

The 73-kDa Heat Shock Cognate Protein Is a CXCR4 Binding Protein that Regulates the Receptor Endocytosis and the Receptor-Mediated Chemotaxis

Yun Ding, Mika Li, Jinwu Zhang, Ningli Li, Zongqin Xia, Yaer Hu, Sunxin Wang, and Guo-Huang Fan

Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and Shanghai Jiaotong University School of Medicine, Shanghai, People's Republic of China (Y.D., G.-H.F., J.Z., N.L.); Department of Biomedical Sciences, Meharry Medical College, Nashville, Tennessee (G.-H.F.); Martin Luther King Jr. Magnet School, Nashville, Tennessee (M.L.); Department of Nuclear Medicine, Shanghai Jiaotong University School of Medicine, Shanghai, People's Republic of China (Z.X., Y.H.); and Department of Chinese Medicine, Shenzhen No. 9 Hospital, Shenzhen, People's Republic of China (S.W.)

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ABSTRACT

The CXCR4 chemokine receptor is a G protein-coupled receptor that plays an important role in leukocyte homing, cancer metastasis, and human immunodeficiency virus infection. In response to ligand stimulation, chemokine receptors undergo endocytosis through clathrin-coated vesicle (CCV). Uncoating of CCV, a process involving heat shock cognate protein and several other proteins, is critical for fusion of CCV to endosomal compartments. The present study demonstrated that CXCR4 was associated with the 73-kDa heat shock cognate protein (Hsc73) in human embryonic kidney 293 cells in response to ligand stimulation. Truncation of the carboxyl terminal domain

of CXCR4 reduced the association with Hsc73 and a glutathione S-transferase-CXCR4 carboxyl terminal fusion protein associated with Hsc73 in vitro, suggesting involvement of the carboxyl terminal domain of the receptor in the interaction. In response to ligand stimulation, CXCR4 underwent internalization and colocalization with Hsc73, but the receptor endocytosis was blocked by knockdown of Hsc73 with RNA interference. Moreover, Hsc73 knockdown significantly reduced the CXCR4-mediated chemotaxis of U87 glioma cell lines. These findings suggest that Hsc73 plays a role in chemokine receptor trafficking and the receptor-mediated chemotaxis.

The CXC chemokine receptor 4 (CXCR4) is a member of the large family of seven-transmembrane domain G protein-coupled receptors (GPCRs) (Haribabu et al., 1997). CXCR4 plays an important role in immune and inflammatory responses by mediating the directional migration and activation of leukocytes (Bleul et al., 1996). It is also a coreceptor for T-cell tropic and dual-tropic human immunodeficiency virus-1 strains (Feng et al., 1996). In addition, CXCR4 is the major chemokine receptor on glioma cells and mediates their survival (Zhou et al., 2002). Its ligand, the CXC chemokine

stromal cell-derived factor 1, also named CXCL12, is a highly efficient chemotactic factor for T cells, monocytes, pre-B cells, dendritic cells, and hematopoietic progenitor cells (Baggio-olini, 1998). Ligand stimulation of CXCR4 induces the activation of a number signaling pathways (Ganju et al., 1998), resulting in cell proliferation and migration. Ligand stimulation also induces CXCR4 endocytosis, a process involving phosphorylation of the receptors by G protein-coupled receptor kinases, followed by binding of β -arrestin (Haribabu et al., 1997). Endocytosis of CXCR4 is a major component of the mechanism of chemokine inhibition of viral infection (Alkhatib et al., 1997), and may be necessary to activate several pathways and functions such as chemotaxis (Guinamard et al., 1999). However, conflicting results have been obtained in which chemotaxis (Arai et al., 1997) has been shown to be independent of receptor endocytosis.

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ABBREVIATIONS: CXCR4, CXC chemokine receptor 4; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; Hsc73, heat shock cognate protein 70; CCV, clathrin-coated vesicle; DMEM, Dulbecco's modified Eagle's medium; RNAi, RNA interference; GST, glutathione S-transferase; EGFP, enhanced green fluorescent protein; FRET, fluorescence resonance energy transfer; HA, hemagglutinin; RIPA, radioimmuno-precipitation assay; FITC, fluorescein isothiocyanate; GFP, green fluorescence protein; PAGE, polyacrylamide gel electrophoresis.

One major route of the internalization of CXCR4 and other chemokine receptors is the clathrin-mediated pathway (Venkatesan et al., 2003). The clathrin coats must be dissociated from clathrin-coated vesicles (CCVs) soon after vesicle formation and reused for subsequent rounds of endocytosis. Although the underlying mechanisms are not fully understood, at least in vitro, clathrin uncoating activity has been associated with the ATPase activity of 73-kDa heat shock cognate protein (Hsc73) (Schlossman et al., 1984). Hsc73 promotes the release of clathrin and other coat proteins from CCVs by binding to clathrin, thus disrupting the clathrin cage concomitant with ATP hydrolysis (Schlossman et al., 1984; Hannan et al., 1998). After uncoating, Hsc73 remains associated with the soluble pool of clathrin (Schlossman et al., 1984).

Despite these advances in understanding the mechanism of Hsc73-mediated clathrin uncoating in vitro, the role of Hsc73 in endocytosis in vivo remains largely unknown. Previous studies have demonstrated that Hsc73 inhibitory antibodies, peptides, or dominant interfering mutants disrupt the internalization and recycling of membrane receptors and neurotransmitters (Honing et al., 1994; Newmyer and Schmid, 2001). Moreover, Hsc73 was identified as a binding protein of A1 adenosine receptor, a member of the GPCR superfamily (Sarrío et al., 2000). Hsc73 partially colocalized with A1 adenosine receptor in internal vesicles, suggesting involvement of Hsc73 in the receptor endocytosis (Sarrío et al., 2000). However, the poor colocalization between Hsc73 and metabotropic glutamate 4 receptor (Sarrío et al., 2000) suggests that not all GPCRs interact with Hsc73. In the present study, we identified Hsc73 as an interacting protein of CXCR4. Hsc73 associated with CXCR4 in internal vesicles after ligand-induced endocytosis, which was blocked by knockdown of Hsc73 with RNA interference (RNAi). Moreover, Hsc73 knockdown with RNAi reduced CXCR4-mediated chemotaxis.

Materials and Methods

Materials. Human embryonic kidney (HEK) 293 cells were from the American Type Culture Collection (Manassas, VA). The wild-type and truncation mutant forms of pcDNA/HA-CXCR4 plasmid were a generous gift from Dr. Gang Pei (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China). Anti-HA monoclonal antibody (12CA5) was purchased from Roche Applied Science (Indianapolis, IN). Anti-CXCR4 monoclonal antibody was purchased from AnaSpec, Inc. (San Jose, CA). Anti-CXCR4 polyclonal antibody was purchased from IMGENEX, Inc. (San Diego, CA). Anti-HA polyclonal antibody, anti-Hsc73 polyclonal antibody, and anti-Hsc73 monoclonal antibody were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). ^{125}I -CXCL12 was from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). SYPRO ruby protein gel stain was from Molecular Probes (Eugene, OR), and lactacystin and calpeptin were from Calbiochem (La Jolla, CA). cAMP radioimmunoassay kit was from BioVision, Inc. (Mountain View, CA).

Construction of RNAi and Hsc73 Plasmids. Two oligonucleotides, one corresponding to the nucleotides 222 to 244 (GGACGCA-GAUUUGAUGAUGCUG), the other corresponding to the nucleotides 325 to 347 (GAGAGACCAAAAGCUUCUAUCC), were selected to generate the Hsc73 RNAi clones 1 and 2 constructs (RNAi-1 and RNAi-2). The oligonucleotides were first inserted into the Apal (blunted) and XhoI sites of BS/U6 vector, a generous gift from Dr. Gang Pei (Institute of Biochemistry and Cell Biology, Shanghai

Institutes for Biological Sciences, Chinese Academy of Sciences). The inverted motif that contains the six-nucleotide spacer and five thymine was then subcloned into the XhoI and EcoRI sites of the intermediate plasmid to generate BS/U6-Hsc73-RNAi-1 and BS/U6-Hsc73-RNAi-2, respectively. For the generation of Hsc73 RNAi construct with an enhanced green fluorescence protein (EGFP), two oligonucleotides, corresponding to the nucleotides 222 to 241 (GGACGCA-GAUUUGAUGAUG) and 325 to 344 (GAGAGAC-CAAAGCUUCUA), respectively, each of which is separated by a nine-nucleotide noncomplementary spacer (TCTCTTGAA) from the reverse complement of the same 19-nucleotide sequence, were inserted into the BglII and HindIII sites of pSUPER.retro.circular.stuffer (OligoEngine, Seattle, WA) backbone to generate pSUPER-Hsc73-RNAi-1 and pSUPER-Hsc73-RNAi-2, respectively. For the construction of Hsc73 into pcDNA3.1, the coding sequence of Hsc73 (GenBank accession no. NM_006597) was amplified by reverse transcription-polymerase chain reaction from HEK293 cells. The amplified product was inserted between EcoRI and HindIII sites of pcDNA3.1 (Invitrogen, Carlsbad, CA).

Cell Culture and Transfection. HEK293 cells and U87 glioma cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 100 U/ml each of penicillin and streptomycin at 37°C in a humidified atmosphere of 95% air/5% CO₂. Cells were transfected with plasmids encoding CXCR4 using Lipofectamine Plus reagent (Invitrogen). Stably transfected HEK293 cells were selected with 560 μg/ml G418 (Geneticin) and evaluated for receptor expression using ^{125}I -CXCL12 binding assay. The transfection of pcDNA3/Hsc73, BS/U6/Hsc73-RNAi, or the pSUPER/Hsc73-RNAi was also performed using the Lipofectamine Plus reagent. Transfection efficiency was evaluated by immunostaining the transfected cells with an antibody against Hsc73. Approximately 70 to 80% of the cells were transfected with the pcDNA3/Hsc73 or the BS/U6-Hsc73-RNAi, whereas approximately 50% of cells were transfected with the pSUPER-Hsc73-RNAi. Therefore, the pSUPER-Hsc73-RNAi was only used for the immunofluorescence experiment, and the BS/U6-Hsc73-RNAi was used in the rest of the experiments.

cAMP Assay. HEK293 cells stably expressing HA-CXCR4 or EGFP-CXCR4 were treated with different concentrations of CXCL12 in the presence of 1 μM forskolin (Sigma) at 37°C for 10 min. The reactions were terminated, and the cAMP levels of each sample were measured using radioimmunoassay as described previously (Fan et al., 1998). The values presented represent the means ± S.E. of at least three experiments, calculated as $100 \times [\text{cAMP}_{(\text{For} + \text{C})} - \text{cAMP}_{(\text{basal})}] / [\text{cAMP}_{(\text{For})} - \text{cAMP}_{(\text{basal})}]$, where cAMP_(For + D) is cAMP accumulation in the presence of forskolin and CXCL12, cAMP_(basal) is cAMP in the absence of forskolin and CXCL12, and cAMP_(For) is cAMP in the presence of forskolin alone.

Glutathione S-Transferase Pull-Down Assay. Bacterial strains (BL21) transformed with plasmids encoding glutathione S-transferase (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 (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. 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 (15,000g, 2 min) and washed four times with RIPA buffer. Bound proteins were released by boiling in SDS-PAGE sample buffer containing 5% β-mercaptoethanol for 5 min, electrophoresed with a 10% polyacrylamide gel, transferred to nitrocellulose membrane, and immunoblotted using anti-Hsc73 monoclonal antibody, horseradish peroxidase-labeled goat anti-

mouse secondary antibodies, and chemiluminescence (Pierce Chemical, Rockford, IL).

Immunoprecipitation. HEK293 cells stably expressing HA-CXCR4 were grown in 15-cm plates to a density of 2×10^6 cells/ml. Cells were treated with carrier buffer or CXCL12 (10 nM) for different time intervals as indicated. As a control, parental HEK293 cells were treated with CXCL12 for 60 min (mock). Cells were harvested by centrifugation, washed two times with ice-cold phosphate-buffered saline, and lysed with 1 ml of RIPA buffer. The cell lysate was centrifuged at 10,000g for 30 min, and the supernatant was pre-cleared by incubation with protein A/G-agarose for 1 h at 4°C. The supernatant was then incubated on a rocker with 10 μ l of anti-HA monoclonal antibody (for proteomic study) or anti-CXCR4 antibody (for coimmunoprecipitation assay) for 1 h at 4°C followed by the addition of 100 μ l of 50% protein G-agarose pre-equilibrated in lysis buffer and 1-h incubation at 4°C. Samples were then centrifuged, and the pellets were washed three times with 1 ml of RIPA buffer and one time with 20 mM Tris-HCl, pH 7.5, and 2 mM EDTA on a rocker for 15 min at 4°C. Bound proteins were eluted by addition of 50 μ l of SDS sample buffer. Samples were electrophoresed on a 10% polyacrylamide gel. Proteins were either stained with Coomassie Blue or SYPRO ruby stain following the manufacturer's instructions, or transferred to nitrocellulose membrane for Western blot analysis.

Mass Spectrometry Analysis. Stained protein bands were excised from the polyacrylamide gel and stored at -20°C until further analysis. Protein bands were digested with 20 ng/ μ l trypsin (Promega, Madison, WI) in 25 mM ammonium bicarbonate buffer for 16 h at 37°C. Mass spectra of tryptic peptides were acquired using surface-enhanced laser desorption/ionization on a hydrophobic H4 chip using a Ciphergen PBS II Instrument (Fremont, CA). Proteins were identified by comparing observed peptide mass fingerprints with those theoretically derived from the NCBIInr database using the Profound database searching algorithm (Rockefeller University, New York, NY).

Densitometry Analysis. The relative amount of the Western blot bands or the autoradiographical bands was measured by densitometry analysis using NIH Image software (<http://rsb.info.nih.gov/nih-image/>). The relative density of the protein bands was calculated in the area encompassing the immunoreactive protein band and subtracting the background of an adjacent nonreactive area in the same lane of the protein of interest.

Confocal Microscopy. Confocal microscopy was performed on a Zeiss LSM-510 laser scanning microscope using a Zeiss 63 \times 1.3 numerical aperture oil immersion lens (Carl Zeiss Inc., Thornwood, NJ), as described previously (Fan et al., 2003). HEK293 cells stably expressing HA-CXCR4 were transiently transfected with pcDNA3.1 vector, pcDNA3.1/Hsc73, BS/U6 vector, or BS/U6-Hsc73-RNAi for 48 h. Cells were treated with carrier buffer or CXCL12 (10 nM) for 30 min and fixed with methanol. Cells were washed with phosphate-buffered saline and incubated with a mixture of a mouse monoclonal anti-HA antibody and a rabbit polyclonal anti-Hsc73 for 30 min. Cells were washed and incubated with a mixture of a Cy3-conjugated anti-mouse and a FITC-conjugated anti-rabbit antibody for 30 min. Colocalization studies were performed using dual excitation (488 nm for EGFP or FITC, 568 nm for Cy3) and emission (515–540 nm for EGFP or FITC, 590–610 nm for Cy3) filter sets. Specificity of labeling and absence of signal crossover were established by the examination of single-labeled samples.

FRET Assay. FRET was measured using confocal microscopy as described previously (Knowles et al., 1999; McLean et al., 2000; Siegel et al., 2000). The energy transfer was detected as an increase in donor fluorescence (FITC or GFP) after complete photobleaching of the acceptor molecules (Cy3). The amount of energy transfer was calculated as the percentage increase in donor fluorescence after acceptor photobleaching. An initial scan was obtained at low laser energy using the 488 line of the krypton-argon laser to record the fluorescein (or GFP) signal. A second scan was performed with the 568 line, and the area of colocalization was noted. A small part of the cell (approximately $5 \times 5 \mu\text{m}$) was then photobleached with brief

exposure to intense 568-nm light (laser power 100%) to destroy the acceptor molecules. The cells were then rescanned using 488-nm light. An increase of the fluorescein (or GFP) within the photobleached area was used as a measure of the amount of FRET present. The ratio $FID2/FID1$ (in which $FID2/FID1$ indicates the ratio of donor fluorescence after photobleaching to donor fluorescence before photobleaching, both corrected for background) was compared with the null hypothesis value of 1.0 by one-group *t* tests. FRET can be detected only if the two fluorophores are in close physical proximity: the distance between fluorophores must be less than 10 nm. To detect CXCR4-Hsc73 interactions, FRET was examined in fixed HEK293 cells overexpressing EGFP-CXCR4. After being treated with or without CXCL12 (10 nM) for 60 min, cells were incubated with a mouse monoclonal anti-Hsc73 antibody (1:50) for 30 min, followed by incubating with a Cy3-conjugated anti-mouse secondary antibody (1:1000) for 30 min. FRET between EGFP-CXCR4 and Hsc73 was examined as outlined above.

A negative control for the FRET experiment is simply the absence of the acceptor (Cy3) fluorophore. Exposing singly labeled cells to 568 nm of light for equivalent times did not alter the amount of fluorescein emission. In this case, $FID2/FID1$ was 1.009 ± 0.06 (mean \pm S.E.), indicating no FRET. Further negative controls for the FRET experiments include the following conditions: cells cotransfected with HA-CXCR4 and myc-CXCR4 showed complete colocalization when immunostained with mouse anti-HA (labeled by Cy3) and rabbit anti-myc (labeled by FITC) antibodies, but no FRET was observed ($FID2/FID1 = 1.006 \pm 0.07$, mean \pm S.E., $n = 10$, not significant). The positive control for the FRET experiment was done in the following way: cells transfected with myc-CXCR4 were fixed and incubated with mouse anti-myc antibody, then labeled with goat anti-mouse IgG conjugated with Cy3, followed by applying the donkey anti-goat IgG-conjugated with FITC. This positive control provides an "upper limit" on the amount of FRET one might measure in these systems: this was shown to be $FID2/FID1 = 1.58 \pm 0.14$ (mean \pm S.E., $n = 10$, $P < 0.0001$).

Chemoinvasion Assay. A 48-well chemotaxis chamber (Neuroprobe Inc., Gaithersburg, MD) was used for the chemoinvasion assay (Fan et al., 2001b). U87 glioma cell lines stably expressing CXCR4 were transiently transfected with pcDNA3.1 vector, pcDNA3.1/Hsc73, BS/U6 vector, or BS/U6-Hsc73-RNAi for 48 h. Cells were removed from the culture dish by trypsinization, washed with Hanks' solution, and incubated in 10% fetal bovine serum/DMEM for 2 h at 37°C to allow time for the restoration of receptors. The cells (5×10^5 cells/ml) were washed with serum-free DMEM buffer and then loaded into the upper chamber. The lower compartment of the chamber was loaded with CXCL12 diluted in the serum-free DMEM (1–100 nM). The chamber was incubated for 4 h at 37°C in humidified air with 5% CO_2 , then the membrane was removed, washed, fixed, and stained with a Diff-Quik kit (American Scientific, Columbus, OH). Cell chemotaxis was quantified by counting the number of migrating cells present in 10 microscope fields ($20\times$ objective lens).

Radioligand Binding Assay. HEK293 cells stably expressing CXCR4 were transiently transfected with vector, pcDNA3.1/Hsc73, or BS/U6-Hsc73-RNAi for 48 h. Cells were harvested and homogenized in 50 mM Tris-HCl, pH 7.4. The homogenates were centrifuged at 38,000g for 15 min at 4°C, and the pellets were resuspended in 50 mM Tris-HCl, pH 7.4, and 0.1% bovine serum albumin (Sigma Chemical, St. Louis, MO). Membrane suspensions (0.5 mg/ml) were incubated with different concentrations of ^{125}I -CXCL12 (2200 Ci/mmol) in 0.4 ml of 50 mM Tris-HCl, pH 7.4, and 1% bovine serum albumin at 30°C for 1 h. The reaction was terminated by filtering through GF/B filters presoaked with 0.1% polyethyleneimine. Radioactivity counts in the vials were determined using a gamma counter. Nonspecific binding was determined using a 500-fold excess of unlabeled CXCL12 (Santa Cruz Biotechnology) and was subtracted from the total binding. Three replicates of each point were performed. Binding affinity and capacity were determined by the program Prism (GraphPad Software Inc., San Diego, CA).

Ligand-Receptor Complex Internalization Assay. The acid-wash technique was used to determine the kinetics of CXCL12-induced internalization of CXCR4 (Fan et al., 2001b). HEK293 cells expressing CXCR4 were grown to confluence on 24-well plates, which were precoated with 0.1 mg/ml poly-L-lysine (Sigma; $M_r = 30,000\text{--}70,000$) for 1 h and washed once with distilled water before use. Cells were incubated at 4°C in 0.5 ml of serum-free DMEM containing 75 nCi/ml ^{125}I -CXCL12 for 1 h. The medium was subsequently removed, 1 ml of ice-cold serum-free DMEM was added carefully into each well and aspirated, and then another 1-ml aliquot of ice-cold serum-free DMEM was added before incu-

bation at 37°C for the indicated time. The medium was removed, and the cells were incubated with 1 ml of ice-cold 0.2 M acetic acid with 0.5 M NaCl for 6 min. After the incubation, the cells were washed once with 1 ml of ice-cold serum-free DMEM and lysed with 1 ml of 1% SDS with 0.1 N NaOH (lysis solution). The radioactive cell lysate was then counted on a gamma counter (Gamma 5500; Beckman Coulter, Fullerton, CA). Total cell surface receptor binding was measured after incubation with ^{125}I -CXCL12 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

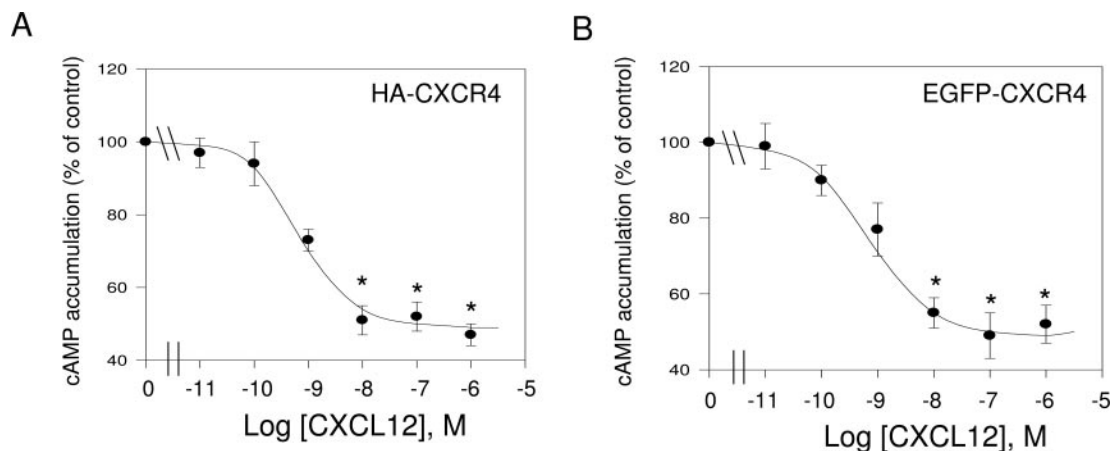


Fig. 1. CXCL12 induced inhibition of cAMP accumulation in HEK293 cells stably expressing HA-CXCR4 or EGFP-CXCR4. HEK293-HA-CXCR4 (A) or HEK293-EGFP-CXCR4 (B) cells were treated with forskolin in the absence or presence of different concentrations of CXCL12. The accumulation of cAMP was measured as described under *Materials and Methods*. Values are mean \pm S.E. of three separate experiments. *, $P < 0.05$, compared with nontreated cells.

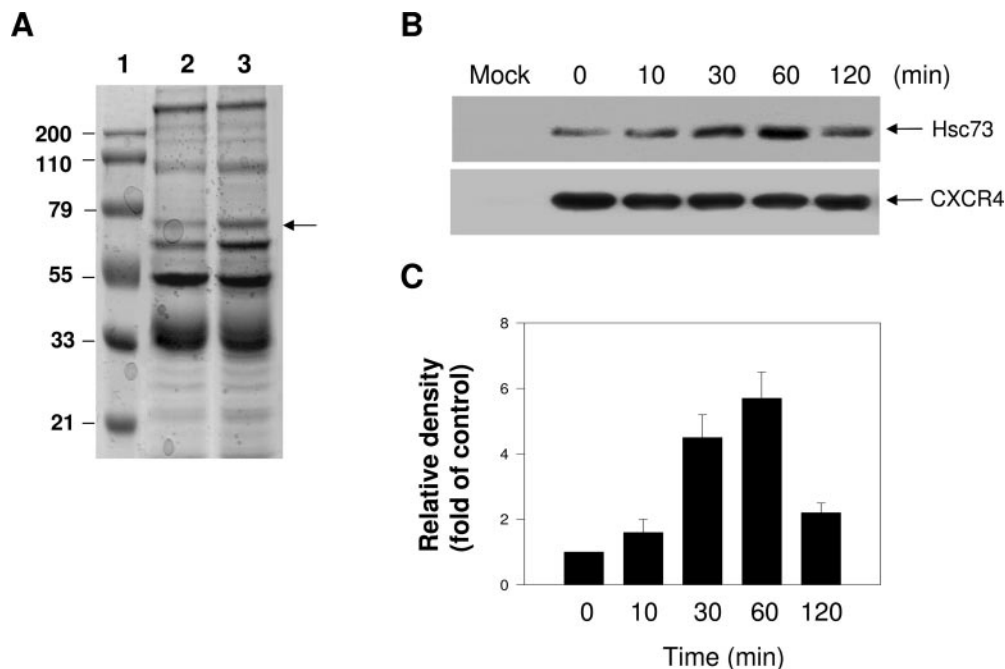


Fig. 2. Identification of Hsc73 as a CXCR4-interacting protein. A, HEK293 cells stably expressing HA-CXCR4 were treated with carrier buffer or CXCL12 (10 nM) for 10 min. HA-CXCR4-interacting proteins were isolated by immunoprecipitation of HA-CXCR4 from HEK293 cell lysates using an anti-HA antibody as described under *Materials and Methods*. The samples were electrophoresed on a 10% SDS-polyacrylamide gel and then stained with SYPRO ruby protein stain. The 70-kDa band (arrow) was excised, digested with trypsin, and analyzed by mass spectrometry. B, HEK293 cells stably expressing HA-CXCR4 were exposed to CXCL12 (10 nM) for the indicated time intervals, and CXCR4 was immunoprecipitated from the cell lysate using a specific CXCR4 antibody. In a parallel experiment, parental HEK293 cells were treated with CXCL12 for 60 min, and immunoprecipitation was performed as described above (mock). Proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Coprecipitated Hsc73 proteins were detected using a monoclonal anti-Hsc73 antibody. The membrane was stripped and reblotted with a specific anti-HA antibody to confirm equal loading. C, quantification of the density of bands representing Hsc73 time-dependently associated with CXCR4 was determined by densitometric scanning. Data are means \pm S.E. from three independent experiments.

^{125}I -CXCL12 in binding medium. Calculation of the percentage of internalized receptor was performed as described before (Fan et al., 2001b).

Statistic Analysis. When means \pm S.D. are given, differences between groups were tested for significance ($P < 0.05$) using Student's *t* test for unpaired samples.

Results

Identification of Proteins Associated with HA-CXCR4 in HEK293 Cells. The signaling and trafficking of CXCR4 are regulated by a number of intracellular proteins after ligand treatment (Fan et al., 2001b, 2002, 2003). Here, we attempted to identify proteins that interact with the ligand-stimulated CXCR4 receptors. The strategy used involved immunoprecipitation of HA-CXCR4 from cell lysates followed by SDS-PAGE and mass spectrometry. Before performing this experiment, the functional expression of HA-CXCR4 in the HEK293-HA-CXCR4 stable cell lines was determined by cAMP assay. CXCR4 is a G_i -coupled receptor, and ligand stimulation of the receptor induced down-modulation of cAMP (Cheng et al., 2000; Dwinell et al., 2004). Therefore, this assay was used to determine the functional expression of CXCR4 in HEK293 cells, which do not endogenously express CXCR4 receptors. The HEK293-HA-CXCR4 stable cell lines were incubated with forskolin (1 μM) in the absence or presence of different concentrations of CXCL12, and cAMP was measured by radioimmunoassay (Fan et al.,

1998). As shown in Fig. 1A, CXCL12 dose-dependently inhibited forskolin-induced cAMP accumulation with an IC_{50} of 1 nM, which is consistent with the previous report (Cheng et al., 2000). These data indicate successful establishment of HEK293-HA-CXCR4 stable cell lines.

To identify CXCR4 interacting proteins, HEK293 cells ($5 \times 10^6/\text{ml}$) stably expressing HA-CXCR4 were treated with carrier buffer or CXCL12 (10 nM) for 30 min. Cells were lysed, and the lysate was incubated with an anti-HA polyclonal antibody. The resulting protein complexes were electrophoresed on a 10% polyacrylamide gel and then stained with Coomassie Blue or SYPRO ruby stain. A strong protein band of 73 kDa was clearly observed to be increased in the CXCL12-treated cells (Fig. 2A, lane 3, arrow) compared with the band of the cells without CXCL12 treatment (Fig. 2A, lane 2). The 73-kDa band was excised from the gel, digested with trypsin, and analyzed by mass spectrometry as described under *Materials and Methods*. The 73-kDa protein was identified as Hsc73 (21 matching peptides and 36% overall sequence coverage).

In Vivo and In Vitro Association of Hsc73 with CXCR4. We next confirmed the ligand-dependent association of CXCR4 with Hsc73 in HEK293 cells by coimmunoprecipitation assay. Cells stably expressing HA-CXCR4 were treated with CXCL12 (10 nM) for different time intervals (0, 10, 30, 60, and 120 min). CXCR4 receptors were immunoprecipitated from the cell lysate using an anti-CXCR4 antibody,

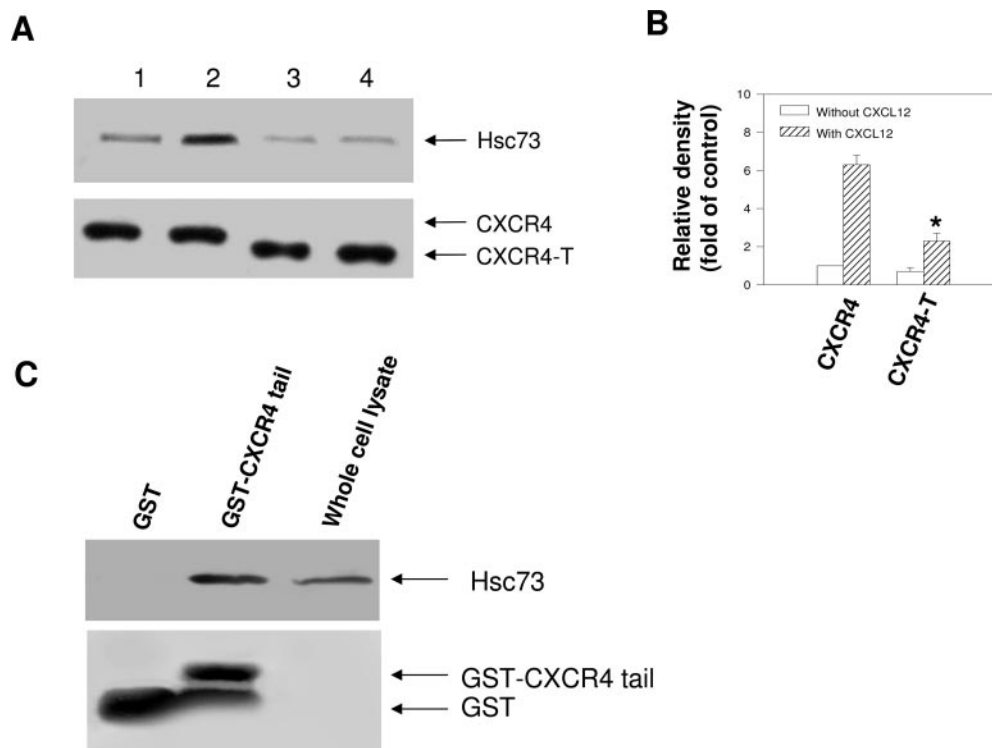


Fig. 3. Requirement of the CXCR4 carboxyl-terminal domain in the interaction with Hsc73. A, HEK293 cells expressing the HA-tagged full-length (lanes 1 and 2) or the carboxyl-terminal truncation mutant ($\Delta 34$) of CXCR4 (lanes 3 and 4) were treated with carrier buffer (lanes 1 and 3) or CXCL12 (10 nM) (lanes 2 and 4) for 30 min. CXCR4 was immunoprecipitated from the cell lysate and coprecipitated Hsc73 proteins were detected by Western blot analysis as described above. B, quantification of the density of bands representing Hsc73 associated with CXCR4 or CXCR4- $\Delta 34$ T was determined by densitometric scanning. Data are mean \pm S.E. from three independent experiments. *, $P < 0.05$ compared with the full-length CXCR4-expressing cells with the same treatment. C, GST or GST-CXCR4 carboxyl terminus fusion proteins were incubated with the cell lysate of HEK293 cells and then adsorbed 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 Hsc73 proteins were analyzed by immunoblotting with a monoclonal Hsc73 antibody. The membrane was stripped and reblotted with a mouse monoclonal GST antibody to confirm equal loading of the protein samples. Shown are representative of three independent experiments with similar results.

and coprecipitated Hsc73 was detected with a specific anti-Hsc73 antibody. To confirm the specificity of the coimmunoprecipitation, in a parallel experiment, parental HEK293 cells were treated with CXCL12 for 60 min (mock), and immunoprecipitation was performed as described above. As shown in Fig. 2, immunoprecipitation of HA-CXCR4 from HEK293 cells revealed a basal association of the receptors with Hsc73, and CXCL12 (10 nM) treatment resulted in a time-dependent increase in the association of Hsc73 with CXCR4, which peaked 30 to 60 min after agonist stimulation (Fig. 2, B and C). However, in the parental HEK293 cells treated with CXCL12 (mock), no Hsc73 was pulled down with the CXCR4 antibody, suggesting specific association of HA-CXCR4 with Hsc73.

To examine whether the carboxyl-terminal domain is required for the association of CXCR4 with Hsc73, a carboxyl-terminal truncation mutant of CXCR4 (CXCR4- Δ 34), which exhibits decreased internalization (Cheng et al., 2000), was used in the coimmunoprecipitation assay. HEK293 cells transiently expressing the full-length HA-CXCR4 or the truncated HA-CXCR4 mutant CXCR4 (Δ 34) were treated with CXCL12 (10 nM) for 30 min. The full-length and the truncated receptors were immunoprecipitated from the cell lysate using a polyclonal anti-HA antibody, and coprecipitated Hsc73 was detected by Western blotting with a specific monoclonal antibody. As shown in Fig. 3, A and B, ligand stimulation induced marked increase in the association of Hsc73 with the full-length CXCR4 but not with the truncation mutant form of CXCR4, suggesting that the carboxyl-terminal domain is required for the receptor association with Hsc73. To confirm the specific biochemical interaction between the carboxyl terminus of CXCR4 and Hsc73, we used an *in vitro* binding assay to examine the direct interaction. GST or GST-CXCR4 carboxyl-terminal fusion protein was purified from *Escherichia coli* as described under *Materials and Methods*. Equal amounts of GST and GST-CXCR4 carboxyl-terminal fusion proteins were incubated with the cell lysate of HEK293 cells, and coprecipitated Hsc73 was detected by Western blotting with a specific anti-Hsc73 antibody. The membrane was stripped and reblotted with a monoclonal anti-GST antibody to confirm equal loading. As shown in Fig. 3C, Hsc73 was coprecipitated with the GST-CXCR4 carboxyl-terminal fusion protein but not with the GST alone, suggesting an association of Hsc73 with the carboxyl-terminal domain of CXCR4.

Colocalization of CXCR4 with Hsc73 in Internal Vesicles. Based on the observation that the interaction between CXCR4 and Hsc73 lasts for at least 60 min, whereas in the same time frame most of the receptors are internalized (Fan et al., 2003), we hypothesize that the interaction between CXCR4 and Hsc73 might occur in internal vesicles. To test this hypothesis, we sought to determine the potential colocalization between EGFP-CXCR4 and Hsc73 in HEK293 cells, which have been confirmed to functionally express EGFP-CXCR4 (Fig. 1B). To determine the colocalization, HEK293-EGFP-CXCR4 cells were treated with CXCL12 (10 nM) for different time intervals. Hsc73 proteins were immunostained with a specific antibody as described under *Materials and Methods*. As shown in Fig. 4, before ligand stimulation, EGFP-CXCR4 receptors were exclusively expressed on the cell surface, whereas Hsc73 proteins were predominantly in the cytoplasm, although a small proportion of

Hsc73 proteins were observed on the cell surface. Ligand treatment for 30 or 60 min induced significant internalization of EGFP-CXCR4, and the internalized receptors colocalized with Hsc73 in internal vesicles (Fig. 4, arrow).

To test whether this colocalization suggested a close intermolecular proximity, we performed FRET assays. FRET, reflected by a ratio of $FID2/FID1$ of greater than 1.0, can only be detected if the fluorophores are within 10 nm of each other. Positive and negative controls are described under *Materials and Methods* and suggest that in this assay, no interaction results in a ratio of 1.0, and a very strong interaction (antibody-antibody binding) gives a ratio of 1.58. FRET was observed between EGFP-CXCR4 and Hsc73 on the cell surface (at time 0) ($FID2/FID1 = 1.21 \pm 0.03$, mean \pm S.E., $n = 10$, $P < 0.001$) (Fig. 5E), indicating that those two molecules directly interact with each other on the cell surface. It is noteworthy that robust FRET was detectable in the internal vesicles during endocytosis at 30 to 60 min ($FID2/FID1 = 1.35 \pm 0.067$, mean \pm S.E., $n = 15$, $P < 0.001$, for 30 min, $FID2/FID1 = 1.31 \pm 0.078$, mean \pm S.E., $n = 15$, $P < 0.001$, for 60 min) (Fig. 5, A–E).

We next determined whether knockdown of Hsc73 by means of RNAi affects the subcellular localization of CXCR4.

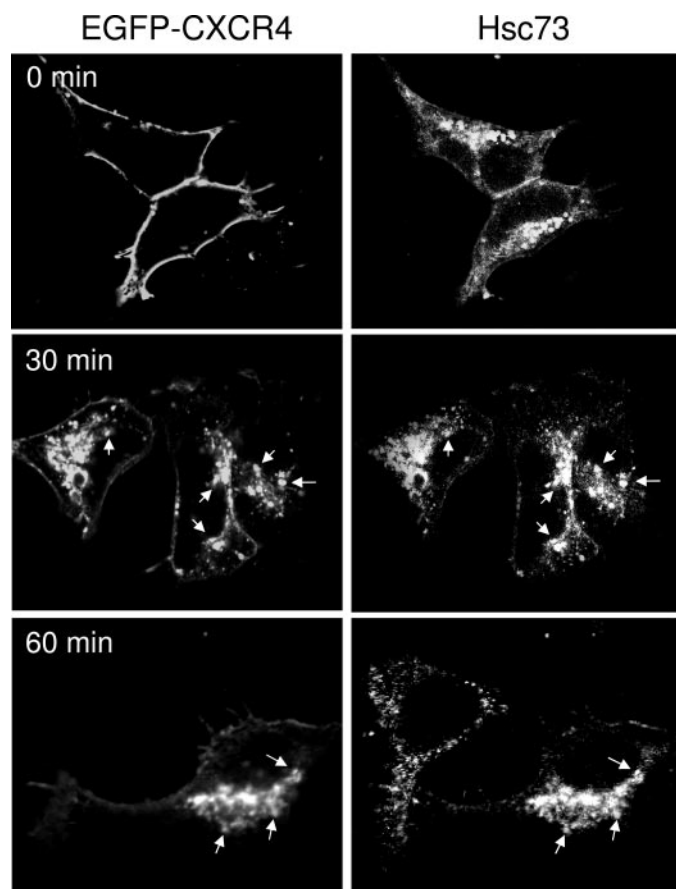


Fig. 4. Colocalization of CXCR4 with Hsc73 in internal vesicles. HEK293 cells stably expressing EGFP-CXCR4 were treated with CXCL12 for different time intervals indicated. Cells were incubated with a mouse monoclonal Hsc73 antibody for 30 min, followed by incubation with a mixture of a Cy3-conjugated anti-mouse antibody for 30 min. Confocal microscopy was performed as described in *Materials and Methods*. Shown are representative images from three independent experiments demonstrating the subcellular localization of EGFP-CXCR4, Hsc73, and colocalization of these three proteins (arrow). Bars, 10 μ m.

We first determined whether Hsc73 expression was specifically knocked down by specific RNAi in pSUPER vector. HEK293 cells stably expressing HA-CXCR4 were transfected with pSUPER vector or pSUPER-Hsc73-RNAi clones 1 and 2 (RNAi-1 and RNAi-2) for different time intervals, and the

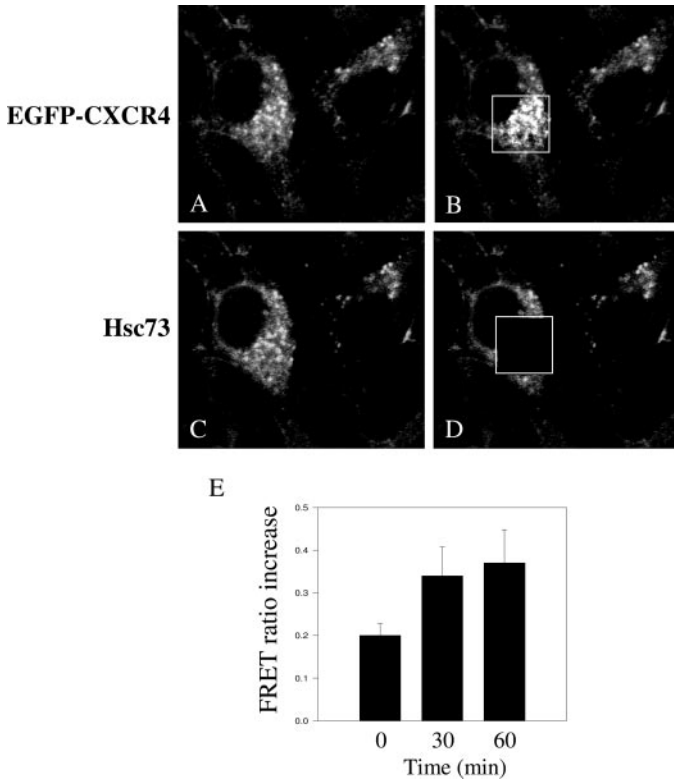


Fig. 5. Example of FRET reflecting the interaction between EGFP-CXCR4 and Hsc73 in HEK293 cells. A to D, example of FRET between EGFP-CXCR4 and Hsc73 in internal vesicles. HEK293-EGFP-CXCR4 cells were treated with CXCL12 for 60 min, immunostained with anti-Hsc73 antibody (labeled by Cy3). Most of the EGFP-CXCR4 signal was found in internal vesicles and colocalized with the Hsc73 signal. Photobleaching of the Cy3 label of Hsc73 (D, boxed area) led to a marked increase in the EGFP-CXCR4 fluorescent signal within the photobleached area, demonstrating FRET (B, boxed area). Bar, 10 μ m. E, FRET ratio increases of *FID2/FID1* after photobleaching between Hsc73 and EGFP-CXCR4 on cell surface and in the internal vesicles are shown. FRET measurements are shown for the interactions after ligand treatment for different time intervals (0, 30, and 60 min). FRET increases on the cell surface and in the internal vesicles were significantly above 1.0 ($P < 0.0001$).

expression of Hsc73 and other Hsp70 family members, including Hsp70, GRP75, and GRP78, was determined by Western blot analysis using specific antibodies, respectively. We observed that transfection of the Hsc73 specific RNAi-1 and RNAi-2 but not the pSUPER vector induced down-regulation of Hsc73, and maximal knockdown was observed after 48 h of the transfection (Fig. 6A). The expression of other Hsp70 family members, including GRP75, HSP70, and GRP78, was not affected by the transfection of the Hsc73 RNAi-1 (Fig. 6B) and RNAi-2 (data not shown).

To assess the role of Hsc73 knockdown in the subcellular localization of CXCR4, HEK293 cells stably expressing HA-CXCR4 were transfected with pSUPER vector (control) or the pSUPER-Hsc73-RNAi (clone 1) for 48 h. Cells were treated with or without CXCL12 (10 nM) for 30 min. The HA-CXCR4 was immunostained as described above. Transfection of the pSUPER vector affected neither cell surface expression of CXCR4 (Fig. 7, A and B) nor ligand-induced CXCR4 internalization (Fig. 7, C and D, small arrows). Transfection of the Hsc73-RNAi did not affect the cell surface expression of CXCR4 (Fig. 7, E and F). However, ligand-induced CXCR4 internalization was significantly inhibited by the expression of the Hsc73-RNAi (Fig. 7, G and H, large arrow). These data suggest that knockdown of Hsc73 blocks CXCR4 endocytosis.

To confirm that Hsc73 is involved in CXCR4 endocytosis, the internalization of radioligand-CXCR4 complex was assessed. HEK293 cells stably expressing CXCR4 were transiently transfected with pcDNA3.1 vector (control), pcDNA3.1/Hsc73, BS/U6 vector, or BS/U6-Hsc73-RNAi clones 1 and 2 (RNAi-1 and RNAi-2) for 48 h. Cells were incubated with 125 I-CXCL12 at 4°C for 1 h before being warmed to 37°C for different time intervals. After acid washing to eliminate the cell surface 125 I-CXCL12, the internalized 125 I-CXCL12 was detected in a gamma counter. Figure 8A shows a time-dependent internalization of 125 I-CXCL12-receptor complex. Overexpression of Hsc73 did not significantly affect the internalization of 125 I-CXCL12-receptor complex. However, in the cells transfected with both of the Hsc73-RNAi plasmids, the internalization of 125 I-CXCL12-receptor complex was attenuated approximately 60% compared with the internalization of 125 I-CXCL12-receptor complex in the control cells ($P < 0.05$). Figure 8B shows that Hsc73 was overexpressed in the pcDNA3/Hsc73-transfected cells and knocked down in the BS/U6-Hsc73-RNAi-1 and

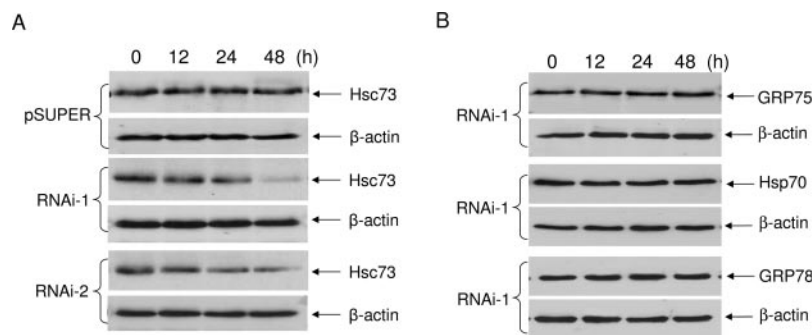


Fig. 6. Hsc73 expression was knocked down by specific RNAi. A, HEK293 cells stably expressing HA-CXCR4 were transfected with pSUPER vector (control), pSUPER-Hsc73-RNAi clone 1 (RNAi-1), or pSUPER-Hsc73-RNAi clone 2 (RNAi-2) for 48 h. Western blot was performed to detect the expression of Hsc73 and β -actin (internal control) using specific antibodies, respectively. B, HEK293 cells stably expressing HA-CXCR4 were transfected with pSUPER-Hsc73-RNAi clone 1 (RNAi-1) for 48 h. Western blot was performed to detect the expression of Hsc73, Hsp70, GRP75, GRP78, and β -actin (internal control) using specific antibodies, respectively. Shown are representatives of three independent experiments with similar results.

BS/U6-Hsc73-RNAi-2 transfected cells. These data together with the data obtained from confocal studies (Fig. 7) suggest that Hsc73 is involved in CXCR4 endocytosis.

Knockdown of Hsc73 Expression Attenuated CXCR4-Mediated Chemoinvasion of Glioma Cells. Previous studies have demonstrated the important role of CXCR4

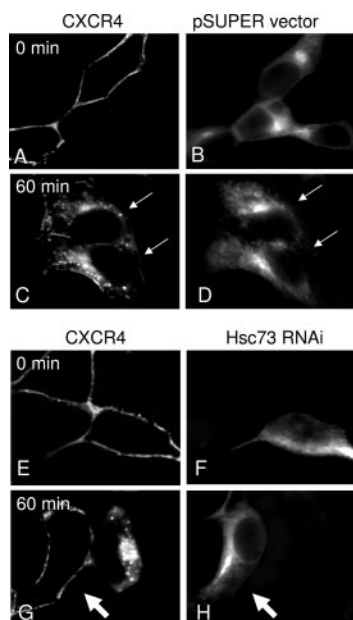


Fig. 7. Knockdown of Hsc73 attenuated CXCR4 endocytosis. HEK293 cells stably expressing HA-CXCR4 were transiently transfected with pSUPER vector (A–D) or pSUPER-Hsc73-RNAi (clone 1) (E–H) for 48 h. Cells were treated with (C, D, G, and H) or without (A, B, E, and F) CXCL12 (10 nM) for 30 min before being fixed in methanol. Cells were incubated with an anti-HA (mouse monoclonal) for 30 min followed by incubation with a Cy3-conjugated anti-mouse secondary antibody. Confocal microscopy was performed as described under *Materials and Methods*. Large arrow, inhibition of CXCR4 internalization in the pSUPER-Hsc73-RNAi-transfected cells. Small arrows, internalization of CXCR4 in the pSUPER vector-transfected cells. Images were processed using Photoshop software (Adobe Systems, Mountain View, CA). Scale bars, 10 μ m.

internalization in the receptor-mediated chemotaxis (Yang et al., 1999; Fan et al., 2001b). We determined whether the overexpression or knockdown of Hsc73 affect CXCR4-mediated chemotaxis. U87 glioma cell lines stably expressing CXCR4 were transiently transfected with pcDNA3.1 vector, pcDNA3/Hsc73, BS/U6 vector, and BS/U6-Hsc73-RNAi clones 1 and 2 (RNAi-1 and RNAi-2) for 48 h. CXCL12-induced chemotaxis was examined using chemotaxis chamber assay as described under *Materials and Methods*. As shown in Fig. 9, A and B, ligand treatment induced a typical bell-shape chemotactic response in the control vector-transfected cells. Overexpression of wild-type Hsc73 did not significantly affect the cell chemotaxis. However, transfection of both the Hsc73 RNAi plasmids (RNAi-1 and RNAi-2) significantly reduced the receptor-mediated chemotaxis. Figure 9C shows that in the cells used for the chemotaxis experiment, Hsc73 was overexpressed in the pcDNA3/Hsc73-transfected cells and knocked down in both the Hsc73 RNAi plasmid-transfected cells.

Effect of Hsc73 on Ligand Binding to CXCR4. We hypothesized that the inhibitory effect of Hsc73 RNAi on CXCR4 endocytosis and CXCR4-mediated chemotaxis might be due to its inhibition of receptor-ligand binding. To test this hypothesis, HEK293 cells stably expressing HA-CXCR4 were transfected with vector (control), pcDNA3/Hsc73, BS/U6 vector, or BS/U6-Hsc73-RNAi (clone 1) for 48 h. Radioligand binding assay was performed as described under *Materials and Methods*, and maximum binding capacity (B_{max}) and dissociation constants (K_d) were calculated. As shown in Table 1, no significant differences in the B_{max} and K_d values were observed among the cells transfected with vectors, pcDNA3/Hsc73, and Hsc73 RNAi plasmids. In a parallel experiment using Western blot analysis to detect the expression of Hsc73 in the cells transfected with the above plasmids, significant overexpression (4.5 times greater than the control) of Hsc73 in the Hsc73-expressing cells and significant knockdown (13% of the control) of Hsc73 in the siRNA-

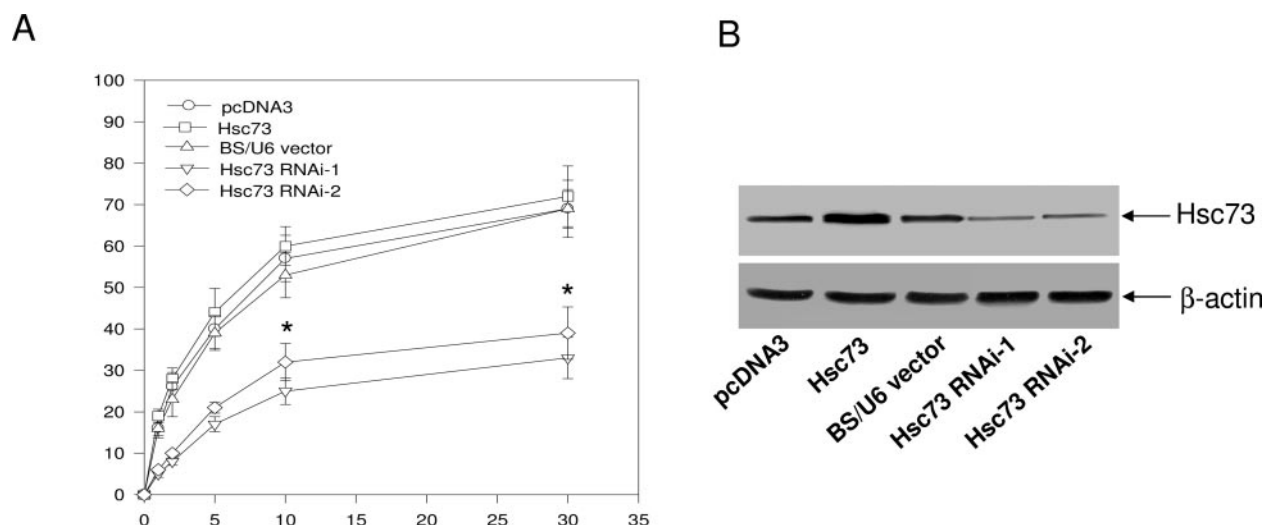


Fig. 8. Knockdown of Hsc73 attenuated ligand-CXCR4 complex internalization. A, HEK293 cells stably expressing HA-CXCR4 were transiently transfected with pcDNA3.1 vector, pcDNA3/Hsc73, BS/U6 vector, BS/U6-Hsc73-RNAi clone 1 (RNAi-1), or BS/U6-Hsc73-RNAi clone 2 (RNAi-2) for 48 h. Cells were incubated with 125 I-CXCL12 at 4°C for 1 h before being warmed to 37°C for different time intervals. After acid washing to eliminate the cell surface 125 I-CXCL12, the internalized 125 I-CXCL12 was detected in a gamma counter. Values represent the mean \pm S.E. of three independent experiments performed in duplicate. The data were analyzed using Student's paired *t* test (*, $P < 0.05$). B, Western blots indicate the expression level of Hsc73 (top) in the cells transiently transfected with different plasmids for 48 h. Western blot of β -actin (bottom) was performed as an internal control.

transfected cells were observed (data not shown). These data suggest that the inhibitory effects of the Hsc73 RNAi on CXCR4 endocytosis and the receptor-mediated chemotaxis are not due to the altered affinity of ligand binding to CXCR4.

Discussion

The internalization and intracellular trafficking of chemokine receptors have important implications for the cellular responses elicited by chemokine receptors. In the present study, we identified Hsc73 as a binding protein of CXCR4, which associated with Hsc73 in a ligand-dependent manner. The receptor carboxyl-terminal domain is required for the interaction, because truncation of the carboxyl terminus of CXCR4 blocked the interaction, and a GST-CXCR4 carboxyl-terminal domain fusion protein associated with Hsc73. The interaction between Hsc73 and CXCR4 has important physiological implications, because knockdown of Hsc73 expression by means of specific RNAi inhibited the receptor endocytosis and the receptor-mediated chemotaxis.

Members of the Hsp70 family interact with a number of proteins, especially the non-native forms of proteins, because of the molecular chaperone function of Hsp70 proteins

(Blond-Elguindi et al., 1993). However, the ligand-dependent association of Hsc73 with CXCR4 and the colocalization between Hsc73 and CXCR4 in internal vesicles suggest that Hsc73 interacts with functional receptors. Although some of the interactions established with Hsc73 seem to be nonspecific, some require a precise structure. Geminard et al. (2004) found that Hsc73 binds to transferrin receptor at a peptide region of YTRFSLARQV (Geminard et al., 2004). However, the carboxyl-terminal region in which Hsc73 binds to CXCR4 does not contain this motif, suggesting that differential binding domains are involved in the association of Hsc73 with CXCR4 and transferrin receptor.

TABLE 1
Effect of Hsc73 on ligand binding to CXCR4
Values are presented as mean \pm S.E.

Transfectants	B_{max} <i>pmol/mg protein</i>	K_d <i>nM</i>
pcDNA3.1 vector	0.97 \pm 0.056	4.4 \pm 0.33
pcDNA3.1/Hsc73	0.92 \pm 0.047	4.6 \pm 0.62
BS/U6 vector	1.02 \pm 0.058	4.7 \pm 0.39
BS/U6-Hsc73-RNAi	0.99 \pm 0.067	3.8 \pm 0.42

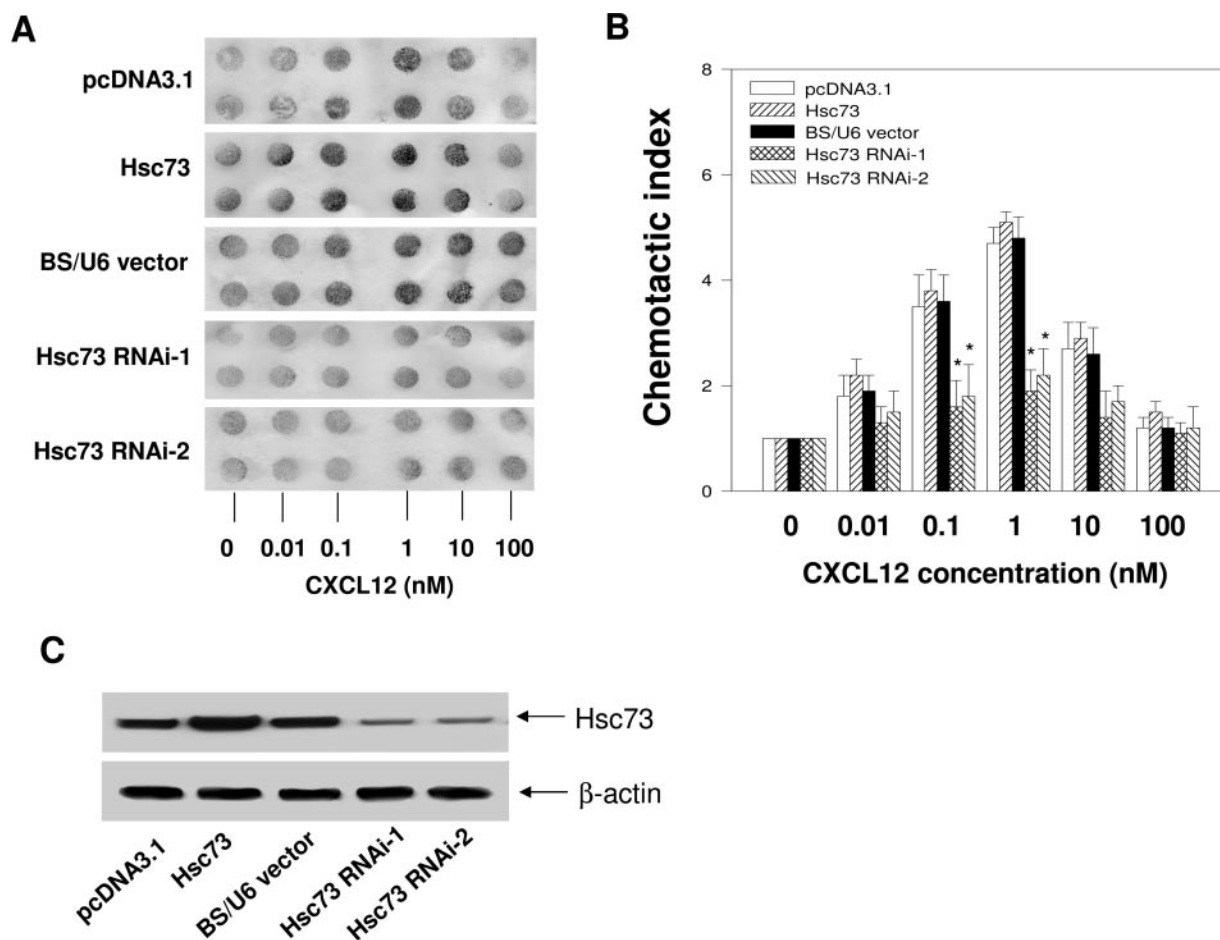


Fig. 9. Involvement of Hsc73 in CXCR4-mediated chemoinvasion. A, pcDNA3.1 vector, pcDNA3/Hsc73, BS/U6 vector, BS/U6-Hsc73-RNAi clone 1 (RNAi-1), or BS/U6-Hsc73-RNAi clone 2 (RNAi-2) for 48 h. CXCL12 (0–100 nM)-induced chemotaxis was examined using chemotaxis chamber assay as described under *Materials and Methods*. Shown is a representation of three independent experiments with similar results. B, cell migration was quantified by counting the number of cells migrated to the other side of the filter in 10 microscope fields (20 \times objective lens). Values represent the mean \pm S.E. of three different experiments. Data were analyzed using Student's paired *t* test. *, *P* < 0.05, compared with the control cells with the same treatment. C, Western blots indicate the expression level of Hsc73 (top) in the cells transiently transfected with different plasmids for 48 h. Western blot of β -actin (bottom) was performed as an internal control.

Consistent with our data showing colocalization of the internalized CXCR4 with Hsc73 in internal vesicles, Sarrio et al. (2000) reported that Hsc73 colocalized with A1 adenosine receptor, a member of GPCR superfamily, in internal vesicles. These findings suggest the involvement of Hsc73 in the endocytosis of certain GPCRs. Furthermore, we demonstrated that the knockdown of Hsc73 expression with specific RNAi inhibited CXCR4 internalization. This provides strong evidence for the involvement of Hsc73 in endocytosis of certain GPCRs in vivo. Studies on other membrane proteins have also demonstrated the involvement of Hsc73 in endocytosis. The endocytosis of transferrin receptors is sensitive to antibodies against Hsc73 (Geminard et al., 2004), and the receptor internalization and recycling are blocked by the overexpression of ATPase-deficient Hsc73 mutants (Newmyer and Schmid, 2001). Although the underlying mechanisms remain to be investigated, it is possible that through association with the internalized receptors Hsc73 is recruited to the CCV. Based on the findings in in vitro studies that Hsc73 broadly modulates clathrin dynamics throughout the CCV cycle by releasing coat proteins from CCV (Hannan et al., 1998), a process required for the fusion of the vesicles with endosomes (Altstiel and Branton, 1983), it is conceivable that in the cells in which Hsc73 is knocked down, fusion of CCV with early endosomes is blocked, resulting in the inhibition of receptor endocytosis. It is unclear why the overexpression of Hsc73 did not affect ligand-CXCR4 complex internalization. One explanation is that Hsc73 is enriched in the cells, and only a proportion of endogenous Hsc73 proteins are used in receptor endocytosis, so the overexpression of Hsc73 does not increase the proportion of Hsc73 involved in the receptor endocytosis.

Increasing lines of evidence support the proposal that endocytosis of chemokine receptors plays a role in the receptor-mediated chemotaxis (Yang et al., 1999; Fan et al., 2001b, 2002). Daaka et al. (1998) reported that truncation of the carboxyl terminus of CXCR4 not only inhibited the receptor internalization but also blocked the receptor-mediated chemotaxis. Compounds that inhibit the endocytosis of CXCR4 also blocked the receptor-mediated chemotaxis (Ji et al., 2005). Consistent with these reports, the present data demonstrated that knockdown of Hsc73 with specific RNAi not only reduced CXCR4 endocytosis but attenuated the receptor-mediated chemotaxis as well. The molecular mechanism underlying the role of receptor endocytosis in chemokine receptor-mediated chemotaxis is not fully understood. It is known that chemokine receptors undergo phosphorylation and desensitization in response to ligand stimulation, followed by receptor internalization (Fan et al., 2001a,b). The internalized receptors are dephosphorylated by protein phosphatases in endosomal compartments, and the dephosphorylated receptors are able to recycle to the cell surface to respond the extracellular ligands again (Fan et al., 2001a). This process, named resensitization, is critical for the cells to continually respond to the gradient concentrations of ligands during chemotaxis. It is therefore conceivable that blocking the receptor internalization may not affect the receptor phosphorylation but inhibits the receptor dephosphorylation and the subsequent resensitization, resulting in the inhibition of chemotaxis. This is supported by our previous results, showing that inhibition of receptor dephosphorylation blocked chemokine-induced chemotaxis (Fan et al., 2001a). It would

be of interest to determine whether knockdown of Hsc73 blocked CXCR4 dephosphorylation in the future studies.

Chemokine receptors play significant roles in tumorigenesis and metastasis (Balkwill, 2004). CXCR4 is one of the major chemokine receptors on certain cancer cells such as glioma and breast cancer cells (Zhou et al., 2002; Helbig et al., 2003). Considering that chemokine receptor-mediated chemotaxis plays a role in the metastasis of cancer cells (Murakami et al., 2004), agents which are able to block chemotaxis are potentially useful for the treatment of cancer metastasis. The RNAi of Hsc73 seems to belong to these agents, because the Hsc73 RNAi blocked CXCR4-mediated glioma cell chemotaxis. Several studies have demonstrated that Hsc73 is up-regulated in certain cancer cells (Strik et al., 2000), suggesting that up-regulation of Hsc73 may play a role in the metastasis or invasion of these cancer cells. Although in the present study, the overexpression of Hsc73 did not significantly enhance CXCR4-mediated chemoinvasion of U87 glioma cells, it cannot rule out the possibility that up-regulation of Hsc73 enhances multiple chemokine-induced cancer metastasis in vivo. This is because single chemokine receptor-mediated chemotaxis involves only a proportion of Hsc73, but multiple chemokine-induced chemotaxis involves more Hsc73 proteins. Nevertheless, in future studies, it would be of interest to investigate the potential therapeutic effect of Hsc73 RNAi on some cancers in which Hsc73 is up-regulated.

Acknowledgments

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References

- Alkhatib G, Ahuja SS, Light D, Mummidi S, Berger EA, and Ahuja SK (1997) CC chemokine receptor 5-mediated signaling and HIV-1 Co-receptor activity share common structural determinants. Critical residues in the third extracellular loop support HIV-1 fusion. *J Biol Chem* **272**:19771–19776.
- Altstiel L and Branton D (1983) Fusion of coated vesicles with lysosomes: measurement with a fluorescence assay. *Cell* **32**:921–929.
- Arai H, Monteclaro FS, Tsou CL, Franci C, and Charo IF (1997) Dissociation of chemotaxis from agonist-induced receptor internalization in a lymphocyte cell line transfected with CCR2B. Evidence that directed migration does not require rapid modulation of signaling at the receptor level. *J Biol Chem* **272**:25037–25042.
- Baggiolini M (1998) Chemokines and leukocyte traffic. *Nature (Lond)* **392**:565–568.
- Balkwill F (2004) Cancer and the chemokine network. *Nat Rev Cancer* **4**:540–550.
- Bleul CC, Farzan M, Choe H, Parolin C, Clark-Lewis I, Sodroski J, and Springer TA (1996) The lymphocyte chemoattractant SDF-1 is a ligand for LESTRE/fusin and blocks HIV-1 entry. *Nature (Lond)* **382**:829–833.
- Blond-Elguindi S, Fourie AM, Sambrook JF, and Gething MJ (1993) Peptide-dependent stimulation of the ATPase activity of the molecular chaperone BiP is the result of conversion of oligomers to active monomers. *J Biol Chem* **268**:12730–12735.
- Cheng ZJ, Zhao J, Sun Y, Hu W, Wu YL, Cen B, Wu GX, and Pei G (2000) β -Arrestin differentially regulates the chemokine receptor CXCR4-mediated signaling and receptor internalization and this implicates multiple interaction sites between β -arrestin and CXCR4. *J Biol Chem* **275**:2479–2485.
- Daaka Y, Luttrell LM, Ahn S, Della Rocca GJ, Ferguson SS, Caron MG, and Lefkowitz RJ (1998) Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. *J Biol Chem* **273**:685–688.
- Dwinell MB, Ogawa H, Barrett KE, and Kagnoff MF (2004) SDF-1/CXCL12 regulates cAMP production and ion transport in intestinal epithelial cells via CXCR4. *Am J Physiol* **286**:G844–G850.
- Fan GH, Lapiere LA, Goldenring JR, and Richmond A (2003) Differential regulation of CXCR2 trafficking by Rab GTPases. *Blood* **101**:2115–2124.
- Fan GH, Yang W, Sai J, and Richmond A (2001a) Phosphorylation-independent

- association of CXCR2 with the protein phosphatase 2A core enzyme. *J Biol Chem* **276**:16960–16968.
- Fan GH, Yang W, Sai J, and Richmond A (2002) Hsc/Hsp70 interacting protein (hip) associates with CXCR2 and regulates the receptor signaling and trafficking. *J Biol Chem* **277**:6590–6597.
- Fan GH, Yang W, Wang XJ, Qian Q, and Richmond A (2001b) Identification of a motif in the carboxyl terminus of CXCR2 that is involved in adaptin 2 binding and receptor internalization. *Biochemistry* **40**:791–800.
- Fan GH, Zhao J, Wu YL, Lou LG, Zhang Z, Jing Q, Ma L, and Pei G (1998) *N*-Methyl-D-aspartate attenuates opioid receptor-mediated G protein activation and this process involves protein kinase C. *Mol Pharmacol* **53**:684–690.
- Feng Y, Broder CC, Kennedy PE, and Berger EA (1996) HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science (Wash DC)* **272**:872–877.
- Ganju RK, Brubaker SA, Meyer J, Dutt P, Yang Y, Qin S, Newman W, and Groopman JE (1998) The chemokine, stromal cell-derived factor-1, binds to the transmembrane G-protein-coupled CXCR4 receptor and activates multiple signal transduction pathways. *J Biol Chem* **273**:23169–23175.
- Geminard C, De Gassart A, Blanc L, and Vidal M (2004) Degradation of AP2 during reticulocyte maturation enhances binding of Hsc73 and Alix to a common site on TFR for sorting into exosomes. *Traffic* **5**:181–193.
- Guinamard R, Signoret N, Masamichi I, Marsh M, Kurosaki T, and Ravetch JV (1999) B cell antigen receptor engagement inhibits stromal cell-derived factor (SDF)-1 α chemotaxis and promotes protein kinase C (PKC)-induced internalization of CXCR4. *J Exp Med* **189**:1461–1466.
- Hannan LA, Newmyer SL, and Schmid SL (1998) ATP- and cytosol-dependent release of adaptor proteins from clathrin-coated vesicles: a dual role for Hsc73. *Mol Biol Cell* **9**:2217–2229.
- Haribabu B, Richardson RM, Fisher I, Sozzani S, Peiper SC, Horuk R, Ali H, and Snyderman R (1997) Regulation of human chemokine receptors CXCR4. Role of phosphorylation in desensitization and internalization. *J Biol Chem* **273**:28726–28731.
- Helbig G, Christopherson KW 2nd, Bhat-Nakshatri P, Kumar S, Kishimoto H, Miller KD, Broxmeyer HE, and Nakshatri H (2003) NF- κ B promotes breast cancer cell migration and metastasis by inducing the expression of the chemokine receptor CXCR4. *J Biol Chem* **278**:21631–21638.
- Honing S, Kreimer G, Robenek H, and Jockusch BM (1994) Receptor-mediated endocytosis is sensitive to antibodies against the uncoating ATPase (Hsc73). *J Cell Sci* **107**:1185–1196.
- Ji LL, Wang Z, Dong F, Zhang WB, and Wang ZT (2005) Andrograpanin, a compound isolated from anti-inflammatory traditional Chinese medicine *Andrographis paniculata*, enhances chemokine SDF-1 α -induced leukocytes chemotaxis. *J Cell Biochem* **95**:970–978.
- Knowles RB, Chin J, Ruff CT, and Hyman BT (1999) Demonstration by fluorescence resonance energy transfer of a close association between activated MAP kinase and neurofibrillary tangles: implications for MAP kinase activation in Alzheimer disease. *J Neuropathol Exp Neurol* **58**:1090–1098.
- McLean PJ, Kawamata H, Ribich S, and Hyman BT (2000) Membrane association and protein conformation of α -synuclein in intact neurons. Effect of Parkinson's disease-linked mutations. *J Biol Chem* **275**:8812–8816.
- Murakami T, Cardones AR, and Hwang ST (2004) Chemokine receptors and melanoma metastasis. *J Dermatol Sci* **36**:71–78.
- Newmyer SL and Schmid SL (2001) Dominant-interfering Hsc73 mutants disrupt multiple stages of the clathrin-coated vesicle cycle in vivo. *J Cell Biol* **52**:607–620.
- Sarrio S, Casado V, Escriche M, Ciruela F, Mallol J, Canela EI, Lluís C, and Franco R (2000) The heat shock cognate protein hsc73 assembles with A₁ adenosine receptors to form functional modules in the cell membrane. *Mol Cell Biol* **20**:5164–5174.
- Schlossman DM, Schmid SL, Braell WA, and Rothman JE (1984) An enzyme that removes clathrin coats: purification of an uncoating ATPase. *J Cell Biol* **99**:723–733.
- Siegel RM, Chan FK, Zacharias DA, Swofford R, Holmes KL, Tsien RY, and Lenardo MJ (2000) Measurement of molecular interactions in living cells by fluorescence resonance energy transfer between variants of the green fluorescent protein. *Sci STKE* **38**:PL1.
- Strik HM, Weller M, Frank B, Hermisson M, Deininger MH, Dichgans J, and Meyermann R (2000) Heat shock protein expression in human gliomas. *Anticancer Res* **20**:4457–4462.
- Venkatesan S, Rose JJ, Lodge R, Murphy PM, and Foley JF (2003) Distinct mechanisms of agonist-induced endocytosis for human chemokine receptors CCR5 and CXCR4. *Mol Biol Cell* **14**:3305–3324.
- Yang W, Wang D, and Richmond A (1999) Role of clathrin-mediated endocytosis in CXCR2 sequestration, resensitization and signal transduction. *J Biol Chem* **274**:11328–11333.
- Zhou Y, Larsen PH, Hao C, and Yong VW (2002) CXCR4 is a major chemokine receptor on glioma cells and mediates their survival. *J Biol Chem* **277**:49481–49487.

Address correspondence to: Dr. Guo-Huang Fan, Department of Biomedical Sciences, Meharry Medical College, Nashville, TN 37208. E-mail: gfan@mmc.edu
