion of the serine-rich domain (amino acids 331-355) in the receptor C-terminus did not affect the interaction, suggesting that the binding domain resides in the region of amino acids 311-330. Neither the bait, PAS2/CXCR2 C-terminus, nor the prey was able to activate transcription of the reporter genes in the presence of only empty prey or bait vectors, respectively (Fig. 1A, left panel). Using the β-galactosidase assay, we observed that only the yeast co-transformed with PACT2/Hip and the PAS2/CXCR2 (311-355) or PAS2/CXCR2 (311-330) displayed LacZ$^+$ (Fig.1A, right panel).

To confirm the specific biochemical interaction between the C-terminus of CXCR2 and Hip, we used an in vitro binding assay to test for the direct interaction. GST or GST fusion proteins of the C-terminus of wild-type or mutant forms of CXCR2 were incubated with the cell lysate of HEK293 cells, and associated Hip was detected by Western blotting. The results
demonstrated that the GST fusion protein of CXCR2 C-terminus, but not the GST alone, associated with Hip. The binding of Hip to the mutant forms of CXCR2 C-terminus, KIL322-324AAA, IH326-327AA, and GLI328-330AAA, was significantly reduced compared to the binding of Hip to wild-type CXCR2 C-terminus, whereas other mutant forms bound a similar amount of Hip as the wild-type CXCR2 C-terminus (Fig. 1B). These data suggest that the Hip binding site resides in the KILAIHGLI motif in the C-terminus of CXCR2.

To identify the CXCR2 binding site in Hip protein, PCR-directed mutagenesis was used to prepare mutant Hip cDNAs for the production of truncated or internally deleted Hip proteins. Fig. 2A presents diagrams of the wild-type and mutant forms of Hip prepared. In addition to the amino and the carboxyl termini, three structurally distinctive regions were targeted for mutagenesis. These regions are (i) the TPR at approximately amino acids 100-200, which is required for Hsc/Hsp 70 binding, (ii) a 50-amino-acid region enriched in charged residues (+-+-), a second binding domain of Hsc/Hsp 70, and (iii) a 33-amino-acid stretch containing degenerate tandem repeats of the sequence glycine-glycine-methionine-proline (G). The His-tagged wild-type and mutant Hip cDNAs in pcDNA3.1 were transiently transfected in HEK293 cells, and the protein expression was determined by Western blotting. Fig. 2B shows the expression of the His-tagged wild-type or mutant forms of Hip in HEK293 cells. To identify the CXCR2 binding domain in the sequence of Hip, the GST fusion protein of CXCR2 C-terminus was incubated with the cell lysate of HEK293 cells over-expressing the His-tagged wild-type or the mutant forms of Hip proteins. Co-precipitated wild-type or mutant Hip proteins were detected by Western blotting using a specific anti-His antibody. As shown in Fig. 2C, only the C-terminal parts of Hip were bound to the GST fusion protein of CXCR2 C-terminus. Truncation of the C-terminal 66 amino acids completely impaired the interaction, suggesting that the
CXCR2 binding site resides in the extreme C-terminal domain of Hip. As expected, the TPR deletion mutant of Hip (ΔTPR) bound an almost equal amount of the GST fusion protein of CXCR2 C-terminus (Fig. 2C).

We next examined whether a functional complex consisting of CXCR2 and Hip could be detected in RBL-2H3 cells stably expressing CXCR2 (generous gift from Dr. Ricardo M. Richardson). Immunoprecipitation of CXCR2 from RBL-2H3 cells revealed a basal association of the receptors with Hip, and CXCL8 (100 ng/ml) treatment resulted in a time-dependent increase in the association of Hip with CXCR2, which peaked 5 min after agonist stimulation (Fig. 3A). Similar time-dependent association of CXCR2 with Hip was also observed in HEK293 cells stably expressing CXCR2 (data not shown). In addition, the agonist-induced increase in the association of CXCR2 with Hip was also observed in human neutrophils in response to CXCL8 treatment for 5 min (Fig. 3B). While in the same time frame, about 50 percent of the receptors have been internalized (15, 23), suggesting that agonist-induced association of CXCR2 with Hip occurs in the internal vesicles, although the basal association suggests that a small proportion of CXCR2 associates with Hip on the cell membrane. To test this hypothesis, an internalization deficient mutant of CXCR2 (IL323, 324AA) (23) was used in the coimmunoprecipitation assay. As excepted, in HEK293 cells stably expressing IL323,324AA, agonist stimulation did not induce an increase in the receptor/Hip association (Fig. 3C). To test whether other chemokine receptors associate with Hip, HEK293 cells transiently expressing myc-tagged CXCR4 were treated with carrier buffer or CXCL12 for 5 min, and myc-CXCR4 was immunoprecipitated. As shown in Fig. 3D, Hip was also coimmunoprecipitated with CXCR4 in an agonist-dependent manner.
To visualize the colocalization of CXCR2 with Hip in intact cells, confocal microscopy was performed. CXCR2 and His-tagged Hip were stained with specific antibodies, respectively, in HEK293 cells stably expressing CXCR2 and transiently expressing His-tagged Hip. As shown in Fig. 4, CXCR2 was expressed exclusively on the cell surface (Fig. 4A), whereas Hip was located largely in the cytoplasm (Fig. 4B). Interestingly, a small proportion of Hip was expressed on the cell surface (Fig. 4B), and colocalized with CXCR2 (Fig. 4C). This may explain the reason for the basal association of CXCR2 with Hip observed in the coimmunoprecipitation assay (Fig. 3). In response to agonist stimulation, a proportion of CXCR2 was internalized in the internal vesicles (Fig. 4D), and the internalized CXCR2 was partially colocalized with Hip (Fig. 4F).

To investigate the potential role of the interaction of CXCR2 with Hip in the receptor signaling and trafficking, a dominant interfering mutant of Hip is needed to block the functions of Hip. Because the major function of Hip characterized so far is to bind Hsc/Hsp 70 and regulate their chaperone activities, we chose to use a mutant Hip that is unable to bind Hsc/Hsp 70. Since Hip regulates the chaperone activities of Hsc/Hsp 70 through its central TPR domain binding to the ATPase domain of Hsc/Hsp 70 (28), and ΔTPR, a dominant interfering mutant form of Hip, has been shown to be unable to bind Hsc/Hsp70 (28), we used ΔTPR as a probe to test the physiological significance of the interaction between CXCR2 and Hip, and to understand the potential role of Hsc/Hsp70 in the signaling and trafficking of CXCR2. HEK293 cells stably expressing CXCR2 were transiently transfected with plasmids encoding wild-type Hip or ΔTPR, and the receptor expression, signaling, and trafficking were assessed. Overexpression of wild-type Hip or ΔTPR did not affect the receptor expression on the cell surface as determined by FACS analysis (data not shown), nor did it significantly affect the receptor-ligand binding as
assessed by $^{125}$I-CXCL8 binding assay (data not shown). In addition, agonist-dependent MAP kinase activation in the CXCR2-expressing cells was not affected by the overexpression of wild-type Hip or ΔTPR (Fig. 5).

To assess whether Hip is involved in the endocytosis of CXCR2, HEK293 cells stably expressing CXCR2 were transiently transfected with plasmids encoding His-tagged wild-type Hip or ΔTPR. Cells were incubated with $^{125}$I-CXCL8 at 4 °C for 1 h, then warmed to 37 °C for different times. After acidic washing to eliminate the cell surface $^{125}$I-CXCL8, the internalized $^{125}$I-CXCL8 was detected in a γ-counter. Fig. 6A shows a time-dependent internalization of $^{125}$I-CXCL8. Overexpression of wild-type Hip did not affect the internalization of $^{125}$I-CXCL8. However, in the cells overexpressing ΔTPR, the internalization of $^{125}$I-CXCL8 was attenuated about 50% compared to the internalization of $^{125}$I-CXCL8 in the control cells (P < 0.05). In addition, the internalization of CXCR4 was also tested in RBL-2H3 cells co-transfected with plasmids encoding CXCR4 and His-tagged wild-type Hip or ΔTPR. Cells were treated in suspension without or with CXCL12 for 30 min, and cell surface expression of CXCR4 was detected by immunofluorescence with anti-CXCR4 using FACS analysis. As shown in Fig. 6B, CXCL12 treatment reduced the cell surface expression of CXCR4 in both the control cells and the cells overexpressing Hip proteins, indicating receptor internalization in these cells. CXCR4 was also internalized in the cells expressing ΔTPR in response to CXCL12 treatment, but the amount of internalization was consistently lower than that observed in the control cells or cells expressing His-tagged Hip. The percentages of CXCR4 internalization from four independent experiments are 75.99 ± 2.45, 81.42 ± 3.54, and 38.29 ± 2.38 for the control cells expressing CXCR4, cells expressing CXCR4 and His-tagged Hip, and cells expressing CXCR4 and His-
tagged ΔTPR, respectively. Statistical analysis demonstrated that the internalization of CXCR4 was significantly reduced in the cells expressing His-tagged ΔTPR (P < 0.05).

Because one of the most important functions for chemokine receptors is to mediate chemotaxis, and previous studies have demonstrated the important role of CXCR2 internalization in the receptor-mediated chemotaxis (16, 23), we examined the potential role of Hip in the chemotaxis of CXCR2 expressing cells. HEK293 cells stably expressing CXCR2 exhibited a typical bell-shape chemotactic response upon treatment by progressive concentrations of CXCL1. Overexpression of wild-type Hip did not affect the cell chemotaxis, whereas overexpression of ΔTPR significantly reduced the receptor-mediated chemotaxis (Fig. 7).

**DISCUSSION**

Chemokine receptor trafficking may play a significant role in the fine tuning of chemokine-induced inflammatory responses, and necessitate a better understanding of the mechanisms responsible for the control of the intracellular trafficking of these receptors. Our present study is the first to provide evidence for novel findings regarding the regulation of chemokine receptor trafficking by Hip, as indicated by the following observations: 1) Hip interacted with CXCR2 through its C-terminal domain binding to the C-terminal leucine-rich domain of CXCR2. CXCR2 and CXCR4 associated with Hip in an agonist-dependent manner in intact cells, and an internalization-deficient mutant of CXCR2, IL323,324AA, failed to associate with Hip; 2) overexpression of a dominant interfering mutant of Hip (ΔTPR) inhibited the internalization of CXCR2 and CXCR4. These observations have important physiological implications because expression of ΔTPR inhibited the migratory responses of the chemokine receptor expressing cells as assessed by agonist-induced chemotaxis.
Hip was first noted as a transient component during the cell-free assembly of progesterone receptor complex (29), and was subsequently found to be associated with Hsp70 and Hsc70 (28, 30), members of a molecular chaperone family that participates in the regulation of protein folding and transport (31). Hip binds the ATPase domain of Hsc/Hsp70 in an ADP-dependent manner (32, 33), through a central TPR motif and a downstream highly charged domain (28, 30). Besides affecting the Hsc/Hsp70 chaperone activities in vitro and in vivo (34, 35), Hip alone can also bind to unfolded proteins and prevent their aggregation. Yet refolding of proteins to their active state requires cooperation with other chaperones (28, 32).

The mechanisms underlying the involvement of Hip in the internalization of chemokine receptors CXCR2 and CXCR4 may be interpreted in two different ways. One interpretation is that through its own chaperone activity Hip may facilitate the conformational change of the receptors that is required for the receptors to undergo internalization. Thus overexpression of the Hip mutant, ΔTPR, which blocks the normal functions of Hip, attenuated the internalization of the chemokine receptors. The second interpretation, which favors an indirect involvement of Hip in the regulation of the chemokine receptor internalization, is that Hip participates in the internalization process of the chemokine receptors by cooperating with Hsc/Hsp70. This is supported by the data that the internalization of CXCR2 and CXCR4 was attenuated by the Hip mutant ΔTPR, which fails to bind the ATPase domain of Hsc/Hsp70 as reported previously (28, 35). Many chemokine receptors including CXCR2 and CXCR4 internalize through CCV (16, 19, 36). This process comprises ligand-receptor formation, aggregation of these complexes in coated pits and formation of CCV (36). Fusion of CCV with endosomes can only occur after the clathrin-containing coat is released from CCV (38). Hsc/Hsp70 have been suggested to broadly modulate clathrin dynamics throughout the CCV cycle by releasing coat proteins from CCV (39-
Studies on the transferrin receptor indicate that the receptor endocytosis is sensitive to antibodies against Hsc70 (42), and the receptor internalization and recycling are blocked by overexpression of ATPase-deficient Hsc70 mutants (43). Although there is no report regarding the direct involvement of Hsc/Hsp70 in the internalization of CXCR2 and CXCR4, a complex formed by CXCR2 and Hsc70 has been observed in intact cells (ongoing study in our laboratory). Moreover, a signal complex containing Hsc70 and CXCR4 (44), and partial colocalization of Hsc73 with the G protein-coupled A1 adenosine receptor in internal vesicles (45) has been demonstrated. These data suggest the potential role of Hsc/Hsp70 in the signaling and trafficking of GPCRs. Further studies regarding the role of Hsc/Hsp70 in the signaling and trafficking of CXCR2 and other chemokine receptors are underway.

Based on the data that overexpression of wild-type Hip or ΔTPR did not affect CXCL8-induced MAP kinase activation in CXCR2 expressing cells, we postulate that the immediate downstream signaling of CXCR2 is not regulated by Hip. Although studies on β2-adrenergic receptor have demonstrated that in HEK293 cells, the β2-adrenergic receptor-mediated MAP kinase activation is inhibited by blocking receptor internalization (46), CXCR2-mediated MAP kinase activation appears to be independent of the receptor endocytosis. This is supported by the present study showing that ΔTPR attenuated CXCR2 internalization but did not inhibit CXCL8-induced MAP kinase activation, and by the previous observation that overexpression of a dominant negative mutant of dynamin I (dynamin I K44A) blocked CXCR2 internalization without affecting the receptor-mediated MAP kinase activation (16). Further evidence suggesting the separation between CXCR2 internalization and immediate downstream signaling events came from the studies of CXCR2 mutants. A mutant of CXCR2 with deletion of the last 31 amino acids at the carboxyl-terminal domain exhibited impaired agonist-induced receptor
endocytosis but not inhibition of adenylyl cyclase or MAP kinase activation (47). Richardson et al. (48) demonstrated that a CXCR2 mutant (331T) exhibiting a deletion of the last 25 amino acid residues of the carboxyl-terminal domain, lost agonist-induced phosphorylation and sequestration when expressed in RBL-2H3 cells. However, the immediate downstream signaling events such as GTPase activity, phosphotidylinositol hydrolysis, and calcium mobilization were not impaired (48). The reasons for the discrepancy in ligand-induced receptor sequestration and MAP kinase activation between CXCR2 and β2-adrenergic receptor are not clear.

The chemotaxis assay is believed to be the most useful assay to evaluate the signal transduction capacity of chemokine receptors, since it measures the ultimate result of a cascade of intracellular events that are activated by ligand-receptor interaction. More importantly, this assay also provides a functional read out for a process composed of sequential desensitization and resensitization events with respect to receptor activation. Our results clearly demonstrate that overexpression of ΔTPR attenuates the ligand-induced chemotaxis of CXCR2 expressing cells. The equal or similar cell surface expression of receptors was confirmed by immunofluorescence assay to exclude the possibility that the reduced chemotaxis in cells expressing ΔTPR is due to reduced receptor expression or cell surface targeting. The mechanism underlying the involvement of Hip in CXCR2-mediated chemotaxis remains elusive. We postulate that the effect of Hip in CXCR2-mediated chemotaxis may result from its regulation of the receptor internalization, although we can not exclude the possibility that Hip directly regulates other proteins involved in the mediation of chemotactic signals. Increasing evidence shows the importance of receptor internalization in receptor-mediated chemotaxis. Studies on CXCR2 have demonstrated that the membrane proximal part of the receptor C-terminal domain consisting of amino acids 317-324 (RHGLLKIL) constituted a minimal requirement for both the receptor
sequestration and the receptor-mediated chemotaxis (25, 49). Combined mutations of the LLKIL motif in the C-terminus of CXCR2 not only impaired the receptor internalization but also blocked agonist-induced chemotaxis (23). Moreover, overexpression of a dominant negative mutant form of dynamin I, dynamin I K44A, blocked CXCR2 internalization as well as the receptor-mediated chemotaxis (16). An antagonist of CCR1 and CCR3 blocked the receptor internalization and the receptor-mediated chemotaxis (50). Since receptor endocytosis permits CXCR2 dephosphorylation and resensitization (10), we therefore postulate that endocytosis, dephosphorylation, and recycling of chemokine receptors are required for gradient response to ligand stimulation. Because ΔTPR attenuated ligand-induced CXCR2 internalization, it is conceivable that this is the mechanism by which ΔTPR inhibited the receptor-mediated chemotaxis. However, it is worthy to note that some other chemokine receptor-mediated chemotaxis is independent of receptor internalization (51). These observations suggest that different chemokine receptors are divergently regulated in this respect. Further studies are required to elucidate whether the regulation of CXCR2-mediated chemotaxis by Hip is a generalized mechanism regarding the chemotactic responses of other chemokine receptors.

In conclusion, the present data demonstrate for the first time that Hip interacts with CXCR2 through its C-terminal domain binding to the receptor C-terminal KILAIHGLI motif. Hip forms a complex with CXCR2 and CXCR4 in intact cells in an agonist-dependent manner. Overexpression of a TPR deletion mutant form of Hip (ΔTPR) significantly attenuates the internalization of CXCR2 and CXCR4. Moreover, overexpression of ΔTPR blocks CXCR2-mediated chemotaxis. Our studies open a possibility that Hip may regulate the signaling and trafficking of chemokine receptors as well as other GPCRs.
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Footnote

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& To whom correspondence should be addressed: Tel.: 615-343-7777; Fax: 615-343-4539.

1 The abbreviations used are: GPCR, G protein-coupled receptor; Hip, heat shock protein-interacting protein; HEK293 cells, human embryonic kidney 293 cells; RBL-2H3 cells, rat basophilic leukemia 2H3 cells; GST, glutathione-S-transferase; DMEM, Dulbecco’s modified essential medium; FBS, fetal bovine serum; CCV, clathrin-coated vesicle; MAP, mitogen-activated protein; TPR, tetratricopeptide repeat; IP, immunoprecipitation; IB, immunoblotting.
Figure legends

Fig. 1. Interaction of CXCR2 C-terminus with Hip. A, yeast strain Y190 co-transformed with PACT2/Hip and PAS2/CXCR2 (311-355) or PAS2/CXCR2 (331-355) were replica-plated on SD medium without tryptophan, leucine, and histidine. The co-transformation of p53 and SV40 provided by the manufacture was performed as a positive control (left panel). A β-galactosidase assay was carried out using a YPD plate replicate (right panel) as described in the EXPERIMENTAL PROCEDURES. B, the indicated GST or GST-CXCR2 C-terminus fusion proteins were incubated with the cell lysate of HEK293 cells, then absorbed onto glutathione-Sepharose beads. After washing, the beads were resuspended in loading buffer. Proteins were separated by 10% SDS-PAGE, and transferred to a nitrocellulose membrane. Coprecipitated Hip proteins were analyzed by immunoblotting with a polyclonal Hip antibody. The membrane was stripped and reblotted with a mouse monoclonal GST antibody to confirm the protein expression and equal loading.

Fig. 2. Identification of the CXCR2 binding domain in Hip. A, diagrams of wild-type and mutant Hip forms, highlighting structurally distinct regions. WT, wild-type. B, the wild-type and mutant Hip forms in pcDNA3.1 were transiently transfected in HEK293 cells. Cells were lysed and proteins were separated by 10% SDS-PAGE. The expression of His-tagged Hip forms were determined by Western blotting using a specific anti-His antibody. C, the GST-CXCR2 C-terminus fusion proteins were incubated with the cell lysate of HEK293 cells transiently expressing His-tagged Hip forms. Coprecipitated Hip forms were analyzed by Western blotting using anti-His antibody as described in Fig.1B legend.
Fig. 3. Association of Hip with CXCR2 or CXCR4 in intact cells. A, RBL-2H3 cells stably expressing CXCR2 were exposed to CXCL8 (100 ng/ml) for the indicated times, and CXCR2 was immunoprecipitated from the cell lysate using a rabbit polyclonal anti-CXCR2 antibody. A preimmune rabbit serum (mock) was used in a parallel experiment to confirm the specificity of the immunoprecipitation. Proteins were separated by 10% SDS-PAGE, and transferred to a nitrocellulose membrane. Co-precipitated Hip proteins were detected using a polyclonal Hip antibody. The membrane was stripped and reblotted with a mouse monoclonal anti-CXCR2 antibody to confirm equal loading. The results represent one of three independent experiments. B, human neutrophils prepared from single donor were treated without (lane 1) or with (lane 2) CXCL8 (100 ng/ml) for 5 min. The cells were lysed and CXCR2 was immunoprecipitated described above. Co-precipitated Hip was blotted using specific anti-Hip antibody. The membrane was stripped and reblotted with a mouse monoclonal anti-CXCR2 antibody (E2, Santa Cruz) to confirm equal loading. C, HEK293 cells stably expressing wild-type (lanes 1 and 2) or IL323,324AA mutant form of CXCR2 (lanes 3 and 4) were treated with carrier buffer (lanes 1 and 3) or CXCL8 (100 ng/ml) (lanes 2 and 4) for 5 min. CXCR2 was immunoprecipitated and co-precipitated Hip proteins were analyzed as described in the Fig. 3A legend. D, HEK293 cells transiently transfected with myc-tagged CXCR4 were treated with carrier buffer (lane 1) or CXCL12 (100 nM) (lane 2) for 5 min. CXCR4 was immunoprecipitated using a monoclonal anti-myc antibody and co-precipitated Hip proteins were analyzed as described in Fig. 3A legend. IP, immunoprecipitation; IB, immunoblotting.

Fig. 4. Colocalization of CXCR2 with Hip. HEK293 cells stably expressing CXCR2 and transiently expressing His-tagged Hip were treated with carrier buffer (A-C) or CXCL8 (B-D)
for 5 min. The cells were then fixed with methanol, and incubated with a mixture of a mouse monoclonal anti-CXCR2 antibody and a rabbit polyclonal anti-His antibody for 30 min, followed by a mixture of a FITC-conjugated anti-mouse antibody and a rhodamine-conjugated anti-rabbit antibody for 30 min. Representative laser-scanning confocal micrographs demonstrating the distribution of CXCR2 (green) (A and D), His-tagged Hip (B and E) and overlay (C, and F) were shown. Images were processed using Photoshop software.

**Fig. 5. Effect of wild-type Hip or ΔTPR on CXCR2-mediated MAP kinase activation.** HEK293 cells stably expressing CXCR2 were transiently transfected with vector (lanes 1 and 2), His-tagged wild-type Hip constructs (lanes 3 and 4), or His-tagged ΔTPR constructs (lanes 5 and 6). Cells were treated with carrier buffer (lanes 1, 3 and 5) or CXCL8 (100 ng/ml) (lanes 2, 4 and 6) for 10 min. 30 µl of protein from clarified cell lysate were loaded on SDS-polyacrylamide gel per lane, electrophoresed, and Western blot analysis was performed with a phosphospecific MAP kinase antibody. The blots were stripped and re-probed with an antibody against ERK-2 and with an antibody against His, respectively, to ensure equal loading and protein expression.

**Fig. 6. Role of Hip in the internalization of CXCR2 and CXCR4.** A, HEK293 cells stably expressing CXCR2 were transiently transfected with pcDNA3.1, His-tagged wild-type Hip, or His-tagged ΔTPR. Cells were incubated with 75 nCi/ml $^{125}$I-CXCL8 at 4 °C for 1 h. Unbound $^{125}$I-CXCL8 was removed by washing at 4 °C. The cells were warmed to 37 °C for the indicated time period. $^{125}$I-CXCL8 remaining at the cell surface was removed with acetic acid (0.2 M, pH 2.5) containing 0.5 M NaCl, and the internalized $^{125}$I-CXCL8 was quantitated on a γ-counter. Values represent the mean ± SEM of three independent experiments performed in duplicate. The
data were analyzed using Student’s paired t-test (*, p < 0.05). B, RBL-2H3 cells stably expressing CXCR2 were transiently transfected with pcDNA3.1, His-tagged wild-type Hip, or His-tagged ΔTPR. Cells were treated with CXCL12 at 37 °C for 30 min, then were incubated with a monoclonal CXCR4 antibody at 4 °C for 1 h. Cells were washed with ice-cold medium followed by incubation with fluorescein isothiocyanate-labeled goat anti-mouse IgG at 4 °C for 30 min. Cells were washed and fixed in 2% formaldehyde in phosphate-buffered saline and analyzed in FACScan. The staining of cells in the absence of primary CXCR4 antibody (thin broken line) or with CXCR4 antibody in cells incubated with (solid line) and without (thick broken line) CXCL12 are shown.

**Fig. 7. Role of Hip in CXCR2-mediated chemotaxis.** HEK293 cells stably expressing CXCR2 were transiently transfected with pcDNA3.1, His-tagged wild-type Hip, or His-tagged ΔTPR. Two days after transfection, cells were compared for chemotaxis in response to CXCL1 stimulation as described under “Experimental Procedures”. Values represent the mean ± SEM of three different experiments. The data were analyzed using Student’s paired t-test (*, P < 0.05).
Fig. 1

A

1. PAS2 + Hip
2. CXCR2 (311-355) + PACT
3. CXCR2 (311-355) + Hip
4. CXCR2 (331-355) + Hip
5. PAS2 + SV40
6. P53 + SV40

β-gal

- - +++ +++

B

GST Wt CXCR2 C-terminus Hip
FRH316-318AAA
GLL319-321AAA
KIL322-324AAA
IH325-327AAA
GLL328-330AAA

← GST fusion proteins
← GST
← Hip
**Fig. 2**

**A**

| Protein | WT | TPR | + | - | G |
|---------|----|-----|---|---|---|
| 1-303   |    |     | + | - |   |
| 1-276   |    |     | + | - |   |
| 1-226   |    |     | + | - |   |
| 1-148   |    |     | + | - |   |
| 1-117   |    |     | + | - |   |
| 226-369 |    |     |   |   |   |
| 276-369 |    |     |   |   |   |
| ΔTPR    |    |     |   |   |   |

**B**

Whole cell lysate

IB: His

**C**

GST pull down

IB: His

IB: GST
Fig. 3

A

|          | Mock | 0 | 1 | 2 | 5 | 10 | 30 | (min) |
|----------|------|---|---|---|---|----|----|-------|
| IP: CXCR2 |      |   |   |   |   |    |    |       |
| IB: Hip  |      |   |   |   |   |    |    |       |
| IP: CXCR2 |      |   |   |   |   |    |    |       |
| IB: CXCR2|      |   |   |   |   |    |    |       |

IP: CXCR2
IB: Hip

IP: CXCR2
IB: CXCR2

B

IP: CXCR2
IB: Hip

IP: CXCR2
IB: CXCR2

1       2

C

IP: CXCR2
IB: Hip

IP: CXCR2
IB: CXCR2

D

IP: c-myc
IB: Hip

IP: c-myc
IB: CXCR4
Fig. 5

IB: p-ERK

1  2  3  4  5  6

P-ERK2

IB: ERK2

ERK2

IB: His

Hip
ΔTPR
Fig. 6

B

![Graph showing internalization of CXCL8](image-url)
Fig. 7
Hsc/Hsp70 interacting protein (Hip) associates with CXCR2 and regulates the receptor signaling and trafficking
Guo-Huang Fan, Wei Yang, Ji-Qing Sai and Ann Richmond

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