Differential Functional Activation of Chemokine Receptor CXCR4 Is Mediated by G Proteins in Breast Cancer Cells

Jane D. Holland,[1] Marina Kochetkova,[1] Chareeporn Akekawatchai,[1] Mara Dottore,[2] Angel Lopez,[2] and Shaun R. McColl[1]

[1]School of Molecular and Biomedical Science, the University of Adelaide and [2]Cytokine Receptor Laboratory, Institute of Medical and Veterinary Science, Adelaide, Australia

Abstract

CXCR4 is a G protein–coupled receptor of considerable biological significance, and among its numerous functions, it is suggested to play a critical role in cancer metastasis. We have investigated the expression and function of CXCR4 in a range of breast cancer cell lines covering a spectrum of invasive phenotypes and found that, while surface levels of CXCR4 were uniform across the entire panel, only highly invasive cells that are metastatic in immunocompromised mice expressed functional receptors. CXCL12/SDF-1 induced cellular responses such as calcium mobilization, actin polymerization, and chemotaxis in metastatic cells, whereas noninvasive cells were unresponsive. Moreover, CXCL12 activated multiple signaling pathways downstream of G proteins in highly invasive cells but failed to activate any of the examined kinase cascades in noninvasive cell lines. This blockade in nonmetastatic cell lines seems to be due to the inability of G protein α and β subunits to form a heterotrimeric complex with CXCR4. Gα and Gβ were able to bind to CXCR4 independently in all cell lines, but the association of G protein $\alpha\beta\gamma$ heterotrimers with the receptor, a prerequisite for signal transduction downstream from G protein–coupled receptors, was only observed in the highly invasive cell lines. Our findings show, for the first time, that CXCR4 function is subject to complex and potentially tightly controlled regulation in breast cancer cells via differential G protein–receptor complex formation, and this regulation may play a role in the transition from nonmetastatic to malignant tumors. (Cancer Res 2006; 66(8): 4117-24)

Introduction

Chemokine receptors and their ligands constitute a family of regulatory proteins controlling numerous physiologic and pathologic processes (1, 2). Chemokines act through specific 7-transmembrane receptors coupled to heterotrimeric G proteins consisting of $\alpha\beta\gamma$ subunits (3). Like all chemokine receptors, CXCR4 initiates signal transduction through the activation of members of the G_i family of G proteins, and when its ligand, CXCL12, is bound the heterotrimeric G_i complex dissociates into its α and $\beta\gamma$ subunits. The released $G\alpha_i$ subunit inhibits adenylyl cyclase and the G$\beta\gamma$ dimer activates two major signaling enzymes, phospholipase Cβ (4, 5) and phosphatidylinositol 3-kinase (6), which initiate the activation of downstream events such as calcium mobilization, actin polymerization, and chemotaxis.

Chemokines have been implicated in tumor development with chemokine networks regulating angiogenesis and leukocyte infiltration (2, 7). Malignant cells themselves may express a wide repertoire of chemokine receptors and respond to chemokines with increased directional migration, and in some cases, proliferation and survival (8–10). Recently, certain chemokine ligands and their G protein–coupled receptors (GPCR), in particular, CXCL12/SDF-1 and CXCR4, have received a great deal of attention due to the discovery of their potential direct involvement in promoting cancer metastatic spread (8, 11–13). Expression of CXCR4 mRNA was detected in all tested breast cancer cell lines and primary tumors, and CXCL12 was shown to mediate invasive activities *in vitro* and *in vivo* in mouse models (11). Furthermore, a link has been proposed between the level of CXCR4 expression and the metastatic status of tumors. Elevated expression of CXCR4 was found on tumor cells in metastatic breast and prostate carcinomas, compared with normal tissues. Interestingly, in breast cancers, the pattern of CXCR4 expression was also significantly correlated with the degree of lymph node metastasis (14–16). On the other hand, Schmid et al. showed that the expression of the CXCR4 is initiated at a very early point in the transition from normal to transformed phenotype in breast epithelium (17). High levels of CXCR4 were detected in 94% of studied cases of atypical ductal hyperplasia, which represents a very early stage in tumorigenic transformation (17). Thus, in spite of these previous studies, the relationship between CXCR4 expression on epithelial cells, including the range of functional responses of breast cancer epithelial cells to CXCL12, and the potential role of CXCR4 in breast cancer metastatic progression, is not clear and requires considerable clarification. Therefore, in the present study, we analyzed CXCR4 expression and function in a panel of cell lines, ranging from nontransformed immortalized breast epithelial cells to highly aggressive, breast cancer cell lines that are metastatic in nude mice, to investigate the relationship between CXCR4 expression/function and the invasive potential of transformed cells. We show for the first time that only highly metastatic breast cancer lines are equipped with functionally active CXCR4 receptors, suggesting that regulatory mechanisms are potentially switched on during the metastatic progression of breast cancer. We also provide evidence that this functional "on-switch" of CXCR4 in breast cancer cells is controlled at the level of the receptor and G-protein subunit interactions that regulate numerous signaling pathways downstream of CXCR4. Our findings point to the existence of a novel mechanism for posttranslational regulation of CXCR4 function, which may have potentially important implications for the acquisition of an invasive, metastatic phenotype by breast cancer cells, and possibly, other tumors.

Requests for reprints: Shaun McColl, Chemokine Biology, School of Molecular and Biomedical Science, the University of Adelaide, Adelaide, South Australia 5005, Australia. Phone: 618-8303-4259; Fax: 618-8303-3337; E-mail: shaun.mccoll@adelaide.edu.au.

doi:10.1158/0008-5472.CAN-05-1631

Materials and Methods

Reagents and cell lines. Synthetic human CXCL12 was provided by Professor Ian Clark-Lewis (Biomedical Research Centre, University of British Columbia, Vancouver, British Columbia, Canada). The antibodies used in this study were polyclonal anti-huCXCR4 (Chemicon International, Inc., Temecula, CA), monoclonal anti-huCXCR4-FITC (clone, 12G5; R&D Systems Minneapolis, MN), monoclonal antimouse β-actin (Sigma, St. Louis, MO), polyclonal anti-$G\alpha_i$, and anti-Gβ (Santa Cruz Biotechnologies, Santa Cruz, CA).

Human breast nonmetastatic cell lines MDA-MB-453, MDA-MB-134, MDA-MB-468, and MCF10A, metastatic cancer cell lines MDA-MB-231 and BT-549, as well as malignant leukemia cell line, Jurkat T cell, were all obtained from the American Type Culture Collection (Manassas, VA). Jurkat T and MDA-MB-231 cells were grown in RPMI supplemented with 10% FCS and 100 units/mL of penicillin/streptomycin. MDA-MB-453, MDA-MB-134, MDA-MB-468, and BT-549 were propagated in DMEM with 20 mmol/L HEPES supplemented with 10% FCS and 100 units/mL of penicillin/streptomycin. MCF10A cells were cultured in a 1:1 mixture of DMEM and Ham's F-12 medium supplemented with 5% FCS, 100 units/mL of penicillin/streptomycin, 10 μg/mL insulin, 1.4 μmol/L cortisol, and 20 ng/mL epidermal growth factor. Cell lines tested in various functional assays were washed with PBS and incubated in serum-free medium for 3 hours before treatment.

SiRNA mediated CXCR4 knockdown. Short hairpin (sh)RNA-containing pro-viruses, targeting nucleotides 470 to 490 in the CXCR4 mRNA or control sequence from the *Renilla* luciferase gene were packaged in retroviral particles and used to infect MDA-MB-231 cells as described in ref. 18. Individual clones were selected in medium with 15 ng/mL puromycin and expanded. Clones with <30% residual CXCR4 expression, estimated by flow cytometry, were used for further analysis. Breast cancer cell lines were stained with monoclonal anti-human CXCR4 antibody for flow cytometry and analyzed as described in ref. 19.

Radiolabeled binding assay (RIA). Two micrograms of CXCL12 was labeled with [^{125}I] using the iodine monochloride method previously

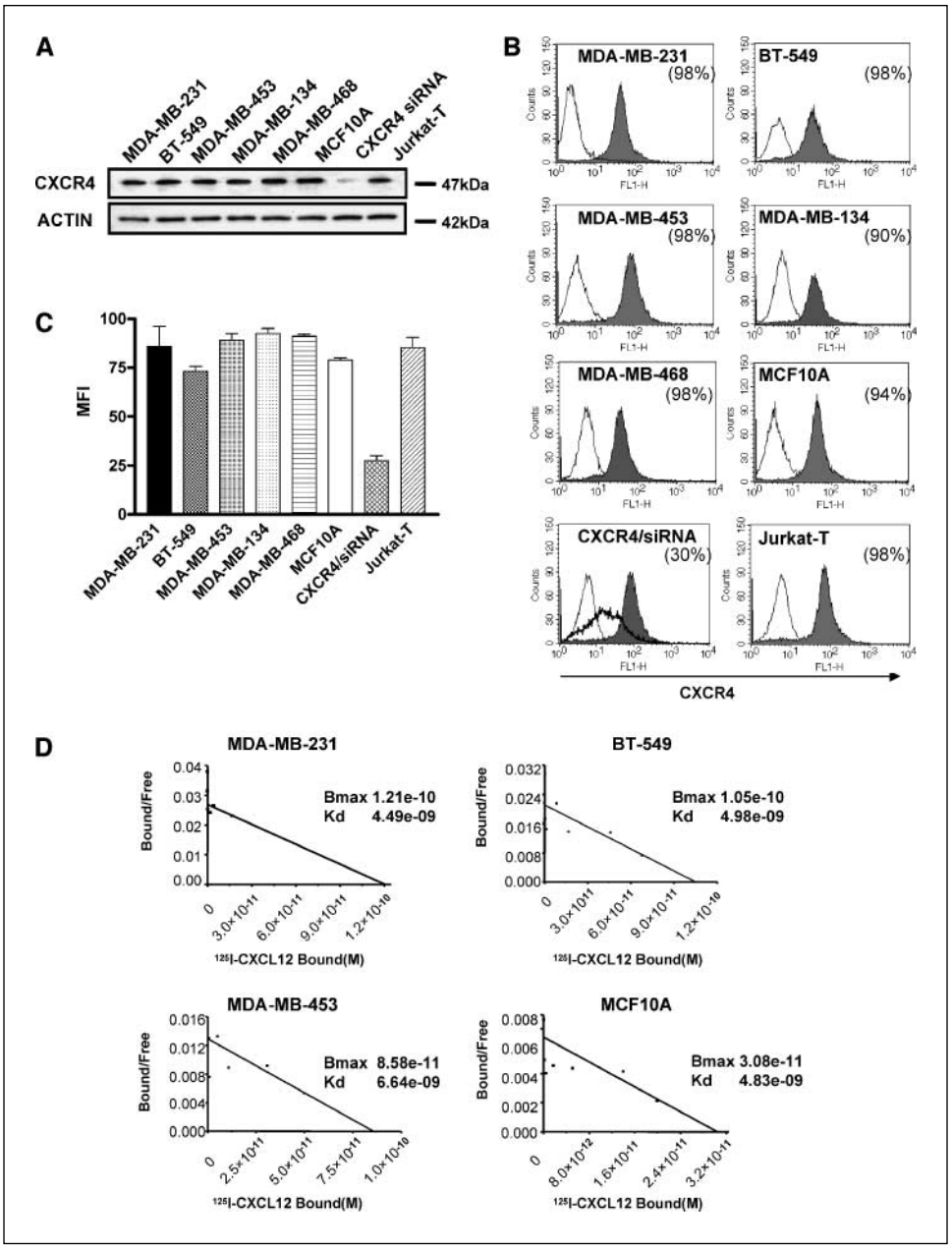

Figure 1. CXCR4 expression in a panel of human breast cancer cell lines. *A,* Western blot analyses of CXCR4 in whole cell lysates. β-Actin was used as a control to ensure equal loading. These blots are representative of three independent experiments done with similar results. *B,* evaluation of CXCR4 cell surface expression by flow cytometry. Histograms are shown for all cell lines used in this study, with the percentage of positive CXCR4 staining in parentheses. *Filled histograms,* CXCR4 labeling; *open histograms,* isotype control staining. MDA-MB-231/CXCR4 siRNA knockdown cell line was included as control for antibody specificity. The histograms in the CXCR4 siRNA knockdown panel are isotype control (*open histogram*), MDA-MB-231 cells expressing a negative control siRNA construct (*filled histograms*), and MDA-MB-231 cells expressing CXCR4 siRNA (*open histogram, bold line*). *C,* comparison of cell surface expression levels of CXCR4 on human breast cancer cell lines presented as the MFI. *Columns,* mean of three independent experiments for all cell lines; *bars,* ±SE. *D,* Scatchard transformation of ^{125}I-CXCL12 binding to breast cancer cells. These data are from one experiment representative of three independent experiments.

described (20). After separation of iodinated CXCL12 from iodide ions on a Sephadex G25 PD-10 column, RIA was done as previously described (21). Briefly, cells (4×10^6/mL) from each cell line were incubated in suspension with concentrations of [^{125}I]CXCL12, ranging from 10 pmol/L to 10 nmol/L in the presence and absence of 100-fold excess of unlabeled CXCL12. Following a 2-hour incubation with shaking at 4°C, each cell suspension was centrifuged through 200 μL of FCS in order to remove free ^{125}I-CXCL12, and the level of radioactivity in cell pellets was determined using a γ-counter. Specific binding was determined by subtracting the counts per minute obtained in the presence of excess unlabeled CXCL12 from the total counts per minute. Scatchard transformation of saturation binding curves was done using Prism 4 software (GraphPad, San Diego, CA).

Actin polymerization. Cells on coverslips, or in suspension, were stimulated with CXCL12 (100 ng/mL), fixed in 3.7% paraformaldehyde and permeabilized in PBS with 0.1% Triton X-100. Fixed cells were stained with NBD-phallacidin (Molecular Probes, Eugene, OR) and analyzed using a FACScan (BD Bioscience, San Jose, CA). Cells grown on glass coverslips were mounted on glass slides with Vecta-shield mounting liquid (Vector Laboratories, Burlingame, CA) containing 4′,6-diamidino-2-phenylindole for nuclear counterstaining and visualized by fluorescent microscopy.

Chemotaxis assay. A chemotaxis assay was done in a 96-well modified Boyden microchamber (Neuro Probe, Gaithersburg, MD) and carried out essentially as described in refs. 16, 18.

Calcium mobilization assay. Cells (10^6/mL) incubated for 15 minutes with 2 μmol/L of Fura-2AM at 37°C (Molecular Probes) were stimulated with CXCL12 (100 ng/mL) and changes in intracellular calcium were quantified using an Aminco-Bowman Series 2 luminescence spectrometer as previously described in ref. 22.

Adenylate cyclase inhibition assay. Breast cancer cells (5×10^4 /mL) in DMEM/HEPES and 10 μmol/L 3-isobutyl-1-methylxanthine (Alexis Biochemicals, Lausen, Switzerland) were either untreated or treated with 5 μmol/L forskolin (Sigma-Aldrich, Castle Hill, Australia) alone or with CXCL12 (100 ng/mL) or phosphatidic acid (100 ng/mL) for 10 minutes. The level of cyclic AMP (cAMP) was assayed in cell lysates using a cAMP Biotrak Enzyme-immunoassay (Amersham Biosciences, Uppsala, Sweden) as recommended by the supplier. Fold induction of cAMP synthesis was determined relative to untreated cells.

Phosphoprotein Bio-Plex assay. Cells stimulated with CXCL12 (100 ng/mL), as well as untreated cells, were lysed in a modified radioimmunoprecipitation assay buffer (1% NP40, 150 mmol/L NaCl, 50 mmol/L Tris-HCl, 10% glycerol, 10 mmol/L sodium vanadate, 10 mmol/L sodium fluoride, 10 mmol/L phenylmethylsulfonyl fluoride, and 10 mmol/L protease inhibitor cocktail). Two hundred micrograms of proteins were analyzed using a 4-Plex microbead-mediated phosphoprotein detection assay kit (Bio-Rad, Hercules, CA). Analysis was carried out using the Bio-Plex suspension array system.

Western blot and magnetic coimmunoprecipitation analysis. Cells were lysed in buffer containing 1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris-HCl, 10 mmol/L sodium vanadate, 10 mmol/L sodium fluoride, 10 mmol/L protease inhibitor cocktail, and 10 mmol/L phenylmethylsulfonyl fluoride (at pH 8.5; Sigma-Aldrich). Fifty micrograms of protein samples were used for Western blotting. Immunocomplexes were formed from 500 μg of protein lysate with 1 μg of the appropriate antibody, captured by incubation with protein G–coupled magnetic beads and separated on a magnetic column (both from Miltenyi Biotech, Bergisch Gladbach, Germany). Columns were washed several times with lysis buffer and bound protein complexes were eluted with preheated sample buffer [50 mmol/L Tris-HCl (pH 6.8), 50 mmol/L DTT, 1% SDS, 0.005% bromophenol blue, and 10% glycerol]. Samples were then separated by SDS-PAGE and subjected to Western blot.

Results

CXCR4 expression is uniform throughout metastatic and nonmetastatic breast cancer cell lines. CXCR4 protein expression was analyzed on a panel of human breast cancer cell lines. The cell lines ranged from untransformed immortalized epithelial cells potentially representing the first step towards malignant transformation (MCF10A), to malignant but noninvasive cells that are not metastatic in nude mice (MDA-MB-453, MDA-MB-134, and MDA-MB-468), to highly invasive breast cancer cells (MDA-MB-231 and BT-549). The invasive phenotypes of these cell lines have been previously characterized from studies *in vitro* and *in vivo* in nude mice (ref. 23; data not shown). Levels of CXCR4 were analyzed by Western blotting. The results show that all tested cell lines expressed comparable levels of total CXCR4 (Fig. 1*A*). The Jurkat T cell line, shown in previous reports to express high levels of CXCR4 protein, was included as a positive control (24, 25), and MDA-MB-231 cells, in which CXCR4 expression has been knocked down using short interfering RNA (siRNA), were used to show antibody specificity.

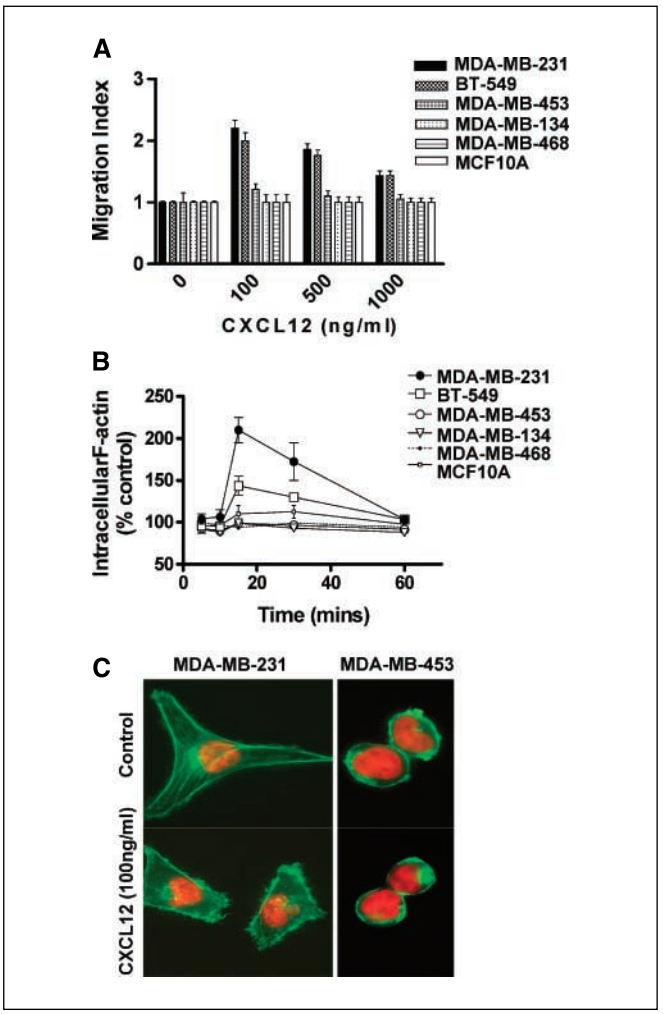

Figure 2. CXCL12 stimulation induces functional responses in metastatic breast cancer cell lines only. *A,* CXCL12-mediated migration. Breast cancer cells were allowed to migrate toward the indicated concentrations of CXCL12 in a 96-well modified Boyden chamber. Ratio of cells that migrated in each well towards CXCL12 relative to media-only controls; *columns,* mean of three independent experiments; *bars,* ±SE. *B,* time course analysis of CXCL12-induced actin polymerization in breast cancer cell lines. Cells were treated with CXCL12 (100 ng/mL) or PBS only, fixed, permeabilized, stained with FITC-conjugated NBD-phallacidin and analyzed by flow cytometry. MFI shown as a measure of F-actin concentration; *points,* mean of all time points plotted relative to the mean fluorescence of the samples treated with PBS; *bars,* ±SE ($n = 3$). *C,* fluorescent microscopy of polymerized F-actin (*green*) in PBS- or CXCL12-treated MDA-MB-231 and MDA-MB-453 cancer cell lines. Cells were counterstained with DAPI to visualize cell nuclei (*red*). The images are representative of two independent experiments done with similar results.

We also evaluated the cell surface expression of CXCR4 by flow cytometry using mouse monoclonal anti-CXCR4 and matched IgG_{2a}-isotype control antibody. The percentages of CXCR4-positive cells and relative mean fluorescence intensities (MFI) were determined. We found that CXCR4 was expressed uniformly across our panel of breast cancer cell lines with 90% to 98% of cells being positive for CXCR4 (Fig. 1*B*). MFIs were similar across the panel, indicating that the levels of CXCR4 surface expression, i.e., surface receptor density, are also uniform in this panel of cell lines (Fig. 1*C*). In addition, radioligand binding experiments using [^{125}I] CXCL12, showed similar receptor numbers and binding affinities for metastatic (MDA-MB-231 and BT-549), untransformed (MCF10A), and nonmetastatic (MDA-MB-453) cells, with K_d being in the nanomolar range (Fig. 1*D*).

CXCL12 induces CXCR4 receptor activation only in metastatic breast cancer cell lines. Given that our studies indicate that CXCR4 receptor expression is not a predictor of metastatic potential, we turned our focus onto the functional responsiveness of CXCR4 receptor by examining the ability of the CXCR4 ligand, CXCL12, to induce migration of the cell lines using a modified Boyden chamber assay. The results of these experiments showed that the highly invasive cell lines, MDA-MB-231 and BT-549, were capable of migrating towards CXCL12 in a dose-dependent manner (Fig. 2*A*). The noninvasive cells were unresponsive, in spite of the fact that all cell lines migrated towards serum used as a positive control (data not shown).

The ability of CXCL12 to stimulate actin polymerization was examined next. Changes in the amount of intracellular filamentous actin (F-actin) induced by CXCL12 were quantified by flow cytometry, the data were normalized to the basal level of untreated cells and presented as a fold-induction. Our results show that after 15 minutes of exposure to CXCL12, the level of F-actin was significantly increased in the metastatic MDA-MB-231 and BT-549 cells. In contrast, the basal levels of F-actin were unchanged in the nonmetastatic cell lines, with CXCL12 having no effect on these cell types (Fig. 2*B*). Furthermore, fluorescence microscopy confirmed CXCL12-induced actin polymerization in the metastatic cells and not in the nonmetastatic cells (Fig. 2*C*). These results indicate that CXCR4 expression and function are uncoupled, at least, at the level of actin polymerization and cell migration in the nonmetastatic cell lines.

Signal transduction blockade occurs at the G protein level. To further study this nonresponsiveness in nonmetastatic cell lines, the signaling events taking place at the G protein level were investigated. First, we measured the ability of CXCL12 to induce intracellular calcium mobilization. Breast cancer cells were labeled with Fura-2AM before the addition of CXCL12. Evaluation of the fluorescence of stimulated cells revealed that CXCL12 induced

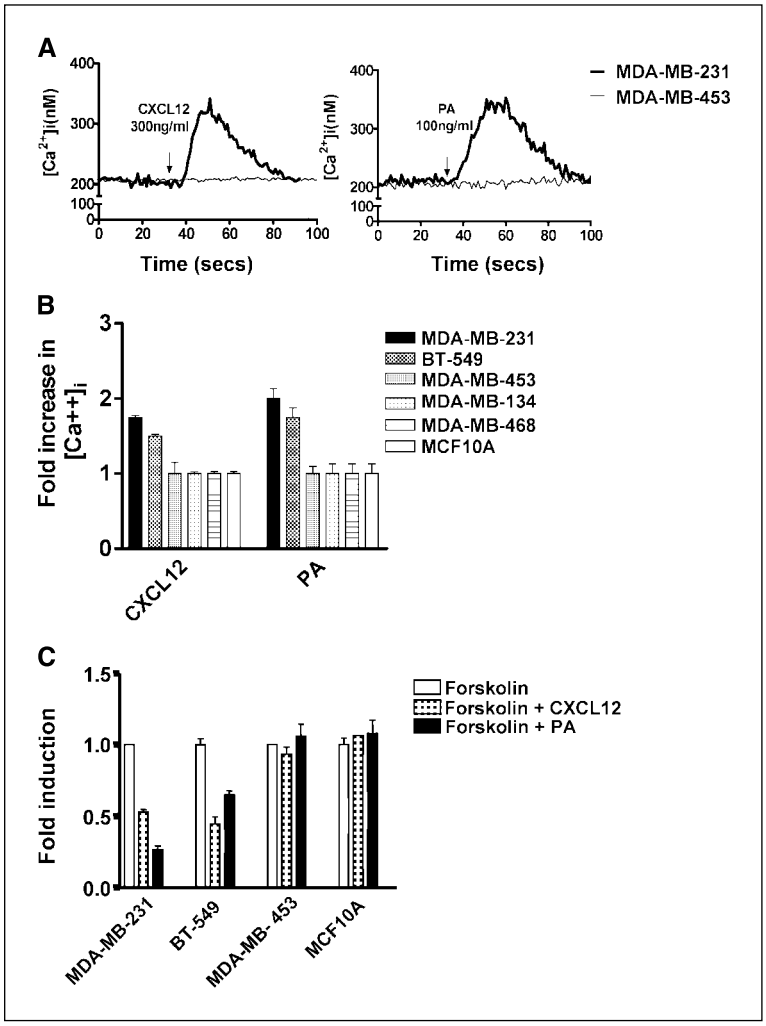

Figure 3. G_i dependent pathways activated by CXCL12 in metastatic breast cancer cell lines only. *A,* CXCL12-induced calcium mobilization in breast cancer cell lines. These graphs are representative of results obtained for metastatic and nonmetastatic cell lines showing the changes in intracellular Ca^{2+} concentrations in real-time following the addition of ligand. The data are representative of the results of four other experiments. *B,* comparison of Ca^{2+} mobilization in human breast cancer cell lines summarized as a fold increase in $[Ca^{2+}]_i$ after stimulation with CXCL12. *Columns,* mean of three independent experiments for all cell lines; *bars,* ±SE. *C,* the inhibition of forskolin-induced (5 μmol/L) cAMP production by CXCL12 or phosphatidic acid (*PA*) was assayed in indicated breast epithelial cell lines using a cAMP ELISA. *Columns,* mean from triplicate measurements; *bars,* ±SE.

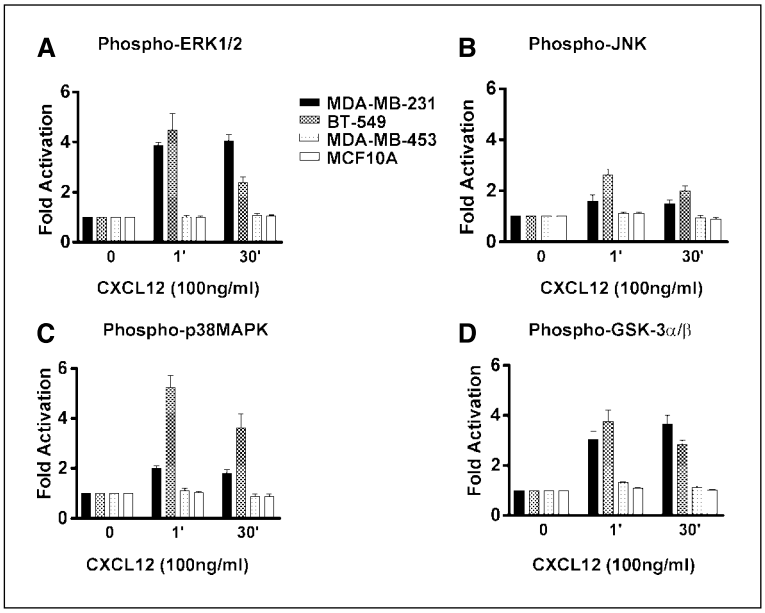

Figure 4. CXCL12 induces the activation of multiple Gβ-dependent signaling pathways in invasive breast cancer cell lines. The levels of phosphorylation of various signaling intermediates were assayed in breast cancer cell lysates. Cells were treated, or not, with CXCL12 (100 ng/mL) as indicated. Cell lysates were prepared and analyzed in a 4-plex phosphoprotein assay which simultaneously measured the activated states of ERK1/2 (*A*), c-*Jun*-NH_2-kinase (*JNK*; *B*), p38MAPK (*C*), and GSK-3α/β (*D*) proteins. The values were expressed as a fold activation relative to the amount of phosphoprotein present in untreated cells. *Columns,* mean of duplicate determinations and are representative of three independent experiments; *bars,* ±SE.

transient increases in intracellular calcium in the metastatic cell lines as shown in Fig. 3*A*, whereas the nonmetastatic cell lines remained unresponsive. The effect of CXCL12 on intracellular Ca^{2+} levels in all of the cell lines examined in this study is shown in Fig. 3*B*, indicating a clear separation between metastatic and nonmetastatic cell lines. A similar profile was observed using the nonchemokine GPCR agonist phosphatidic acid.

Next, the ability of CXCL12 to inhibit forskolin-induced cAMP production in MDA-MB-231, BT-549, MDA-MB-453, and MCF10A was examined. The cells were incubated with forskolin alone, or in combination with CXCL12 or phosphatidic acid, which was used as a positive control for the inhibition of cAMP production (26). As shown in Fig. 3*C*, both CXCL12 and phosphatidic acid significantly inhibited forskolin-induced cAMP accumulation in the metastatic MDA-MB-231 and BT-549 cells, whereas noninvasive cells, MDA-MB-453 and MCF10A, were unresponsive to either agent.

Gβγ subunits have been shown to mediate the activation of numerous kinase cascades downstream of GPCRs (27). We therefore assayed the phosphorylation of a number of downstream signaling molecules induced by CXCL12 (28) as a measure of Gβγ subunit functionality using a multiplex phosphoprotein assay. Lysates were prepared from cells that were serum-starved for 4 hours and treated with chemokine ligand for 1 minute and 30 minutes. CXCL12 induced rapid and sustained 2- to 5-fold activation of ERK1/2, p38 mitogen-activated protein kinase (MAPK), c-*Jun*-NH_2-kinase, and GSK-3α/β in the metastatic cell lines MDA-MB-231 and BT-549 (Fig. 4). However, no significant response was detected in the MDA-MB-453 or MCF10A cell lines. Together, these data suggest that CXCR4 function is blocked at the level of G protein activation.

Differences in G protein subunit coupling to CXCR4 are responsible for functional activation in metastatic breast cancer cell lines. To further investigate the molecular mechanisms leading to the inhibition of G protein activation, we examined the expression of the G protein subunits $G\alpha_i$ and Gβ in selected breast cancer cell lines. Both G protein subunits were detected by Western blot in breast cancer cell lysates. The levels in $G\alpha_i$ expression were consistently lower in the nonmetastatic cell lines, whereas expression of Gβ was approximately the same in all cell lines (Fig. 5*A*). These observations were consistent over three independent experiments.

CXCR4/G protein coupling was then compared in resting or CXCL12-activated MDA-MB-231 and MDA-MB-453 cells. Cellular lysates were precipitated with polyclonal anti-$G\alpha_i$ antibodies and the immunocomplexes were analyzed for the presence of CXCR4 or Gβ by Western blot (Fig. 5*B*). The results of these experiments show that, first, although $G\alpha_i$ binds to CXCR4 in both resting metastatic MDA-MB-231 and noninvasive MDA-MB-453 cells (compare *lanes 2* and *4*, Fig. 5*B*), uncoupling of $G\alpha_i$ from CXCR4 following ligation of CXCL12, a necessary step for signal transduction, occurs only in MDA-MB-231 cells (compare *lanes 3* and *5*, Fig. 5*B*). Second, whereas $G\alpha_i$ and Gβ form strong complexes in resting MDA-MB-231 cells (*bottom*; *lane 2*, Fig. 5*B*) and the association of Gβ with $G\alpha_i$ is decreased following the treatment of MDA-MB-231 cells with CXCL12 (*bottom*; compare *lanes 2* and *3*, Fig. 5*B*), in contrast, $G\alpha_i$ and Gβ do not associate in MDA-MB-453 cells (*bottom*; *lanes 4* and *5*, Fig. 5*B*), despite the fact that Gβ binds to CXCR4 in those cells (*lanes 4* and *5*, Fig. 5*C*).

Analysis of the association between $G\alpha_i$ and Gβ in the entire panel of breast epithelial cell lines confirmed that $G\alpha_i$ and Gβ dimers associated with CXCR4 are detected only in the metastatic MDA-MB-231 and BT-549 cells (*lanes 3* and *4*, Fig. 5*D*), which respond to CXCL12 stimulation, but not in noninvasive MCF10A and MDA-MB-453, MDA-MB-134, or MDA-MB-468 cell lines (*lanes 5, 6, 7* and *8*, Fig. 5*D*). The differences in $G\alpha_i$ and Gβ binding observed throughout the panel of breast cancer cell lines were not due to the absence of Gβ protein because all cells express the Gβ subunit (*bottom*, Fig. 5*D*).

Discussion

In this study, we present three novel observations with respect to the role of the chemokine receptor CXCR4, and its ligand CXCL12, in cancer progression and metastasis. First, we show that the expression of CXCR4 by breast cancer epithelial cells does not simply correlate with function of CXCR4. Second, functional CXCR4 expression on breast cancer epithelial cells seems to

correlate with the invasive potential of the cells. Third, the acquisition of functional CXCR4 on metastatic breast cancer epithelial cells involves altered G protein subunit coupling to CXCR4 in those cells. These observations have potentially significant implications for our understanding of the role of this receptor and its ligand in cancer.

It is becoming increasingly clear that chemokines regulate important processes promoting tumor progression such as cancer cell growth, angiogenesis, host immune responses against malignant cells, and metastasis (2, 29–31). A large and growing body of evidence supports an important and potentially crucial role for CXCR4 and CXCL12 in breast, gastric, ovarian, prostate, colon, and many other cancers (11, 32–35).

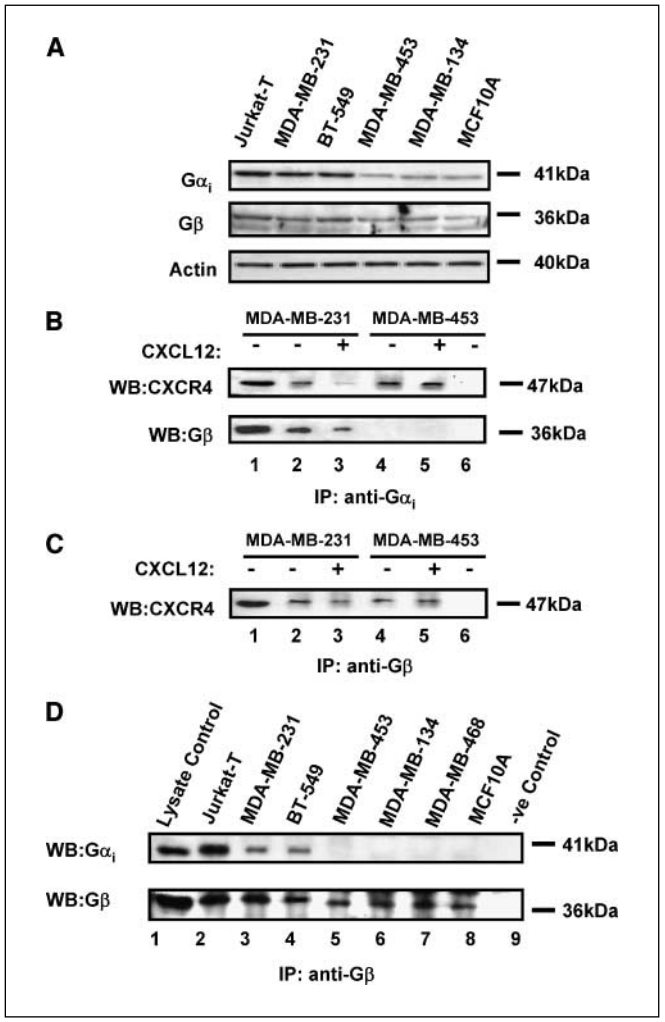

Figure 5. Differential G protein subunit coupling in breast cancer cell lines. *A,* Western blot analysis of $G\alpha_i$ and Gβ subunits expression in whole cell lysates. Jurkat T cell lysates were included as a positive control whereas β-actin levels were assessed to evaluate protein loading. *B,* association of $G\alpha_i$ with CXCR4 or Gβ in MDA-MB-231 (*lanes 2* and *3*) and MDA-MB-453 cells (*lane 4* and *5*). Cells were incubated with CXCL12 (100 ng/mL) or PBS for 15 seconds and lysed in a modified Triton X buffer. The immunocomplexes were precipitated with anti-$G\alpha_i$ antibodies (*lanes 2-5*) or with anti-β-actin antibodies included as a negative control (*lane 6*). MDA-MB-231 whole cell lysates were used as a positive control (*lane 1*). The samples were subjected to SDS-PAGE followed by Western blot analysis for CXCR4 (*top*) and Gβ (*bottom*). *C,* association of Gβ with CXCR4 in MDA-MB-231 and MDA-MB-453 cells. Cell lysates prepared as described in (*B*) were immunoprecipitated with anti-Gβ polyclonal antibodies and analyzed by Western blot analysis with anti-CXCR4; control *lanes 1* and *6* are similar to (*B*). *D,* association between $G\alpha_i$ and Gβ in the panel of breast cancer cell lines. Whole cell lysates were prepared from unstimulated cells, immunoprecipitated with anti-Gβ antibodies, and subjected to Western blot analysis with either $G\alpha_i$ or Gβ antibodies. Representative of three independent experiments each done with similar results. Immunoprecipitation with anti-β-actin antibodies were used as a negative control (*lane 6*). *WB,* Western blot; *IP,* immunoprecipitation.

A positive correlation between the level of CXCR4 expression in tumor tissues and tumor metastatic potential in breast and other cancers has been suggested. However, the precise molecular and cellular function of this chemokine receptor has not yet been established. It has been shown that CXCR4 is highly expressed in ductal carcinoma *in situ*, which is believed to be a precursor of invasive ductal carcinomas (17). Moreover, similar levels of CXCR4 are present in atypical ductal hyperplasia, potentially the first clonal preneoplastic expansion of ductal epithelial cells, representing a very early step toward malignant transformation. Therefore, these previous observations raise the possibility that expression of CXCR4 is not restricted to the more malignant forms of breast cancer.

Our data provide significant further insight into this issue. They show a clear demarcation with respect to functional activation of CXCR4 in a panel of breast cancer epithelial cells, at least with respect to the assays undertaken in this study. Although CXCR4 expression was uniform on all of the cell lines examined with similar binding affinities for its ligand in untransformed, metastatic, and nonmetastatic cells, CXCL12-induced migration, intracellular Ca^{2+} mobilization, actin polymerization, phosphorylation of a range of intracellular signaling intermediates, and the inhibition of cAMP production could only be detected in highly invasive cell lines that have been characterized in our laboratory, and those of others, as metastatic in nude or severe combined immunodeficiency mice. These observations are important because they indicate that the detection of CXCR4 expression in cell lines and tissue sections cannot be taken as indicating the involvement of CXCR4 in the pathologic process being investigated. In the case of breast cancer, although our data and those of others indicate expression of CXCR4 throughout cell lines and normal and pathologic tissue, our results clearly show that functional CXCR4 may only be associated with the metastatic process.

Although the phenomenon of nonfunctional expression of chemokine receptors is not common in the literature, it has been previously described for a subset of NKT cells displaying unique chemotactic responses (36). In a recent study, Trentin et al. also reported differential function of CXCR4 and other chemokine receptors in non–Hodgkin lymphomas. Their findings show expression of nonresponsive CXCR4 in normal B cells (with respect to chemotaxis and Ca^{2+} mobilization) and fully functional receptors in leukemic cells (37). Nonfunctional CXCR4 was also found on cerebellar granule cells, in contrast to glial cells and cortical neurons, which responded to CXCL12 stimulation in terms of chemotaxis and Ca^{2+} mobilization (38). Differences in CXCR4 functionality were also shown in normal human hematopoietic cells with high expression levels of the receptor (25). These findings, together with our observations, strongly point to the existence of distinct mechanisms regulating the activation of CXCR4, which may be cell type–dependent. It is plausible to hypothesize that function, and not the expression of chemotactic receptors, CXCR4 in particular, is regulated by specific cellular processes, such as differentiation and transformation among others. In the case of cancer progression, it is possible that cells which acquire functional chemokine receptors receive selective advantages specifically at distant sites of colonization.

We have examined a range of points in the signaling pathway downstream of CXCR4 and discovered a difference between

nonmetastatic and metastatic cell lines at the level of G protein subunit coupling. Chemokine receptors are generally, although not exclusively, coupled to the G_i subclass of G proteins (3). Indeed, in responsive cells, CXCL12-induced actin polymerization and cell migration were inhibited by prior treatment of the metastatic cells with pertussis toxin, an inhibitor of $G\alpha_i$ (data not shown). Analysis of the CXCR4-mediated signaling events downstream of the G protein α and $\beta\gamma$ subunits revealed that the receptor function in nonresponsive cells is blocked at the level of G protein activation. We found that whereas $G\alpha_i$ and $G\beta$ subunits interact with CXCR4 in both noninvasive and metastatic breast cancer cells, the dissociation of both subunits from the receptor on stimulation with CXCL12 occurred only in the responsive, metastatic cells. Furthermore, $G\alpha_i$ and $G\beta$ could be coprecipitated only from the cells with functional CXCR4 receptors. This novel finding indicates that in noninvasive cells with nonfunctional CXCR4, $G\alpha_i$ and $G\beta\gamma$ do not form the functional heterotrimeric structure which is critical for GDP to GTP transfer, and thus, activation of signaling pathways downstream of G proteins (39). Although at this point in time, no further insight into the molecular mechanism involved is available, the results of recent studies suggest that various α, β, and γ subunits form preferred G protein heterotrimers which in turn form complexes with GPCRs that are specific for these trimers (40). Therefore, lack of trimer formation in the noninvasive cells may be due to the expression of "incompatible" α and $\beta\gamma$ subunits in those cells. It is noteworthy that the family of heterotrimeric G proteins consist of 27 α, 5 β, and 14 γ subunits, which leads to a very high number of possible $\alpha\beta\gamma$ subunit combinations of varying affinity for a multitude of GPCRs (41). Another plausible explanation for the inability of $G\alpha$ and $G\beta\gamma$ subunits to form stable complexes in selective cell lines may be the expression of one or more inhibitory molecules. Of relevance, Soriano et al. recently found that SOCS3 up-regulation by cytokines led to functional inactivation of CXCR4 via blockade of the $G\alpha_i$ pathway (42). Whereas these previous studies raise some insight into our observations, determination of the precise molecular basis underlying CXCR4 blockade in the noninvasive cells will require further experimentation which is currently under way.

Although the major focus of the present study was on CXCR4, the question of whether other GPCRs are similarly regulated in these cell lines is still open. Interestingly, we and others (26) observed a distinction between nonmetastatic and metastatic cells in terms of their ability to respond to the lysophosphatidic acid precursor, phosphatidic acid. Lysophosphatidic acid has been implicated in a host of important biological and pathologic effects, including cancer progression (43). This raises the possibility that other GPCRs, including other chemokine receptors, may be subject to the same control mechanism.

In summary, our data provide several important and novel observations with respect to the acquisition of the invasive phenotype in breast and potentially other cancers (we have made similar observations in colon cancer cell lines; data not shown). Our results show that the level of CXCR4 expression may not be an adequate marker of metastatic potential. Indeed, our findings imply that there is a specific mechanism in place to prevent the activation of CXCR4 in noninvasive cells, which is potentially altered during the process of cellular progression to the more invasive metastatic phenotype. Identification of specific molecular intermediates participating in the transition of the CXCR4 chemokine receptor from a nonactive to a functional status may offer novel targets for the therapeutic intervention and/or early detection and prevention of metastatic breast disease, the most common and severe malignancy in women.

Acknowledgments

Received 5/12/2005; revised 12/30/2005; accepted 2/8/2006.

Grant support: National Health and Medical Research Council of Australia.

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Jane D. Holland, Marina Kochetkova, Chareeporn Akekawatchai, et al.

Cancer Res 2006;66:4117-4124.