CXCL12 Induces Tyrosine Phosphorylation of Cortactin, Which Plays a Role in CXC Chemokine Receptor 4-mediated Extracellular Signal-regulated Kinase Activation and Chemotaxis*

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The CXC chemokine receptor 4 (CXCR4) plays a role in the development of immune and central nervous systems as well as in cancer growth and metastasis. CXCR4-initiated signaling cascades leading to cell proliferation and chemotaxis are critical for these functions. The present study demonstrated that stimulation of CXCR4 by its ligand, CXCL12, induced transient translocation of cortactin from endosomal compartments to the cell periphery where it colocalized with CXCR4 followed by internalization of CXCR4 together with cortactin into endosomes. Cortactin was co-immunoprecipitated with CXCR4 in response to CXCL12 treatment in a time-dependent manner. Ligand stimulation induced phosphorylation of cortactin at tyrosine 421, and the phosphorylation was both c-src- and dynamin-dependent. Cortactin overexpression promoted CXCR4 internalization and recycling. However, overexpression of a cortactin mutant in which tyrosine 421 was replaced with alanine (cortactin-Y421A) or knockdown of cortactin with RNA interference (RNAi) reduced CXCR4 internalization in response to CXCL12. CXCR4-mediated activation of extracellular signal-regulated kinases 1 and 2 was significantly prolonged by overexpression of wild-type cortactin but not by the cortactin-Y421A mutant and was inhibited by cortactin knockdown with RNAi. Moreover, CXCL12-induced chemotaxis was enhanced by cortactin overexpression, reduced by overexpression of the cortactin-Y421A mutant, and blocked by cortactin knockdown with RNAi. These data provide strong evidence for an important role of cortactin in CXCR4 signaling and trafficking as well as in the receptor-mediated cell migration.

* This work was supported by a merit grant from the Department of Veterans Affairs (to G.-H. F.), by Research Centers in Minority Institutions, National Institutes of Health Grant RR03032-19, and by a grant from Science and Technology Commission of Shanghai Municipality (project 04DZ14902). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: CXCR4, CXC chemokine receptor 4; HEK293 cells, human embryonic kidney 293 cells; ERK1 and -2, extracellular signal-regulated kinases 1 and 2; siRNA, short interference RNA; SH3, Src homolog three; EGFP, enhanced green fluorescence protein; HA, hemagglutin.
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tactin directly associates with dynamin, a large GTPase that is involved in the liberation of clathrin-coated pits from the plasma membrane via its Src homolog 3 (SH3) domain and proline-rich domain, respectively (22). Electron microscopy revealed that cortactin associated with endosomes along with the Arp2/3 complex (26). More recently it has been shown that cortactin is distributed over the surface or base of clathrin lattices as well as actin filaments associated with the clathrin-coated pits (27). The inhibitory effect of microjection of anticitin antibody or transfection of a plasmid encoding the cortactin SH3 domain on endocytosis further supports the role of cortactin in receptor-mediated endocytosis (27). Cortactin, originally identified as a substrate for Src kinase (28), is directly phosphorylated by Src on three tyrosine residues, tyrosine 421, tyrosine 466, and tyrosine 482 (29,30). However, little is known about the role of each of these phosphorylations in cortactin functions.

In this study we demonstrated that CXCL12 activation of CXCR4 leads to cortactin translocation from endosomes to the cell periphery. Ligand stimulation also induced tyrosine phosphorylation of cortactin in a Src- and dynamin-dependent manner. Overexpression of wild-type cortactin, but not the phosphorylation-deficient cortactin mutant, resulted in enhanced CXCR4 recycling, prolonged ERK1/2 activation, and enhanced CXCR4-mediated chemotaxis. In contrast, RNA interference of cortactin significantly inhibited CXCR4 internalization and reduced CXCR4-mediated ERK1/2 activation and chemotaxis. These data indicate that cortactin plays important roles in CXCR4 signaling and trafficking as well in the receptor-mediated chemotaxis.

EXPERIMENTAL PROCEDURES

Plasmids and Short Interference RNAs—The plasmids encoding HA-CXCR4 and Myc-CXCR4 were obtained from Dr. Gang Pei (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences). The wild-type and dominant negative mutant forms of c-Src and dynamin constructs were kindly provided by Dr. Mark Caron (Duke University). For the construction of cortactin in pcDNA3.1, the full-length cDNAs were amplified by using a reverse transcriptase-PCR. Oligonucleotide primers specific for cortactin were designed according to cortactin cDNA sequence from GenBank [accession number AF054619]. The PCR fragments were inserted into the BamHI and XhoI sites of the eukaryotic expression vector pcDNA3.1 (Invitrogen). Mutagenesis of specific cortactin tyrosine 421 to alanine (Y421A) was conducted by using of the Gene-Editor site-directed mutagenesis system (Promega). The full-length cortactin cDNAs in pcDNA3.1 were used as the template for the mutagenesis. The cortactin-specific short interference RNA (siRNA) (sc-35093) that specifically knocks down cortactin gene expression and control siRNA (sc-37007) that consists of a scrambled sequence that will not lead to the specific degradation of any cellular message were purchased from Santa Cruz Biotechnology, Inc. CXCR2 construct in pRC/CMV vector was constructed as described previously (17).

Cell Culture and Transfection—Human embryonic kidney (HEK) 293 cells or HeLa cells were grown in Dulbecco’s modified essential medium containing 10% fetal bovine serum, 100 units of penicillin/ml, and 100 μg of streptomycin/ml in 5% CO2, 95% air at 37 °C. Cells were cultured in P-100 dishes or on 22 × 22-mm glass coverslips for transfections and immuno-fluorescence microscopy, respectively. Transfection was performed with Lipofectamine Plus reagent (Invitrogen). Cells stably expressing CXCR4 or CXCR2 were selected with 560 μg/ml Geneticin and evaluated for receptor expression by radioligand binding assay using 125I-labeled CXCL12 or 125I-labeled CXCL8 (Amersham Biosciences).

Confocal Microscopy—Cells grown on coverslips for 1 or 2 days were prepared as described previously (31). HEK293 cells stably expressing Myc-CXCR4 were treated with carrier buffer or CXCL12 for various time intervals and fixed with methanol. Cells were washed with phosphate-buffered saline and incubated with an antibody mixture containing a mouse monoclonal anti-Myc antibody (Santa Cruz Biotechnology) and a rabbit polyclonal cortactin antibody (Santa Cruz Biotechnology) for 30 min. Cells were washed and incubated with an antibody mixture containing a CY3-conjugated anti-mouse antibody (Molecular Probes) and a fluorescein isothiocyanate-conjugated anti-rabbit antibody (Molecular Probes) for 30 min. HeLa cells transfected with enhanced green fluorescence protein (EGFP)-Rab5 were treated with CXCL12 for 5 or 30 min before being fixed in methanol. Cells were incubated with a rabbit polyclonal cortactin antibody for 30 min followed by a CY3-conjugated anti-rabbit antibody for 30 min. Confocal microscopy was performed on an LSM-510 laser scanning microscope (Carl Zeiss, Thornwood, NY) with a 63 × 1.3 numerical aperture oil immersion lens using dual excitation (488 nm for fluorescein isothiocyanate (FITC) or EGFP, 568 nm for Cy3) and emission (515–540 nm for FITC or EGFP, 590–610 nm for Cy3) filter sets. All digital images were captured at the same settings to allow direct quantitative comparison of staining patterns. Final images were processed using Adobe Photoshop software. Confocal colocalization between two fluorescent staining was quantified using Optimas 5.2 image analysis software. Briefly, matching green and red images of individual cells were opened sequentially. Fluorescence thresholds were applied to each image to exclude diffuse labeling in the cytoplasm. Punctate sites of positive staining were identified, and each image was converted to binary form. Matching binary images derived from CXCR4, and cortactin images were then subjected to an “and” operation, resulting in the identification of sites containing both CXCR4 and cortactin staining. The following equation was used to determine percentage colocalization per cell: % colocalization = (no. of punctate sites containing both CXCR4 and cortactin) × 100/(no. punctate of sites containing CXCR4). Means ± S.E. were determined and plotted using SigmaPlot software.

Co-immunoprecipitation and Western Blot—For the co-immunoprecipitation of CXCR4 and cortactin, HEK293 cells stably expressing HA-CXCR4 were treated with CXCL12 (10 nm) for various time intervals, washed 3 times with ice-cold phosphate-buffered saline, and lysed in 1 ml of radioimmune precipitation assay buffer containing phosphate-buffered saline (pH 7.0), 0.1% sodium deoxycholate, 0.01% SDS, and 1% Nonidet P-40. The cell debris was removed by centrifugation (15,000 × g, 15 min). The supernatant was preclarified by incu-
bation with 40 μl of protein A/G-agarose (Pierce) for 1 h at 4 °C to reduce nonspecific binding. After removing the protein A/G-agarose by centrifugation (15,000 × g, 1 min), the cleared supernatant was collected, and 10 μl of mouse monoclonal anti-HA antibody (Santa Cruz Biotechnology) was added for overnight precipitation at 4 °C. Protein A/G (40 μl) was then added, and incubation was continued at 4 °C for 2 h. The protein A/G-antibody-antigen complex was collected by washing three times with ice-cold immunoprecipitation buffer. The final pellet was resuspended in 40 μl of SDS sample buffer containing 5% β-mercaptoethanol and heated to 50 °C for 10 min. Forty microliters of this preparation was separated by 10% SDS-PAGE, and the proteins on the gel were transferred to nitrocellulose membranes (Bio-Rad). The co-immunoprecipitated cortactin was detected by Western blotting using a rabbit anti-cortactin antibody (Santa Cruz Biotechnology).

**Cortactin Phosphorylation Assay**—HEK293 cells stably expressing HA-CXCR4 were grown in 6-well plates. Cells were treated with CXCL12 for different time intervals before being lysed in radioimmune precipitation assay buffer. Lysates containing equal amounts of protein were subjected to SDS-PAGE. Phosphorylated cortactin was detected by Western blot analysis with phosphospecific cortactin antibodies against tyrosine 421, tyrosine 466, and tyrosine 482 (Santa Cruz Biotechnology).

**Mitogen-activated Protein Kinase Assay**—HEK293 cells stably expressing HA-CXCR4 and transiently transfected with vector or cortactin plasmids were grown in 6-well plates. Agonist-treated cells were lysed by radioimmune precipitation assay buffer. Lysates containing equal amounts of protein were subjected to SDS-PAGE. Phosphorylated ERK1/2 was detected by a phosphospecific ERK1/2 antibody (Santa Cruz Biotechnology).

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

**Chemotaxis Assay**—A 96-well chemotaxis chamber (Neuroprobe, Gaithersburg, MD) was used for chemotaxis assays, and the lower compartment of the chamber was loaded with 400-μl aliquots of 1 mg/ml ovalbumin/Dulbecco’s modified essential medium (chemotaxis buffer) or CXCL12 diluted in the chemotaxis buffer (0.001–10 nM). 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, and then stored at 4 °C overnight. To prepare HEK293 cells for chemotaxis assay, cells were removed from the culture dish by trypsinization, washed with Hanks’ solution, and incubated in 10% fetal bovine serum/Dulbecco’s modified essential medium for 2 h at 37 °C to allow time for restoration of receptors. Cells (5 × 10^5 in 100 μl) were loaded into the top of each well of a 96-well chemotaxis chamber. The bottom of each well contained 600 μl of prewarmed chemotaxis buffer with different concentrations of ligand. The plate was then incubated for 240 min at 37 °C in a 5% CO2 atmosphere, and cells migrating to the lower chamber were counted after being stained with a Diff-Quik kit. Cell chemotaxis was quantified by counting the number of migrating cells present in 10 microscope fields (20× objective).

**Internalization and Recycling Assays**—HEK293 cells stably expressing HA-CXCR4 and transiently transfected with vector or cortactin plasmids were grown in 24-well plates that were precoated with 0.1 mg/ml poly-l-lysine (Sigma, M, 30,000–70,000). For the internalization assay cells were incubated at 4 °C in 0.5 ml of serum-free Dulbecco’s modified essential medium containing 125I-labeled CXCL12 (75 nCi/ml) at 4 °C for 1 h with or without excess unlabeled CXCL12 for the determination of nonspecific binding. After removing unbound ligand, the cells were shifted to 37 °C. At each time point, the medium was removed, and the cell surface 125I-labeled CXCL12 was removed by incubating with 1 ml of ice-cold 0.2 M acetic acid and 0.5 M NaCl for 6 min. Internalized CXCL12 (acid-resistant, cell-associated cpm) was determined with a γ-counter. For the recycling assay cells were incubated with unlabeled CXCL12 (10 nM) for 1 h at 4 °C. After removing unbound ligand, cells were shifted to 37 °C. At each time point, residual bound CXCL12 was removed by acid wash, and surface receptor levels were assessed by binding of 125I-labeled CXCL12.

**Fluorescence-activated Cell Sorting Analysis**—HEK293 cells stably expressing CXCR4 were transfected with vector (control), cortactin plasmids, control siRNA, or cortactin specific siRNA. Cells were incubated with a monoclonal phycoerythrin-conjugated CXCR4 antibody (BD PharMingen, San Diego, CA) at 4 °C for 60 min. In a parallel experiment, parental HEK293 cells were incubated with the phycoerythrin-conjugated CXCR4 antibody as a control. Cells were washed and fixed in 2% formaldehyde in phosphate-buffered saline and analyzed in a FACScan equipped with CellQuest software (BD Biosciences).

**Intracellular Calcium Mobilization Assay**—HEK293 cells stably expressing CXCR4 were transfected with vector (control), cortactin plasmids, control siRNA, or cortactin specific siRNA. Cells were released by shaking, collected by centrifugation at 300 × g for 5 min, and washed with incubation buffer (Hanks’ buffer containing 5 mM HEPES). Cells were resuspended at 2 × 10^6 cells/ml and incubated with 2.5 μM Fluo-3 (Molecular Probes) for 30 min at 37 °C. After incubation the cells were washed once with the incubation buffer containing 2 mM CaCl2. The cells were finally adjusted to 2 × 10^6 cells/ml. Cells were stimulated with CXCL12 (10 nM), and intracellular Ca2+ mobilization experiments were performed as described previously (17). The time taken to recover 80% of the mobilized Ca2+ (t0.80) was used as a measure of Ca2+ removal.

**RESULTS**

Previous studies have shown that cortactin is located in endosomal compartments (26) and that it can be translocated from the cytoplasm to the periphery after growth factor signaling, integrin activation, and bacteria entry (32–34). To determine whether stimulation of CXCR4 induces cortactin translocation, HeLa cells, which endogenously express CXCR4 (35), were transfected with EGFP-Rab5, an early endosomal marker (36). Cells were treated with CXCL12 (10 nM) for different time intervals, and confocal microscopy was performed to visualize...
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FIGURE 1. CXCL12 induced cortactin translocation from endosomes to cell periphery. HeLa cells transiently expressing EGFP-Rab5 were stimulated with CXCL12 (10 nM) for different time intervals (0, 5, 30 min) before being fixed. Cells were immunostained for cortactin. The subcellular localization of cortactin (red) and EGFP-Rab5 (green) was observed under a confocal microscope. Colocalization of cortactin with EGFP-Rab5 was seen in the cytoplasmic punctate structures before and after CXCL12 treatment, and cortactin cell surface translocation was seen at 5 min of CXCL12 treatment. Shown are representative images from three independent experiments with similar results. Bars, 10 μm.

The subcellular localization of cortactin and EGFP-Rab5. Before ligand stimulation, cortactin was localized predominantly in cytoplasmic punctate structures and co-localized in part with the EGFP-Rab5, suggesting its early endosomal localization, which also occurs after growth factor signaling (26). Treatment of cells for 5 min with CXCL12, however, induced a quick translocation of cortactin to the cell surface. The plasma membrane translocation of cortactin appeared to be transient, since after 30 min of ligand treatment most cortactin proteins returned to the Rab5-positive endosomes (Fig. 1).

Cortactin is known to associate with clathrin-coated pits (27) through which chemokine receptors undergo internalization. The translocation of cortactin to the surface membrane encouraged us to determine whether cortactin colocalizes with CXCR4 in response to CXCL12 treatment. To visualize CXCR4 we used HEK293 cells stably expressing Myc-CXCR4. Cells were treated with CXCL12 for different time intervals, and subcellular localization of cortactin and CXCR4 was observed by confocal microscopy. As shown in Fig. 2, CXCR4 receptors were exclusively localized on the cell surface, whereas cortactin was predominantly in the cytoplasm before CXCL12 stimulation. Ligand stimulation for 5 min, however, resulted in translocation of cortactin to the cell surface, where it appeared to co-localize with CXCR4. After ligand treatment for 30 min, a large proportion of CXCR4 receptors were internalized, and the internalized receptors co-localized with cortactin in the cytoplasm. Because our findings demonstrate that cortactin localizes in Rab5-positive endosomes, as shown previously in studies of growth factor signaling (37), it is conceivable that the internalized CXCR4 colocalized with cortactin in early endosomes.

The ligand-dependent colocalization of CXCR4 with cortactin suggests that these two proteins may interact with each other. To assess this probable interaction, we treated HEK293 cells stably expressing CXCR4 with CXCL12 (10 nM) for different time intervals, immunoprecipitated CXCR4 from the cell lysate, and assessed the presence of cortactin by Western blot. We observed a basal association of CXCR4 with cortactin before ligand treatment that increased significantly in a time-dependent fashion, peaking at 5 min and lasting for ~60 min (Fig. 3). By comparing the density of the immunoblots for cortactin coprecipitated with CXCR4 from 1 ml of cell lysate and the immunoblots of cortactin in 40 μl of cell lysate, we estimated that maximally 10% cortactin proteins were co-immunoprecipitated with CXCR4. Because glutathione S-transferase-CXCR4 did not pull down cortactin in the in vitro binding assay (data not shown), we propose that cortactin may interact with CXCR4 indirectly via association with other proteins in a CXCL12-modulated CXCR4 signaling complex.

Based on the previous findings that CXCR4 signaling involves Src (38), which directly phosphorylates cortactin at tyrosine residues (tyrosine 421, tyrosine 466, and tyrosine 482) (29), we determined whether stimulation of CXCR4 with specific ligand affects cortactin tyrosine phosphorylation. HEK293 cells stably expressing HA-CXCR4 were treated with CXCL12 for different time intervals. Cortactin tyrosine phosphorylation was determined by Western blot analysis using phospho-specific cortactin antibodies against the tyrosine 421, tyrosine 466, and tyrosine 482. As shown in Fig. 4, A and B, stimulation of the CXCR4-expressing cells resulted in cortactin phosphorylation at the tyrosine 421 but not at the tyrosine 466 or tyrosine 482. Overexpression of wild-type c-Src resulted in slight enhancement of CXCR4-mediated cortactin phosphorylation at the tyrosine 421 but not at the tyrosine 466 or tyrosine 482. Overexpression of c-Src (K295R/Y527F) significantly reduced CXCL12-induced cortactin tyrosine phosphorylation (Fig. 4, C and D). These findings suggest that c-Src is involved in CXCR4-mediated cortactin phosphorylation.

The GTPase dynamin is a cortactin-binding protein that may play a role in recruitment of cortactin to clathrin-coated pits (27). Consequently, we determined whether CXCR4-mediated
Contactin tyrosine phosphorylation requires dynamin. As shown in Fig. 4, E and F, overexpression of the wild-type dynamin II did not affect CXCR4-mediated contactin tyrosine phosphorylation, whereas overexpression of the dominant negative mutant dynamin II (K44A) inhibited tyrosine phosphorylation. These data suggest that normal function of dynamin is required for CXCR4-mediated contactin tyrosine phosphorylation.

CXCR4-mediated activation of mitogen-activated protein kinase kinase and ERK1/2 signaling cascades plays an important role in cell proliferation and survival (39). Some signaling regulators such as Src have been implicated in control of ERK1/2 activation (40). These findings encouraged us to determine whether contactin is involved in CXCR4-mediated ERK1/2 activation. HeLa cells transiently transfected with vector alone (control) or contactin-expressing vector were treated with CXCL12 for different time intervals, and phosphorylation of ERK1/2 was detected by Western blot analysis. As shown in Fig. 5, A and B, ligand stimulation of control cells induced ERK1/2 activation in a time-dependent manner, which peaked at 5–10 min and went down to basal level after 30 min of incubation. In contrast, in cells overexpressing contactin, phosphorylation of ERK1/2 was sustained throughout the experimental period (60 min). These data indicate that overexpression of contactin prolongs CXCR4-mediated ERK1/2 activation. To determine the role of contactin phosphorylation in CXCR4-mediated ERK1/2 activation, we generated a contactin mutant in which tyrosine 421 was replaced by alanine (contactin-Y421A) and transfected this mutant into HeLa cells before ERK1/2 phosphorylation was assessed. In the contactin-Y421A mutant-expressing cells, CXCL12 treatment induced a similar time course of ERK1/2 phosphorylation as that in the control cells, but the peak phosphorylation was lower than that in the control cells (Fig. 5, A and B). Interestingly, in contrast to that observed in the wild-type contactin expressing cells, we did not observe a sustained ERK1/2 phosphorylation in the contactin-Y421A overexpressing cells, suggesting an important role of contactin tyrosine phosphorylation in CXCR4-mediated ERK1/2 activation.

Because contactin overexpression prolonged CXCR4-mediated ERK1/2 activation, we proposed that knock-down of contactin with RNA interference may exert an opposite effect. To test this hypothesis, HeLa cells transiently transfected with control siRNA or contactin-specific siRNA were treated with CXCL12 for different time intervals, and phosphorylation of ERK1/2 was assessed. As shown in Fig. 5, C and D, in cells transfected with the contactin-specific siRNA, ligand stimulation resulted in a modest ERK1/2 phosphorylation. However, cells transfected with the control siRNA exhibited similar time-dependent ERK1/2 activation as the cells transfected with the empty vector (control) as described above. Western blot of contactin showed that the expression level of contactin was robustly reduced in cells transfected with the specific siRNA compared with cells transfected with the control siRNA. These data suggest that normal contactin expression is critical for CXCR4 signaling.

To determine whether other chemokine receptor-mediated ERK1/2 activation is affected by contactin overexpression or...
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A

**FIGURE 3. Cortactin co-immunoprecipitated with CXCR4.** A, 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 10 min, and immunoprecipitation (IP) was performed as described above (mock). Proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane, and coprecipitated cortactin proteins were detected by Western blotting (IB). The membrane was stripped and reblotted with a specific anti-HA antibody to confirm equal loading. B, quantification of the density of bands representing cortactin time-dependently associated with CXCR4 was determined by densitometric scanning. Data are the means ± S.E. from three independent experiments.

knockdown, we transfected HEK293 cells stably expressing CXCR2, another chemokine receptor, with empty vector (control), cortactin expressing vector, control siRNA, or cortactin specific siRNA. After incubating the cells with or without CXCL8 (10 nM) for 10 min, ERK1/2 phosphorylation was detected by Western blot analysis as described above. As shown in Fig. 5, E and F, CXCL8 treatment induced a marked phosphorylation of ERK1/2 in the control cells and in the cortactin-overexpressing cells but not in the cells with cortactin knockdown. These data suggest that other chemokine receptor-mediated ERK1/2 activation is similarly regulated by cortactin.

One possible mechanism for the effect of cortactin on chemokine receptor-mediated ERK1/2 activation is that cortactin overexpression or knockdown alters the cell surface expression of chemokine receptors. To test this hypothesis we transfected HEK293 cells stably expressing CXCR4 with empty vector (control), cortactin expressing vector, control siRNA, or cortactin-specific siRNA. After the transfection we determined cell surface CXCR4 expression by fluorescence-activated cell sorting analysis using a phycoerythrin-conjugated CXCR4 antibody. As shown in Fig. 6 the cells transfected with cortactin construct or siRNA exhibited similar CXCR4 expression levels, suggesting that cortactin overexpression or knockdown does not affect CXCR4 expression on the cell surface.

Using a radioligand binding assay that allows assessment of cell surface bound versus internalized ligand, we observed that overexpression of cortactin did not affect the maximal CXCR4 internalization (~75%) but significantly increased the rate of the receptor internalization within 10 min (Fig. 8A). Interestingly and in contrast, overexpression of the cortactin-Y421A mutant significantly reduced ligand-induced CXCR4 internalization (Fig. 8A). These data suggest that cortactin is important for ligand-evoked CXCR4 internalization, and Src phosphorylation of cortactin appears to play a role in CXCR4 internalization.

Based on the early/sorting endosomal localization of cortactin (26), we proposed that cortactin may also affect CXCR4 recycling subsequent to internalization. To test this hypothesis, HEK293 cells stably expressing HA-CXCR4 were transiently transfected with empty vector (control) or cortactin-expressing vector. CXCR4 recycling was assessed. As shown in Fig. 8B, after an initial phase of CXCR4 internalization and recovery for 60 min, the reappearance of CXCR4 at the cell surface was observed in both the control and the cortactin-overexpressing cells, presumably due to the recycling of endocytosed receptors. However, more receptors appeared to be re-expressed on the cell surface in the cortactin-overexpressing cells compared...
with the control cells. These data suggest that cortactin overexpression promotes CXCR4 recycling.

Although cortactin is known to be involved in cell motility (41), the effect of cortactin on chemokine receptor-mediated chemotaxis has not been addressed. Therefore, we sought to determine whether overexpression of cortactin affects CXCR4-mediated chemotaxis. HEK293 cells stably expressing CXCR4 and transiently transfected with empty vector or cortactin-expressing vector were examined for CXCL12-induced chemotaxis using a Boyden chamber assay. As shown in Fig. 9, A and C, CXCL12 stimulation resulted in a typical bell-shape dose-dependent chemotactic response in the control cells, and maximal chemotactic response (5-fold over basal) was induced by 1 nM CXCL12. The chemotactic response went down when the concentration of CXCL12 was increased to 10 nM and returned to baseline when CXCL12 concentration was 100 nM. In contrast, cells overexpressing cortactin exhibited significantly enhanced bell-shape chemotactic response to CXCL12 stimulation, with a maximal chemotactic index of ~9.5-fold above basal. Interestingly, compared with the control cells, which exhibited no chemotactic response in response to high concentrations of CXCL12 (10 nM), the cortactin-overexpressing cells still exhibited chemotactic response (3-fold above control) in response to 10 nM CXCL12. These data suggest that overex-

FIGURE 4. CXCL12 induced cortactin tyrosine phosphorylation. A, HEK293 cells stably expressing HA-CXCR4 were treated with CXCL12 for different time intervals. Cortactin tyrosine phosphorylation was determined by Western blot analysis using phospho-specific cortactin antibodies against tyrosine 421, tyrosine 466, and tyrosine 482, respectively. B, quantification of the density of bands representing cortactin phosphorylation, which was normalized with the density of the total cortactin bands, was performed based on three independent experiments; the mean ± S.E. is shown. C, HEK293 cells stably expressing HA-CXCR4 were transiently transfected with vector (control), c-Src construct, or dominant negative mutant c-Src construct (K295R/Y527F). Cells were treated with CXCL12 for different time intervals, and cortactin tyrosine phosphorylation (P-cortactin) was determined by Western blot analysis using phospho-specific (anti-Tyr-421) cortactin antibody. D, quantification of the density of bands representing cortactin phosphorylation, which was normalized with the density of the total cortactin bands. Data are the mean ± S.E. of three independent experiments. E, HEK293 cells stably expressing HA-CXCR4 were transiently transfected with vector (control), wild-type dynamin II construct, or a dominant negative mutant dynamin II construct (K44A). Cells were treated with CXCL12 for 10 min, and cortactin tyrosine phosphorylation was determined by Western blot analysis using phospho-specific (anti-Tyr-421) cortactin antibody. F, quantification of the density of bands representing cortactin phosphorylation, which was normalized with the density of the total cortactin bands. Data are the mean ± S.E. of three independent experiments. *, p < 0.05 compared with control cells with the same treatment.
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A

|       | Vector | Cortactin | Cortactin (Y421A) |
|-------|--------|-----------|-------------------|
| 0     |        |           |                   |
| 2     |        |           |                   |
| 5     |        |           |                   |
| 10    |        |           |                   |
| 30    |        |           |                   |
| 60    |        |           |                   |

B

Relative image density of phosphorylated ERK1/2

Time (min)

C

|       | Control siRNA | Cortactin siRNA |
|-------|--------------|-----------------|
| 0     |               |                 |
| 2     |               |                 |
| 5     |               |                 |
| 10    |               |                 |
| 30    |               |                 |
| 60    |               |                 |

D

Relative image density of phosphorylated ERK1/2

Time (min)

E

|       | Vector | Cortactin | Control siRNA | Cortactin siRNA |
|-------|--------|-----------|---------------|-----------------|
| -     |        |           |               |                 |
| +     |        |           |               |                 |
| -     |        |           |               |                 |
| +     |        |           |               |                 |

F

Relative image density of phosphorylated ERK1/2

- + + + + +
pression of cortactin can elicit enhanced and prolonged chemotactic response to CXCL12 stimulation. We also determined the effect of overexpression of the cortactin tyrosine phosphorylation-deficient mutant (cortactin-Y421A) on CXCR4-mediated chemotaxis in HEK293 cells stably expressing CXCR4. Overexpression of the cortactin mutant resulted in a reduced chemotactic response compared with that of the control cells, and the chemotactic response was not prolonged as the wild-type cortactin-overexpressing cells. These data suggest that cortactin phosphorylation is important for chemotaxis.

Based on the above finding that the level of cortactin parallels the extent of CXCR4-mediated chemotaxis, we proposed that knockdown of cortactin may block chemotaxis. To test this hypothesis HEK293 cells stably expressing CXCR4 were transiently transfected with control siRNA or cortactin-specific siRNA, and chemotaxis was assessed. As shown in Fig. 9, B and C, the control siRNA transfected cells exhibited a similar chemotactic response to CXCL12 stimulation as nontransfected control cells. However, the cortactin-specific siRNA-transfected cells, which manifest significant reduction in cortactin expression, exhibited modest chemotactic response.

**DISCUSSION**

The present study demonstrates that CXCL12 stimulation of CXCR4 induces cortactin translocation from early endosomes to the cell surface in parallel with the association of cortactin in a complex with CXCR4. Phosphorylation of the tyrosine 421 residue of cortactin in both Src- and dynamin-dependent manner appears to be functionally relevant for the cortactin-regulated events since overexpression of a cortactin-Y421A mutant significantly blocked cortactin-dependent CXCR4 recycling and prolongation of CXCR4-mediated ERK1/2 activation. CXCR4-mediated chemotaxis was also inhibited by cortactin Y421A mutant. CXCR4 internalization, receptor-mediated ERK/12 activation, and chemotaxis were inhibited by knockdown of cortactin with RNA interference.

These studies provide the first evidence for the translocation of cortactin from endosomal compartments to cell surface in response to chemokine stimulation. Previous studies have shown that cortactin undergoes membrane translocation in response to other stimuli, such as growth factor signaling, integrin activation, and bacteria entry (32–34). Our data also demonstrate that the translocated cortactin forms a complex with CXCR4. However, our data suggest that cortactin does not bind directly to CXCR4 since we did not obtain evidence for the direct interaction in our in vitro binding (glutathione S-transferase pull down) studies (data not shown). These findings suggest that cortactin associates with and likely modulates a CXCR4-containing complex. There is such a scenario for the somatostatin receptor subtype 2 (SST2) where the cortactin C-terminal SH3 domain binds several intracellular proteins, one of which is cortactin-binding protein 1 (42).

Cortactin translocation to the cell surface is likely required

**FIGURE 5.** Cortactin was involved in CXCR4- or CXCR2-mediated ERK1/2 activation. A, HeLa cells were transiently transfected with vector (control), a wild-type cortactin construct, or a cortactin mutant (cortactin-Y421A) construct. Cells were treated with CXCL12 (10 nM) for different time intervals, and ERK1/2 phosphorylation (P-ERK1/2) was determined by Western blot analysis using phospho-specific antibody. The membrane was stripped and reblotted with cortactin to confirm the overexpression of the wild-type or mutant cortactin. B, quantification of the density of bands representing ERK1/2 phosphorylation, which was normalized with the density of the total ERK2 bands. Data are the mean ± S.E. of three independent experiments. C, HeLa cells were transiently transfected with a control siRNA or a cortactin-specific siRNA. Cells were treated with CXCL12 (10 nM) for different time intervals, and ERK1/2 phosphorylation was determined by Western blot analysis using phospho-specific antibody. The membrane was stripped and reblotted with cortactin to confirm the knockdown of cortactin. D, quantification of the density of bands representing ERK1/2 phosphorylation, which was normalized with the density of the total ERK2 bands. Data are the mean ± S.E. of three independent experiments. E, HEK293 cells stably expressing CXCR2 were transiently transfected with vector (control), cortactin construct, control siRNA, or cortactin specific siRNA. Cells were treated with or without CXCL8 (10 nM) for 10 min, and ERK1/2 phosphorylation was determined as described above. F, quantification of the density of bands representing ERK1/2 phosphorylation was performed as described above. Data are the mean ± S.E. of three independent experiments. *, p < 0.05 compared with control cells with the same treatment.
for its Src-dependent tyrosine phosphorylation because Src is a membrane-associated tyrosine kinase and because cortactin phosphorylation occurred within 5 min, which is in the same time frame as the cortactin cell surface translocation. Previous studies have shown that Src directly phosphorylates cortactin in vitro (29) on three tyrosine residues (tyrosine 421, tyrosine 466, and tyrosine 482) located within the proline-rich domain (30). However, CXCR4-mediated cortactin phosphorylation appeared to only occur at the tyrosine 421 residue. The underlying mechanism remains unknown, but the presence of a consensus Src SH2 binding sequence next to tyrosine 421 raises the possibility that this residue may be initially phosphorylated by Src, thereby allowing for the stabilization of Src with cortactin through an interaction between the Src SH2 domain and phosphorylated tyrosine 421. Stabilization of Src at tyrosine 421 may act to further facilitate the phosphorylation of cortactin at tyrosines 466 and 482 (43). Therefore, we cannot exclude the possibility that other tyrosine residues (tyrosine 466 and tyrosine 482) are phosphorylated after the phosphorylation of tyrosine 421 over prolonged CXCL12 treatment. This is highly possible because we observed that once cortactin was phosphorylated by CXCL12 stimulation within 5 min, the phosphorylation lasted over the entire experimental period (60 min), with no sign of decrease. Previous studies have shown that stimulation by the growth factors fibroblast growth factor-1 or epidermal growth factor leads to a rapid induction of cortactin tyrosine phosphorylation (within 5 min) that decreased within 1 h but increased again within 4–7 h and was sustained for 12–24 h (44), presumably due to the dynamic association of cortactin with c-Src (45). In addition to the involvement of Src, dynamin appears to play a role in CXCL12-induced cortactin phosphorylation, since a dominant negative mutant of dynamin (K44A) blocked cortactin phosphorylation. The underlying mechanisms remain unknown. We propose that dynamin may be required for cortactin membrane translocation to interact with c-Src, or it may be critical for the kinase activity of c-Src. It would be worthwhile to investigate if other chemokine receptors also...

FIGURE 7. Effect of cortactin on CXCR4-mediated intracellular Ca\(^{2+}\) mobilization. HEK293 cells stably expressing CXCR4 were transiently transfected with empty vector (control), cortactin construct, control siRNA, or cortactin-specific siRNA. Cells were loaded with Fluo-3 (2.5 \(\mu\)M) for 30 min. CXCL12 (10 nM)-induced intracellular Ca\(^{2+}\) mobilization was measured. Shown are representatives of three independent experiments. The arrows indicate the addition of CXCL12. Shown are representative of three independent experiments with similar results.

FIGURE 8. Cortactin was involved in CXCR4 internalization and recycling. A, HEK293 cells stably expressing CXCR4 were transiently transfected with a vector alone (control), a cortactin expression vector, a cortactin-Y421A mutant expression vector, a control siRNA, or a cortactin-specific siRNA. After transfection, cells were subjected to internalization assays. B, HEK293 cells stably expressing CXCR4 were transiently transfected with a vector alone (control) or a cortactin expression vector. After transfection, cells were subjected to internalization assays. *p < 0.05; **p < 0.01 compared with control.
mediate cortactin tyrosine phosphorylation, but it is likely true that not all G protein-coupled receptors mediate cortactin tyrosine phosphorylation, as demonstrated by the previous study that stimulation of β2-adrenergic receptor induced Src activation but not cortactin phosphorylation (46).

Cortactin phosphorylation at tyrosine 421 likely plays a role in CXCR4 internalization based on the observation that overexpression of the cortactin-Y421A mutant blocked CXCR4 internalization. It is known that chemokine receptors and many other G protein-coupled receptors undergo internalization through clathrin-coated pits, which invaginate and pinch off of the plasma membrane to form clathrin-coated vesicles. The latter are fused to early endosomal compartments after the vesicles have been uncoated. The mechanism underlying the involvement of cortactin in CXCR4 internalization is not fully understood. One possibility is that as a dynamin-binding protein cortactin may play a role in the formation of clathrin-coated vesicles. Dynamins comprise a family of large GTPases that play a fundamental role in the fission and recycling of membrane vesicles during receptor-mediated endocytosis (47). The SH3 domain of cortactin can interact with two different proline motifs within the dynamin 2 proline-rich domain (22). Stimulation of the cells with growth factor induced dynamin membrane translocation, which was inhibited in cells overexpressing a cortactin mutant lacking the SH3 domain, pointing to a functional requirement for cortactin in targeting dynamin to sites of membrane ruffling (22). However, whether overexpression of the cortactin-Y421A mutant interferes with dynamin functions remains to be investigated.

We demonstrated that CXCL12-induced cortactin tyrosine phosphorylation is important for CXCR4-mediated activation of ERK1/2, which is considered to play an important role in cell proliferation and survival. This is based on the observation that overexpression of wild-type cortactin but not the cortactin-Y421A mutant prolonged CXCR4-induced ERK1/2 activation, whereas knockdown of cortactin blocked the receptor-mediated ERK1/2 activation. We showed that the cell surface CXCR4 expression and ligand-induced early signaling such as Ca²⁺ mobilization were not affected by either cortactin overexpression or cortactin knockdown, suggesting other mechanisms are involved in the effect of cortactin of chemokine receptor-initiated ERK1/2 activation. Recent studies suggest involvement of Src kinase in CXCR4-mediated ERK1/2 activation (39). Because cortactin is a direct substrate of Src kinase, we propose that CXCR4-mediated cortactin tyrosine phosphorylation may link Src with the downstream effector cascades, leading to ERK1/2 activation. In addition, studies on epidermal growth factor receptors have suggested that overexpression of cortactin induced prolonged ERK1/2 activation likely through preventing receptor degradation and promoting receptor recycling, resulting in more receptors on the cell surface to respond to ligand (48). We cannot exclude this possibility in our system since we also observed that overexpression of cortactin promoted CXCR4 recycling. Interestingly, previous studies have demonstrated that cortactin is phosphorylated by ERK1/2 (49), and ERK1/2-mediated cortactin phosphorylation plays an important role in actin polymerization (50). These together with our findings suggest that cortactin and ERK1/2 are mutually regulated, and this mutual regulation plays a critical role in fine tuning actin cytoskeleton.

FIGURE 9. Cortactin was involved in CXCR4-mediated chemotaxis. A, HEK293 cells stably expressing CXCR4 were transiently transfected with vector alone, a cortactin expression vector, and a cortactin-Y421A mutant expression vector. After transfection cells were subjected to chemotaxis assay as described under “Experimental Procedures.” Values represent the mean ± S.E. of three independent experiments. The data were analyzed using Student’s paired t test. B, HEK293 cells stably expressing CXCR4 were transiently transfected with a control siRNA or a cortactin-specific siRNA. Cells were subjected to chemotaxis assay. Values represent the mean ± S.E. of three independent experiments. The data were analyzed using Student’s paired t test. p < 0.05 (*) and p < 0.01 (**) compared with control. C, representative images showing chemotaxis of the above transfected cells in response to 1 nM of CXCL12 treatment.
The evidence for the involvement of cortactin in CXCR4-mediated chemotaxis comes from our observation that overexpression of cortactin robustly enhanced chemotaxis, whereas knockdown of cortactin blocked chemotaxis. This can be explained in different ways. The role of cortactin in chemotaxis may be due to the dual role of cortactin in endocytosis of the receptor, terminating response, and in recycling, resulting in accelerated restoration of receptors to the cell surface to respond to the CXCL12 gradients. Second, cortactin may act as a linker between clathrin-dependent endocytosis and the actin network, thereby regulating CXCR4-mediated chemotaxis. Cortactin is known to be localized in dynamic-actin assembly sites, such as lamellipodia, endosomes, podosomes, and invadopodia (51). Recent studies have shown that cortactin binds to and activate Arp2/3 complex (24), which binds to the side of an actin filament and nucleates daughter filaments (52), to form branched-actin network that produces protrusive force necessary for directed cell movement. It is conceivable that overexpression of cortactin may enhance the activation of Arp2/3 proteins and, thus, results in enhanced chemotaxis, whereas cells deficient in cortactin have a selective defect in the persistence of lamellipodial protraction, with impaired cell migration and invasion (41). The precise mechanisms for the involvement of cortactin in CXCR4-mediated chemotaxis remain to be revealed; it should be noted that cortactin tyrosine phosphorylation is important for the receptor-mediated chemotaxis based on the result that overexpression of the cortactin-Y421A reduced chemotaxis significantly. Because cortactin is a direct substrate for Src family kinases, this result strongly supports the previous findings regarding the important role of Src family kinases in CXCR4-mediated chemotaxis (53, 54).

Taken together this study provides new and important evidence that CXCL12 ligand stimulation of CXCR4 induced tyrosine phosphorylation of cortactin, which plays a role in CXCR4 internalization and recycling, CXCR4-mediated ERK1/2 activation, and chemotaxis. Considering the important roles of CXCR4 in the development of immune system and central nervous system and in cancer growth and metastasis, these data may provide significant insight into understanding the mechanisms underlying CXCR4 functions, particularly since cortactin is overexpressed in many cancer types (28, 55, 56) in which CXCR4 is expressed or up-regulated (57, 58).

Acknowledgments—We thank Dr. Lee Limbird in the Department of Biomedical Sciences of Meharry Medical College for helpful discussion. We thank Dr. Gang Pei in Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences for the generous gifts of the CXCR4 plasmids. We thank Dr. Mark Caron (Duke University) for the generous gifts of the wild-type and dominant negative mutant forms of c-Src and dynamin constructs. We thank Dr. Yanyun Zhang in the Institute of Health Sciences, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences for fluorescence-activated cell sorting analysis. We also thank Dr. Sam Wells of Vanderbilt University Ingram Cancer Center for confocal analysis.

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