Carboxyl-Terminal Src Kinase Homologous Kinase Negatively Regulates the Chemokine Receptor CXCR4 through YY1 and Impairs CXCR4/CXCL12 (SDF-1α)–Mediated Breast Cancer Cell Migration

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Abstract

Using microarray gene analysis, we found that carboxyl-terminal Src kinase homologous kinase (CHK) regulated the expression of the chemokine receptor, CXCR4. Northern blot and fluorescence-activated cell-sorting analyses showed that CHK down-regulated CXCR4 mRNA and protein levels, respectively. Mutated CHK, which contains a mutation within the ATP binding site of CHK, failed to inhibit CXCR4 expression, thus suggesting that CHK kinase activity is involved in the regulation of CXCR4. Results from gel shift analysis indicated that CHK regulates CXCR4 transcriptional activity by altering YY1 binding to the CXCR4 promoter. Whereas CHK had no significant effects on the expression of YY1, c-Myc, Max, and other YY1-binding proteins, CHK was found to modulate the YY1/c-Myc association. Furthermore, CHK inhibited CXCR4-positive breast cancer cell migration. Taken together, these studies show a novel mechanism by which CHK down-regulates CXCR4 through the YY1 transcription factor, leading to decreased CXCR4-mediated breast cancer cell motility and migration. (Cancer Res 2005; 65(7): 2840-5)

Introduction

The carboxyl-terminal Src kinase (Csk) family of non–receptor-type tyrosine kinases consists of Csk and the Csk homologous kinase, CHK (1). Despite their structural similarities, Csk homologous kinase (CHK) and Csk show striking differences. Whereas Csk is ubiquitously expressed, CHK expression is limited to brain and hematopoietic cells. Moreover, whereas CHK knockout mice do not show any apparent abnormal phenotype, Csk knockout mice present with a defect in neural tube formation and death at E11.5 (2). CHK has been suggested to play a specific role as a novel negative growth regulator of human breast cancer based on the following observations: (*a*) Unlike Csk, which is ubiquitously expressed, CHK is specifically expressed in primary breast cancer specimens but not in normal breast tissues (3–5). (*b*) Unlike Csk, which cannot associate with ErbB-2, CHK binds directly to phospho-Tyr^{1248} of the ErbB-2/neu receptor kinase upon heregulin stimulation and inhibits Src kinase activity (4). (*c*) Overexpression of CHK in breast cancer cells markedly inhibits the cell growth, transformation, and invasion induced by heregulin and causes a significant delay of cell entry into mitosis (5). Thus, CHK not only inhibits breast cancer cell proliferation and transformation but also may inhibit tumor cell invasion, suggesting its possible role in cell motility and metastasis in breast cancer.

There is now accumulating evidence of a role for chemokines and their receptors in cancer cell motility and metastasis (6–10). Whereas most chemokine/chemokine receptor studies have focused on leukocytes, recent studies have highlighted another important role for chemokines/chemokine receptors in the metastasis of breast cancer, especially showing CXCR4 as an important mediator of breast cancer metastasis (9). These results indicate that the elevated expression of CXCR4 is correlated with a more metastatic breast cancer cell phenotype. The potential contribution of CXCR4 to cancer metastasis is further supported by recent studies demonstrating a high level of CXCR4 expression in malignant ovarian cancers and glioblastomas (11, 12).

YY1 is a 65-kDa DNA-binding protein, which can either repress or activate transcription depending on the context (13). YY1 represses the promoter activity of CXCR4 through its binding to the upstream region of the CXCR4 promoter (14, 15).

In this study, we identified CHK as a negative regulator of CXCR4, which is an important signature molecule in breast cancer metastasis. CHK down-regulated the level of CXCR4 expression through YY1, a potent negative regulator of CXCR4. This suppressive effect was attenuated by a mutation at position 262 in the ATP-binding site of the kinase domain of CHK (kinase-dead CHK), suggesting that CHK kinase activity is involved in this regulatory mechanism. We also showed that CHK expression impaired the transmigration of breast cancer cells *in vitro*. This study provides novel insights into the regulation of CXCR4 by the nonreceptor tyrosine kinase, CHK.

Materials and Methods

Materials. Antibodies and reagents were obtained from the following sources: polyclonal anti-YY1 (H414), polyclonal anti-CSK (c-20), polyclonal anti-Lsk, polyclonal p300, and monoclonal CBP were from Santa Cruz Biotechnology (Santa Cruz, CA). pGL-CXCR4 (−375) was a generous gift from Dr. Moriuchi (Division of Medical Virology, Nagasaki University Hospital, Nagasaki, Japan) and contains CXCR4 between −357 and +51 relative to the transcription site followed by the luciferase gene (14).

Cell culture and transfections. MDA-MB-231 cells were cultured in DMEM with 10% FCS. MCF-7 cells were grown in RPMI containing 10% FCS. These cell lines were cultured at 37°C in 5% CO_2. Transfections were done using LipofectAMINE Plus according to the manufacturer's instructions (Life Technologies, Inc, Grand Island, NY). For stable clones, the transfectants were selected with G418 (Life Technologies). The expression of CHK in the G418 selected clones was confirmed by immunoblotting with anti-Lsk polyclonal antibody (Santa Cruz Biotechnology).

Fluorescence-activated cell-sorting analysis. Cells were briefly trypsinized and resuspended in PBS supplemented with 0.1% bovine serum

Note: B. Lee and T. Lee contributed equally to this work.

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albumin. Cells were incubated with anti-CXCR4 monoclonal antibody (clone 12G5, R&D Systems, Minneapolis, MN) for 30 minutes on ice followed by a 30 minutes incubation on ice with anti-mouse secondary antibody conjugated to FITC or TRITC (Jackson ImmunoResearch, West Grove, PA). After the final wash, cells were fixed in 1% paraformaldehyde and analyzed using a FACScan (Becton Dickinson, San Jose, CA).

Chemotaxis assay. The chemotaxis assay was done as described (16). Briefly, MDA-MB-231 cells were starved overnight in serum-free medium before application to an 8-μm pore-size fibronectin (50 μg/mL)-coated transwell insert (Costar, Cambridge, MA). Cells were suspended into the upper chamber at a final concentration of 50×10^4 cells/mL in 200 μL of RPMI 1640, and then recombinant CXCL12 (SDF-1α, 10-20 ng/mL) was added to the lower chamber. After 3 to 6 hours of incubation, the cells on the upper surface of the filters were removed by wiping with cotton swabs, and the migrated cells on the lower chamber were fixed and stained using a Hema3 kit (Biochemical Sciences Inc, Swedesboro, NJ) according to the manufacturer's instructions. Cellular transmigration was enumerated in four separate microscopic fields per field.

Western blot analysis and immunoprecipitation. Western blot analysis and immunoprecipitation were done as described (17).

Electrophoretic mobility shift assay. Nuclear extracts were prepared using MCF-7 cells as described (15). Complementary oligonucleotides corresponding to YY1 (5′-TAGCAAGGATGGACGCGCCACAGAGAGAC-3′) were annealed and end-labeled with [γ-^{32}P]ATP. Competition experiments were done with 1:50 ratios of labeled to nonlabeled oligonucleotides. The binding reactions were done and analyzed on 5% nondenaturing gel as described (15). For the interference assay, 1 μL of YY1 polyclonal antibody (H414, Santa Cruz Biotechnology) was preincubated with the cell extracts before addition of the appropriate radiolabeled oligonucleotide.

Luciferase assay. Cells were plated at (2 to 3) $\times 10^5$ cells per 60-mm dish 1 day before transfection with the promoter-luciferase construct. Cells were transfected with LipofectAMINE (Life Technologies, Rockville, MD) and with the reporter constructs, CHK and β-galactosidase. After a 24-hour transfection, cells were harvested for the luciferase assay. Luciferase activity was measured using the Luciferase assay system (Promega, Madison, WI). In all of the cotransfection experiments, transfection efficiency was normalized by assaying β-galactosidase activity using the β-galactosidase gene under the control of the SV40 early promoter as an internal control.

Northern blot analysis and quantitation of CXCR4 mRNA. Total RNAs were prepared from MDA-MB-231 and MCF-7 cells using RNAStat60 reagent (Tel-Test, Friendswood, TX). Twenty micrograms of each RNA sample were electrophoresed on a 1% formamide-agarose gel and transferred to nylon membranes (Hybond-N+, Amersham Biosciences, Piscataway, NJ). The membranes were cross-linked under UV light, prehybridized, and then hybridized in Expresshyb hybridization solution (Clontech, Palo Alto, CA). The hybridized membranes were exposed to X-ray film at −80°C. CXCR4 mRNA of individual stable clones was quantified using the biotin-labeled CXCR4 oligonucleotide probe and standard provided in the Quantikine mRNA probes and calibrator kit (R&D Systems) according to the manufacturer's instructions. The minimum detectable dose of the kit ranged from 0.78 to 4.0 amol/mL.

Microarray analysis. The Human Cytokine Expression array was purchased from R&D Systems. The membrane consists of 847 genes, which includes cytokines, chemokines, and other immunomodulatory factors, as well as the receptors for these categories of molecules. Probe synthesis and hybridization were done according to the manufacturer's specifications. Quantitation of array images was determined using the ArrayVision software (Imaging Research, Inc., Ontario, Canada).

Results

Regulation of CXCR4 expression by CHK. Our previous results indicated that a nonreceptor tyrosine kinase, CHK, inhibits the invasion/migration of breast tumor cells *in vitro* (5). Thus, we sought to determine whether CHK inhibits breast cancer cell invasion/migration by modulating chemokines, chemokine receptors, or cytokines because chemokines and their receptors have emerged as key molecules implicated in breast cancer cell migration and metastasis (6, 9, 18). Using cDNA probes derived from MDA-MB-231 cells transfected with CHK or with control vector, we did microarray analysis. The array represents a comprehensive collection of cytokines, chemokines, and other immunomodulatory factors and their receptors. Whereas most metastasis-related genes, including matrix metalloproteinases (MMP) and integrins, did not show significant changes in their expression, CXCR4 was found to be significantly suppressed by CHK (Fig. 1*A*). To confirm the microarray results, mRNA extracted from CHK stable transfectants of the MDA-MB-231 and MCF-7 clones was analyzed by Northern blot analysis. The expression of CXCR4 mRNA was suppressed in wild-type CHK-transfected cell lines (W1 and W2 of MCF-7 stable clones and W1 of the MDA-MB-231 stable clone; Fig. 1*B*). Interestingly, enhanced CXCR4 mRNA expression was observed in mutant CHK (A262G, ATP binding site) transfected clones (M1 and M2 of MCF-7 clones and M1 of MDA-MB-231 clones), suggesting that CHK kinase activity might be involved in the transcriptional regulation of CXCR4. The CXCR4 regulation shown is not due to the homologous kinase, Csk, because our Western blot (Fig. 1*C*) and Northern blot (data not shown) analyses did not show any differences in Csk expression between the mock- and CHK-transfected clones. To confirm that these regulatory effects of CHK were not due to clonal variation, we established additional stable clones in MDA-MB-231 cells (n = 7), and quantitated the CXCR4 mRNA levels. Most of the clones tested showed an ∼50% to 60% decrease in CXCR4 mRNA (Fig. 1*D*). To analyze whether CHK regulates CXCR4 transcriptional activity, we transfected CXCR4-luciferase reporter constructs into MDA-MB-231 cells, and then assessed the effects of CHK. The presence of the wild-type CHK caused a significant reduction (at least 3-fold) in CXCR4 promoter activity in both transiently transfected and stably cloned MDA-MB-231 cells as compared with the mock-transfected cells (vector; Fig. 1*E*). We also found similar effects of wild-type CHK in the MCF-7 cells (data not shown), although the extent of suppression was not as significant as in the MDA-MB-231 cells.

CHK mediated CXCR4 regulation via YY1. To understand more fully the molecular mechanism of CHK-induced transcriptional repression of the CXCR4 promoter, we embarked on studies of the cellular DNA-binding proteins that interact with the CXCR4 promoter elements. The CXCR4 promoter contains several cis-acting regulatory elements including E box family proteins and a YY1 binding site (14). YY1 represses the promoter activity of CXCR4 through its binding to the upstream region of the CXCR4 promoter (14, 15). Therefore, we examined the possibility of whether a similar regulatory mechanism is also involved in CHK-mediated CXCR4 modulation. Stably transfected MCF-7 cells were harvested, and the binding of YY1 to the CXCR4 promoter was visualized by electrophoretic mobility shift assay. As shown in Fig. 2, when analyzed with nuclear extracts from stably transfected MCF-7 cells, a sequence-specific DNA protein complex was detected. The formation of the complex corresponding to YY1 binding was competed by an excess of the YY1-unlabeled probe, but not by an unrelated double-stranded oligonucleotide, AP1. In the presence of anti-YY1 antibody, but not control normal rabbit immunoglobulin (NRIgG), the YY1 complex was disrupted, demonstrating that the indicated band was due to the binding of YY1. This suggests that the observed

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binding activity resulted from the specific binding of YY1 in the extracts to the DNA probe. Interestingly, mutation in the ATP binding site sequence of CHK alleviated YY1 binding to the CXCR4 promoter elements (Fig. 2, *lane 3*).

CHK has no effects on YY1, c-Myc, or Max expression but down-regulates CXCR4 expression via modulation of the YY1-c-Myc interaction. c-Myc associates with YY1, which mutually inhibits their biological functions (19). Overexpression of c-Myc protein is often found in poorly differentiated and highly proliferative breast cancer cells (18–21). We therefore analyzed whether CHK down-regulates c-Myc in breast cancer, reducing YY1/c-Myc complexes, and thus allowing more YY1 to bind to the CXCR4 promoter. To test this hypothesis, we measured the level of c-Myc in the stable clones. As shown in Fig. 3*A* (*top*), CHK had no significant effect on the expression of c-Myc. In addition, the ectopic expression of CHK did not have any direct effects on the endogenous level of YY1 as shown in Fig. 3*A* (*middle*). Although Max does not directly associate with YY1, it does associate with c-Myc (22). Thus, changes in Max levels could alter the Max/c-Myc association followed by alteration of the c-Myc and YY1 association. However, as shown in Fig. 3*A* (*bottom*), CHK had no significant effect on the expression of Max.

Previous studies showed that human herpesvirus 6 (HHV-6) infected cells alter the association of c-Myc with YY1, thus up- or down-regulating CXCR4 promoter activity (15). We therefore assessed whether CHK regulates the interaction between YY1 and c-Myc. To test whether CHK regulates the YY1/c-Myc association, 293T cells were cotransfected with wild-type or mutant CHK together with c-Myc, and then analyzed for c-Myc and YY1 association. As shown in Fig. 3*B*, the levels of c-Myc associated with YY1 were lower in the wild-type CHK-transfected cells as compared with the mutant CHK-transfected cells. The endogenous

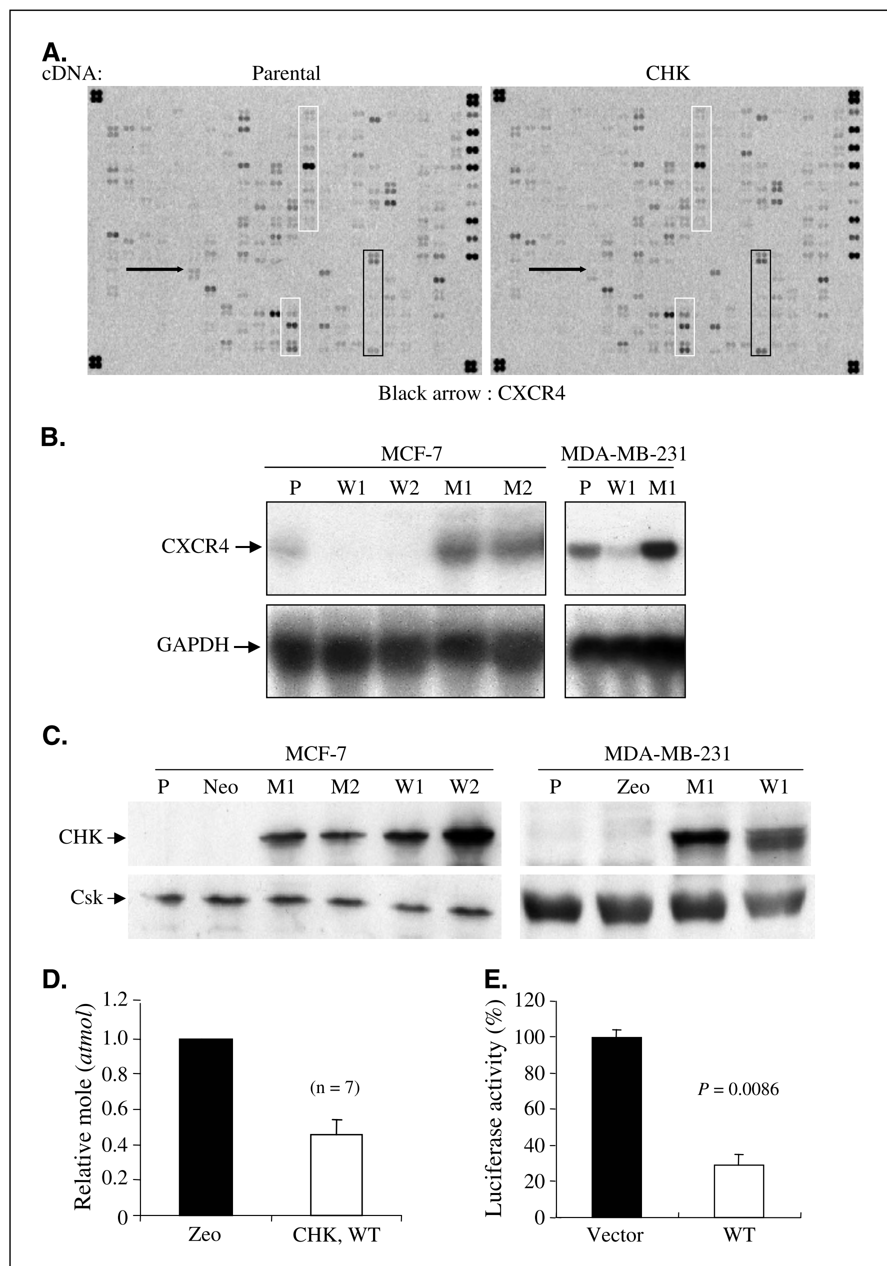

Figure 1. *A,* cDNA microarray analysis of parental and CHK-transfected MDA-MB-231 breast cancer cells. Gene expression was measured by cDNA array hybridization (Human Cytokine Array, R&D Systems) of RNA from each of the indicated cell lines. *Arrow,* indicates the location of CXCR4. The locations of integrin (□) and MMPs (■) are highlighted. *B,* Northern blot analysis. Total RNA (20 μg) was size-fractionated, blotted onto a nylon membrane, and then hybridized to the ^{32}P-labeled CXCR4 cDNA probe. Hybridization with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as a control for RNA loading (*bottom*). *P,* parental cell line; *W,* wild-type CHK; *M,* mutant CHK. *Number,* independent clone. *C,* CHK expression in stably transfected MDA-MB-231 and MCF-7 breast cancer cells. Whole-cell lysates were probed for CHK by Western blotting. *Lanes, P,* parental cell line; *Neo* and *Zeo,* mock-transfected; *M,* kinase-dead CHK-transfected; *W,* wild-type CHK-transfected. *Number,* independent clone. Note that Csk expression was not affected by ectopic CHK expression. *D,* quantitation of human CXCR4 mRNA in several independent MDA-MB-231 stable clones. Stable clones were selected in the presence of G418. Total RNA was extracted from each clone (n = 7) and analyzed for the quantitation of CXCR4 mRNA using a Quantikine CXCR4 mRNA kit (R&D Systems). *Zeo,* mock-transfected cells. *E,* CHK (*WT*) represses CXCR4 promoter activity. The effects of wild-type CHK (*WT*) on CXCR4 promoter activity were measured in MDA-MB-231 breast cancer cells. Cells were cotransfected with CXCR4 promoter luciferase constructs and the wild-type CHK expression vector (*WT*). Twenty-four hours after transfection, luciferase assays were done. Results are expressed as the percent of luciferase activity, which was calculated relative to the activity of the same reporter construct cotransfected with an empty expression vector. Representative of four independent experiments with similar results. *Columns,* mean of three experiments; *bars,* SD. *P* value is shown in the upper right corner.

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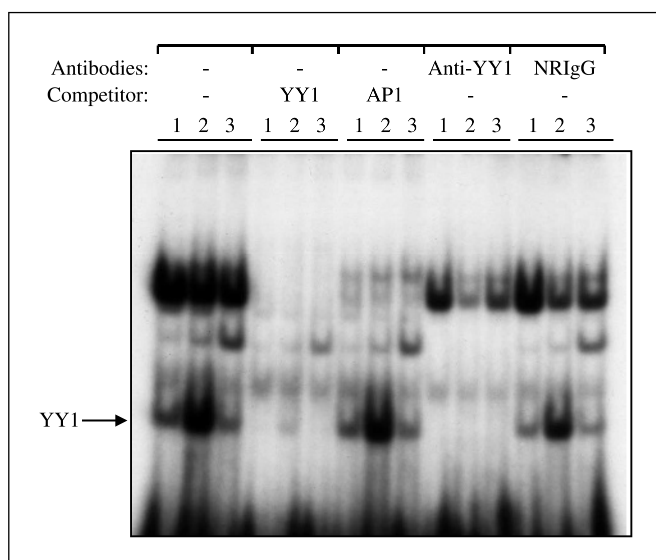

Figure 2. CHK regulates YY1 binding to CXCR4 promoter elements. *A,* ^{32}P-end-labeled oligonucleotide, corresponding to the sequence from −49 to −71 of the CXCR4 promoter, was incubated with the nuclear cell extracts of MCF-7 stable clones: *1,* vector alone; *2,* wild-type CHK; *3,* mutant CHK. The specificity of the complexes was assessed by competition experiments using a 50-fold molar excess of the corresponding unlabeled YY1 oligonucleotide or an unrelated oligonucleotide (*AP1*). *Arrow,* YY1 complexes. The YY1 complex was disrupted by the anti-YY1 polyclonal antibody (*anti-YY1*), but not by the normal rabbit immunoglobulin (*NRIgG*). Representative results from three independent experiments.

association of YY1/c-Myc was also measured using stable clones, yielding similar results (data not shown). Thus, CHK regulates CXCR4 transcriptional activity by altering the physical association of YY1/c-Myc.

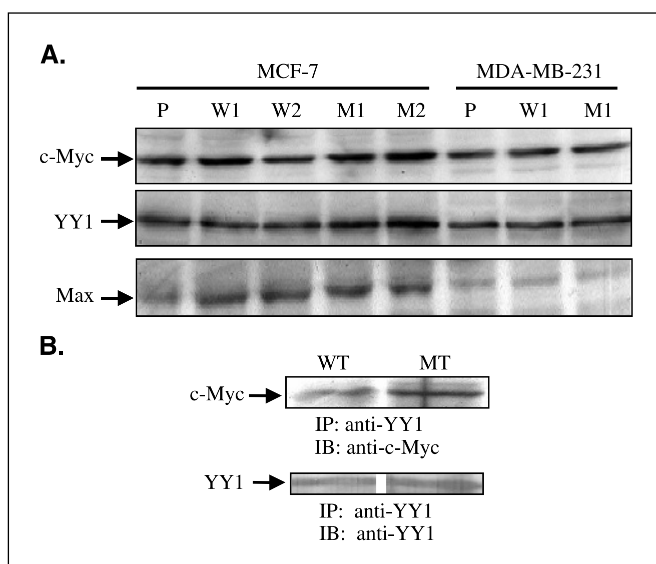

Figure 3. CHK does not affect the protein synthesis of YY1, c-Myc, or Max, but alters the *in vivo* association of c-Myc with YY1. *A,* the amount of YY1, c-Myc, or Max in the stable clones was determined by immunoblotting with their corresponding antibodies. Representative of five independent experiments with similar results. *B,* YY1 was immunoprecipitated from the whole-cell lysates of wild-type (*WT*) or mutant (*MT*) CHK-transfected MDA-MB-231 cells using anti-YY1 antibody. The immunoprecipitates (*IP*) were then analyzed by immunoblot analysis (*IB*) with anti-c-Myc antibodies (*top*). For the loading control, the level of YY1 protein in the immunoprecipitated cell lysates was determined with anti-YY1 antibody (*bottom*). Representative of three independent experiments with similar results.

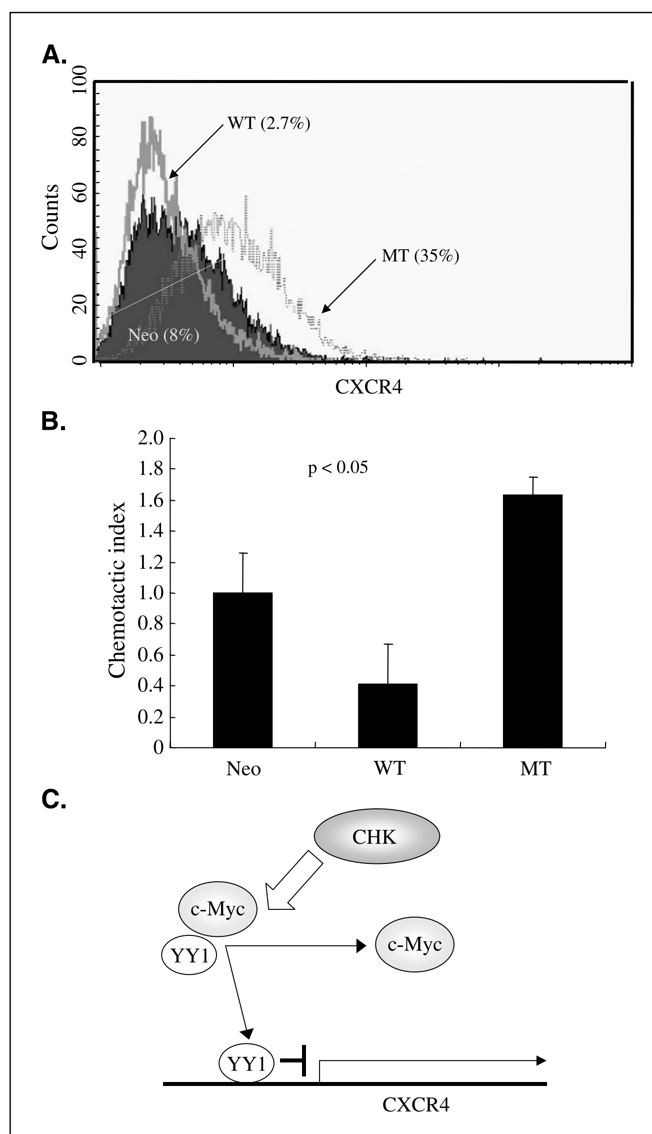

Figure 4. CHK overexpression impaired transmigration of the CXCR4-positive breast cancer cell line, MDA-MB-231, toward CXCL12. *A,* cell surface expression of CXCR4 in stably transfected MDA-MB-231 cells. CXCR4 expression was determined as described in Materials and Methods. Representative of three independent experiments with similar results. For the histograms: *Neo,* mock-infected; *WT,* wild-type CHK-infected; *MT,* mutant CHK-transfected. *B,* cell migration was evaluated using fibronectin-coated 8-μm transwells. Migration of the cells was enumerated as described in Materials and Methods. Representative of three independent experiments with similar results. Results are shown as the mean ± SD. The statistical significance was analyzed using the Student's *t* test (two-tailed distribution). Differences were considered to be significant when $P < 0.05$. *C,* model for CXCR4 repression mediated by CHK. CHK alters the association of c-Myc with YY1, thus interfering with the ability of YY1 to regulate CXCR4 transcription.

Effects of CHK on the migration of breast cancer cells. Next, we tested whether CXCR4 expression is also regulated at the protein level. As the functionality of CXCR4 is correlated with its expression on the cell surface, stable clones were analyzed for their cell surface CXCR4 expression. Similar to the Northern blot analysis results, CXCR4 protein expression was decreased in wild-type CHK-transfected MDA-MB-231 cells (Fig. 4*A*). Whereas MDA-MB-231 cells transfected with wild-type CHK showed a significant decrease from 8% to 2.7% in CXCR4 protein expression, as compared with

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the mock-transfected cells (neo), MDA-MB-231 cells transfected with mutant CHK expressed more CXCR4, with expression increasing from 8% to 35%. These results show that CHK not only regulates CXCR4 at the transcriptional level but also regulates CXCR4 at the protein level. To define the role of CHK in breast cancer cell migration, we carried out a transmigration assay of stably transfected breast cancer cell lines in the presence of CXCL12 (SDF-1). Because MDA-MB-231 cells have been widely used to study breast cancer cell migration and *in vivo* metastasis, we used MDA-MB-231 cells for the transmigration assay. Whereas wild-type CHK inhibited MDA-MB-231 cell migration toward CXCL12 as compared with the mock-transfected cells (~60% inhibition), mutant CHK increased the migration of the cells (Fig. 4*B*). We did not observe any differential chemokinesis between the clones that we tested (data not shown).

Discussion

CXCR4 expression was found to be correlated with the malignant progression of tumors (10, 12, 23, 24). A recent study showed that expression of the chemokine receptor, CXCR4, is tightly correlated with the metastatic properties of breast cancer cells (8, 9).

Whereas CHK exerts its tumor suppressive effects by down-regulating ErbB-2/neu–activated Src kinases (5), our present study revealed another novel link between CHK and CXCR4. Northern blot analysis, a promoter assay, as well as fluorescence-activated cell-sorting (FACS) analysis, showed that wild-type CHK down-regulated the level of CXCR4 in human breast cancer cells. Interestingly, point mutation at the ATP-binding site of the CHK kinase domain attenuated the suppressive effect of CHK on CXCR4 expression, suggesting that its kinase activity is involved in this regulatory mechanism.

Previous studies showed that a nonreceptor tyrosine kinase, ZAP-70, regulates the transmigration of T lymphocytes toward CXCL12 (25, 26). ZAP-70 is a protein tyrosine kinase that associates with the ζ subunit of the T-cell antigen receptor and undergoes tyrosine phosphorylation following T-cell receptor stimulation (27, 28). The Tyr^{292} in the interdomain B region of ZAP-70 negatively regulates ZAP-70–dependent effects on T-cell migration. More recently, the upstream molecule, Lck, was also shown to regulate the T-cell chemotaxis induced by CXCL12, suggesting that the ZAP-70/Lck signaling pathway may have a critical role in T-lymphocyte migration (29). However, the lower migration of ZAP-70- and Lck-deficient cells was not due to the regulation of CXCR4 receptor expression, as determined by FACS analysis.

Although cytokines, including interleukin 2 (30), transforming growth factor β and IFN-γ (31, 32), have been shown to modulate the level of CXCR4 expression, the detailed mechanisms involved in the regulation of its expression are unknown. Moreover, all of these studies were done in T lymphocytes.

Increased Src activity is frequently associated with the metastasis of cancer cells. Src activation by SDF-1α also contributes to this metastasis (33–35). Src is one of the downstream molecules of CHK, and its kinase activity is often increased significantly in advanced cancer (36). Here, we have tested whether Src is involved in the regulation of CXCR4 expression. However, the repression of CXCR4 is not likely due to negative regulation of Src kinase activity because the treatment of breast cancer cell lines with Src kinase inhibitors did not show significant effects on CXCR4 transcription.[3]

Rather than the factors described above, our study revealed a novel pathway by which CHK negatively down-regulates CXCR4, through the transcriptional factor YY1. YY1 binds to the upstream region of the CXCR4 promoter and negatively regulates CXCR4 promoter activity (14). Our gel shift assay indicated that wild-type CHK regulates the DNA binding activity of YY1. Furthermore, mutated CHK attenuated the ability of YY1 to bind DNA, suggesting that the kinase activity of CHK plays a role in modulating the DNA-binding ability of YY1. Although CHK does not modulate the level of YY1, c-Myc, or Max, our results suggest that CHK affects c-Myc and YY1 association by a mechanism similar to that shown in HHV-6–infected T lymphocytes (15). Our findings are summarized in Fig. 4*C*. In addition, we have tested whether CHK regulates other YY1-binding proteins, such as CBP and p300. We found that CHK does not show any effects either on the levels of CBP and p300 or on their association with YY1.[3] The ectopic expression of CHK in the metastatic breast cancer cell line, MDA-MB-231, significantly impaired its ability in the *in vitro* transmigration assay, suggesting the potential role of CHK in regulating tumor cell motility and metastasis.

Taken together, our study shows a novel mechanism by which CHK regulates the level of CXCR4, through the YY1 transcription factor, and impairs CXCR4/CXCL12-mediated breast cancer cell migration.

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[3] Lee et al., unpublished data.

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