CXCL-12/Stromal Cell–Derived Factor-1α Transactivates HER2-neu in Breast Cancer Cells by a Novel Pathway Involving Src Kinase Activation

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Abstract

Experimental evidence suggests that CXCR4, a G_i protein–coupled receptor for the ligand CXCL12/stromal cell–derived factor-1α (SDF-1α), plays a role in breast cancer metastasis. Transactivation of HER2-neu by G protein–coupled receptor activation has been reported as a ligand-independent mechanism of activating tyrosine kinase receptors. We found that SDF-1α transactivated HER2-neu in the breast cancer cell lines MDA-MB-361 and SKBR3, which express both CXCR4 and HER2-neu. AMD3100, a CXCR4 inhibitor, PKI 166, an epidermal growth factor receptor/HER2-neu tyrosine kinase inhibitor, and PP2, a Src kinase inhibitor, each blocked SDF-1α–induced HER2-neu phosphorylation. Blocking Src kinase, with PP2 or using a kinase-inactive Src construct, and inhibiting epidermal growth factor receptor/HER2-neu signaling with PKI 166 each inhibited SDF-1α–stimulated cell migration. We report a novel mechanism of HER2-neu transactivation through SDF-1α stimulation of CXCR4 that involves Src kinase activation. (Cancer Res 2005; 65(15): 6493-7)

Introduction

HER2-neu (ErbB2) is recognized as an indicator of poor prognosis in breast cancer, and substantial evidence exists to suggest that signaling through this receptor contributes to malignant progression. Unlike other members of this receptor family [HER1 (ErbB1 or epidermal growth factor receptor, EGFR), HER3 and HER4] that bind specific ligands [the epidermal growth factor receptor (EGF)-related peptide growth factors] and form heterodimers with HER-2neu, HER2-neu does not seem to have a ligand (1). Increasing evidence suggests that the primary function of HER2-neu is that of a coreceptor (2). Transactivation of EGFR and HER2-neu by endothelin-1, thrombin, and lysophosphatidic acid through activation of seven-transmembrane G protein–coupled receptors has been reported as a ligand-independent mechanism in various types of cancer cells (3). In several studies, the phosphorylation of EGFR by activation of certain G protein–coupled receptors was found to depend on Src kinase activity (4). Chemokine receptors are also members of the G protein–coupled receptor family, which initiate chemotactic and growth signals following interaction with their ligands. Stimulation of CXCR-1/2 chemokine receptors by interleukin-8 has been shown to induce transient phosphorylation of EGFR in ovarian cancer cells, causing rapid activation of the p44/42 mitogen-activated protein kinase (5). Another chemokine receptor, CXCR4, a pertussis toxin–sensitive, G_i protein–coupled receptor for CXCL12/stromal cell–derived factor-1α (referred to as SDF-1α), has recently been shown to have an important role in breast cancer metastasis (6). Given what has been reported on G protein–coupled receptor transactivation, we investigated whether SDF-1α-CXCR4 interactions would increase HER2-neu signaling. We report in this study that SDF-1α induced HER2-neu tyrosine kinase transactivation in breast cancer cells, and that this transactivation involved the activation of Src kinase.

Materials and Methods

Cell culture and reagents. Breast cancer cell lines MDA-MB-361, SKBR3, T47D, and MCF-7 were obtained from the American Type Culture Collection (Manassas, VA). Human recombinant SDF-1α was purchased from R&D Systems (Minneapolis, MN); EGF, pertussis toxin and AMD3100 were purchased from Sigma Chemical Co. (St. Louis, MO); PP2 was purchased from Calbiochem (La Jolla, CA). PKI 166 was provided by Novartis Pharmaceutical (through Dr. I.J. Fidler, University of Texas M.D. Anderson Cancer Center).

Immunohistochemistry for CXCR4 and immunofluorescence staining for CXCR4 and HER2-neu. Primary tumor tissue samples of invasive ductal carcinoma were obtained after informed consent, and approval from institutional ethics committees. Deparaffinized slides of tissues were incubated with mouse anti-human CXCR4 monoclonal antibody (44173.111, IgG_{2b}, R&D Systems) at 1:150 dilution for 18 hours at 4°C. Color was developed with diaminobenzidine after incubation with a rat anti-mouse-IgG_{2b}-horseradish peroxidase (Serotec, Inc., Raleigh, NC). CXCR4 expression was considered high when >50% of tumor cells showed strong cytoplasmic staining.

For immunofluorescence double staining, samples were incubated with the primary antibody as described above, and then with goat anti-mouse antibody conjugated to Alexia-488-FITC (Molecular Probes, Eugene, OR) at 1:400 dilution. The sections were then incubated with rabbit anti-human HER2-neu (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution, then with goat anti-rabbit antibody conjugated with Alexia-549 (Molecular Probes) at 1:600 dilution at room temperature for 1 hour. Immunofluorescence staining of MDA-MB-361 cells cultured on glass slides, then fixed in acetone, used the same primary antibodies and Cy5-conjugated anti-mouse IgG, and Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Confocal microscopy was used to examine the localization of CXCR4 and HER2-neu. As negative controls for all staining, replicate samples were incubated with protein-blocking solution instead of primary antibodies.

The HER2-neu-expressing cell lines MDA-MB-361 and SKBR3 were analyzed for CXCR4 and HER2-neu expression by flow cytometry using

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anti-HER2-neuFITC and anti-human CXCR4CyChrome and the corresponding isotypic monoclonal control antibodies (BD Biosciences, San Jose, CA).

Quantitative real-time PCR. Total RNA was isolated using TriReagent (Sigma) and reverse-transcribed with random primers from the high capacity cDNA archive kit (Applied Biosystems, Foster City, CA). cDNA was amplified in duplicate samples using the ABI 7000 sequence detection system for the expression of CXCR4 and 18S using predeveloped TaqMan assay reagents (Applied Biosystems) following the manufacturer's recommended amplification procedure. Results were recorded as mean threshold cycle, and relative expression was determined using the comparative threshold cycle method, using human placenta RNA (Promega, Madison, WI) as a calibrator sample.

Immunoblot analysis for HER2-neu, epidermal growth factor receptor, Src, extracellular signal-regulated kinase-1/2, and Akt phosphorylation. Breast cancer cells were plated in culture plates and grown to 50% to 80% confluence. The cultures were serum-starved for 48 hours then stimulated with SDF-1α (10-50 ng/mL) after treatment with AMD3100 (10 μmol/L), pertussis toxin (0.25 μg/mL), PKI 166 (0.5 μmol/L), or PP2 (1 and 2.5 μmol/L) in different combinations. Lysates were prepared from the cells, and aliquots of protein were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were hybridized with antibodies in 5% bovine serum albumin (BSA) in TBS and 0.1% Tween 20. Antibodies against phospho-HER2/ErbB2 (Tyr^{1248}), HER2/ErbB2, phospho-EGFR (Tyr^{845}), EGFR, phospho-extracellular signal–regulated kinase (ERK)-1/2, ERK1/2, phospho-Akt, Akt and phospho-Src (Tyr^{416}) were purchased from Cell Signaling Technology (Beverly, MA); antibodies against phospho-Src (Tyr^{215}) and pan-Src were purchased from Biosource (Camarillo, CA). The secondary antibody was horseradish peroxidase–conjugated goat anti-rabbit (Amersham Corp., Arlington Heights, IL), which was detected with the Amersham enhanced chemiluminescence system, following the manufacturer's recommended procedure. Immunoreactive bands were quantified by densitometry using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Migration assays. SDF-1α-induced migration was measured using 24-well cell culture inserts with membranes with 8 μm pores (Becton Dickinson, Bedford, MA). Breast cancer cells were suspended in serum-free medium with 0.1% BSA and 1×10^5 cells in 0.5 mL plated in the top part of the insert. PP2 (2.5 μmol/L), PKI 166 (0.5 μmol/L), or DMSO (0.01% v/v) was added to the cell suspension; DMSO was the solvent for stock solutions of the inhibitors. The inserts were placed in wells containing serum-free medium with 0.1% BSA, with or without SDF-1α. After incubation at 37°C for 24 hours, residual cells were wiped off the top of the membranes with cotton swabs, and migrated cells on the underside of the membranes were fixed and stained using the HEMA-3 kit (Fisher Diagnostics, Middletown, VA). Cells were counted in 10 microscope fields of each filter, from three inserts per experimental condition.

SDF-1α-induced migration was measured in MDA-MB-361 cells infected with adenovirus expressing a kinase inactive Src (7), or with a control adenovirus Ad PU1 (8). Cells plated in 35 mm plates were infected with adenovirus at the indicated multiplicity of infection, as described previously (8), then harvested after 48 hours of incubation for the migration assays.

Results

The expression of CXCR4 and HER2-neu in MDA-MB-361 and SKBR3 breast cancer cells was evaluated with double-staining flow cytometry. MDA-MB-361 showed the highest expression of CXCR4 and HER2-neu (Fig. 1*A*). We also examined expression in primary tumor specimens from patients with stage II or stage III breast cancer by immunohistochemistry. Four of 24 patients (17%) showed high CXCR4 and HER2-neu expression. Expression of

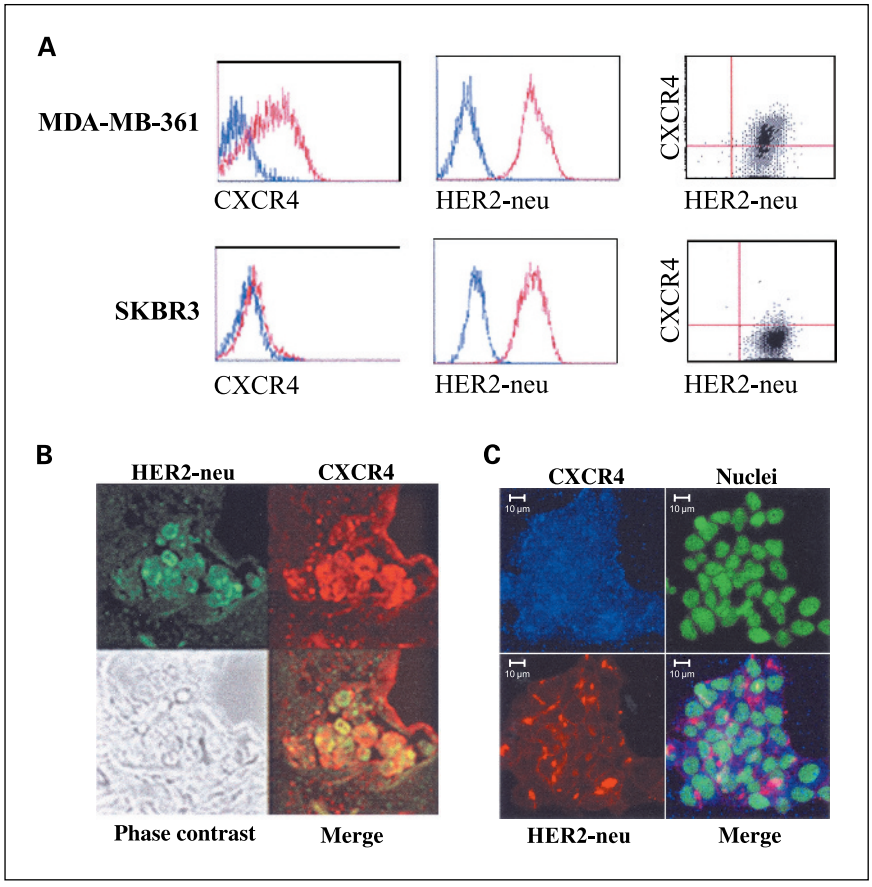

Figure 1. Coexpression of CXCR4 and HER2-neu in breast cancer cells. *A,* flow cytometric analysis shows coexpression of CXCR4 and HER2-neu in MDA-MB-361 and SKBR3, cells with the high CXCR4 and HER2-neu expression. Blue lines, cells incubated with control IgG; red lines, cells incubated with the indicated antibody. *B,* confocal microscopy series of a representative slide showing CXCR4 and HER2-neu coexpression in cells from a primary breast tumor by double immunofluorescence staining. HER2-neu expression was indicated by green fluorescence, and CXCR4 by red fluorescence. Bottom right, yellow fluorescence shows coexpression of the proteins (180× original magnification). *C,* confocal microscopy showing CXCR4 and HER2-neu coexpression in MDA-MB-361 cells *in vitro.* Diffuse and punctate CXCR4 expression is shown by blue fluorescence and HER2-neu expression is shown by red fluorescence. Nuclei were stained with Cytox green (green fluorescence).

CXCR4 and HER2-neu in breast cancer cells in a sample from a patient who developed bone and lung metastases is shown in Fig. 1*B*. Confocal microscopy of MDA-MB-361 cells showed coexpression but not colocalization of HER2-neu and CXCR4 (Fig. 1*C*). This result was the same whether or not the cells were stimulated with SDF-1α (data not shown).

MDA-MB-361 cells were used for *in vitro* studies of responses to SDF-1α as these cells had the highest expression of CXCR4 of a panel of breast cancer cell lines, measured by flow cytometry (Fig. 1*A*) and real-time PCR (data not shown); the PCR results were consistent with a previous report using some of the same cell lines (6). Stimulation with SDF-1α increased the phosphorylation of HER2-neu and Akt in serum-starved cells (Fig. 2*A* and *E*). To show whether the activation of HER2-neu occurred through G protein–dependent mechanisms, MDA-MB-361 cells were preincubated with pertussis toxin (0.25 μg/mL) for 18 hours; this inhibited the SDF-1α-induced HER2-neu phosphorylation (Fig. 2*A*).

To investigate the participation of Src kinase activity in SDF-1α-induced HER2-neu phosphorylation, we examined the phosphorylation of Src in lysates of SDF-1α-treated MDA-MB-361 cells. SDF-1α stimulation increased phosphorylation of Src-Tyr^{416} and Src-Tyr^{215} (Fig. 2*B*), in parallel with induction of HER2-neu phosphorylation. Pretreatment with pertussis toxin inhibited the SDF-1α-induced Src phosphorylation. In separate experiments, treating MDA-MB-361 cells with the Src kinase inhibitor PP2 for 1 hour before stimulation with SDF-1α led to inhibition of SDF-1α-induced HER2-neu phosphorylation (Fig. 2*C*). To confirm the specificity of the action of the SDF-1α and CXCR4, we added AMD3100, an inhibitor of ligand binding to CXCR4 (9), which inhibited the SDF-1α/CXCR4-mediated transactivation of HER2-neu (Fig. 2*D*).

SDF-1α-induced transactivation of receptor tyrosine kinases was shown in SKBR3 cells (Fig. 3*A* and *B*), in which both HER2-neu and EGFR showed increased phosphorylation following treatment with the chemokine. These cells express moderate levels of CXCR4, compared with MDA-MB-361 cells and both EGFR and HER2-neu (10). Stimulation with SDF-1α also increased the phosphorylation of ERK1/2 (Fig. 3*C*). When SKBR3 cells were stimulated with both SDF-1α and EGF, the level of HER2-neu phosphorylation was more than that in cells treated with either ligand alone. Addition of the tyrosine kinase inhibitor PKI 166 inhibited the HER2-neu phosphorylation induced in cells stimulated with either EGF or

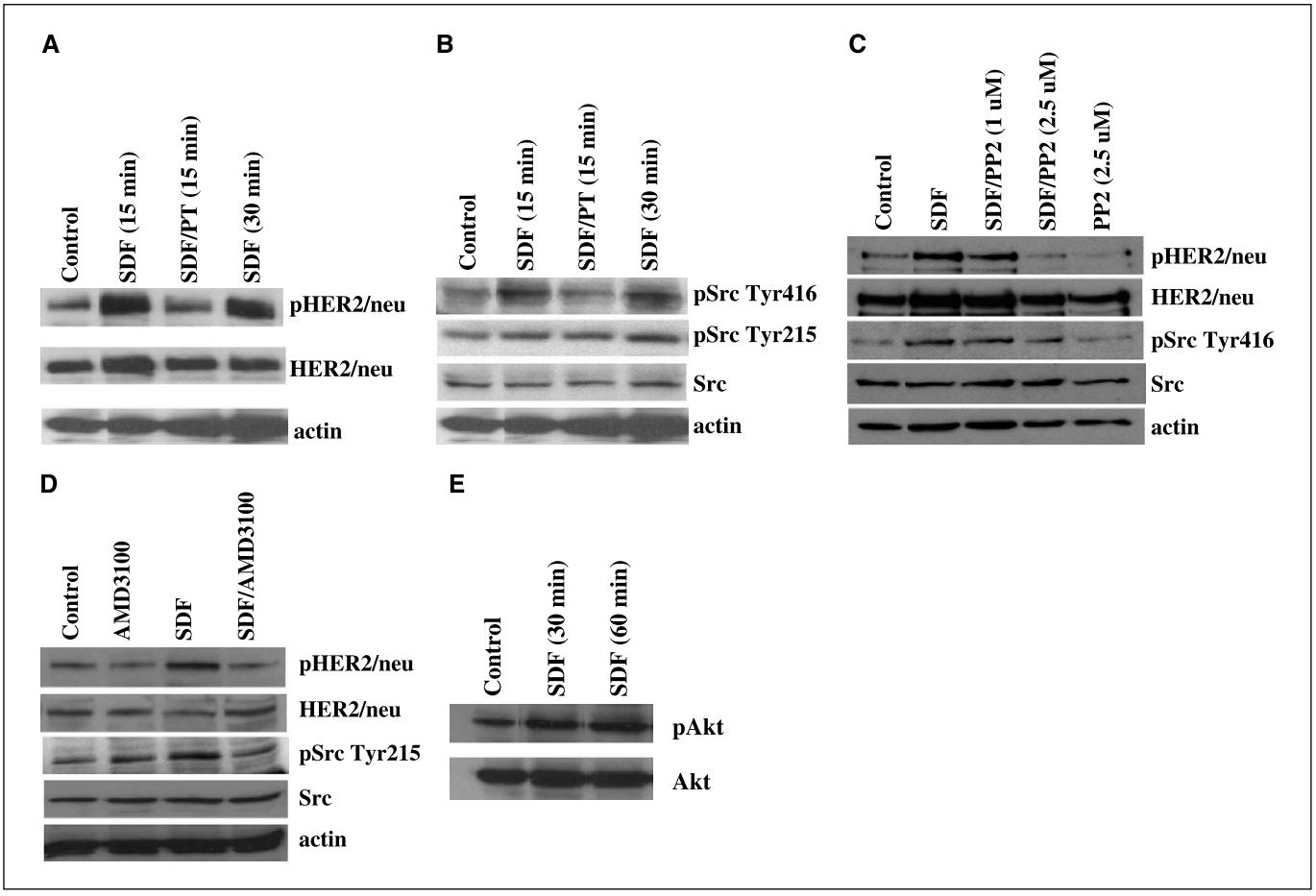

Figure 2. *A,* the effect of pertussis toxin (PT), G_i protein inhibitor, on SDF-1α-induced HER2-neu phosphorylation in MDA-MB-361 breast cancer cells. Western blot analysis shows that phosphorylation of HER2-neu by SDF-1α (10 ng/mL) was inhibited by pretreatment with pertussis toxin (0.25 μg/mL). SDF-1α increased the phosphorylation of HER2-neu by a factor of 7 and 4.1, at 15 and 30 minutes incubation, respectively, based on densitometric analysis; pretreatment with pertussis toxin abrogated this response. *B,* phosphorylation of Tyr^{416} and Tyr^{215} of Src kinase upon stimulation with SDF-1α in MDA-MB-361 cells. The addition of SDF-1α increased the phosphorylation of Src Tyr^{416} by a factor of 6.5, and Tyr^{215} by a factor of 2.4, compared with control. *C,* pretreatment of MDA-MB-361 cells with 1 or 2.5 μmol/L PP2, a Src kinase inhibitor, inhibited SDF-1α-induced phosphorylation of HER2-neu and Src (Tyr^{416}) in MDA-MB-361 cells. *D,* pretreatment of MDA-MB-361 cells with 10 μmol/L AMD3100, a CXCR4 inhibitor, for 1 hour inhibited SDF-1α-induced phosphorylation of HER2-neu and Src (Tyr^{416}). *E,* the addition of SDF-1α increased the phosphorylation of Akt (Ser^{473}) by a factor of 1.6 to 2 in MDA-MB-361 cells after 30 and 60 minutes, respectively.

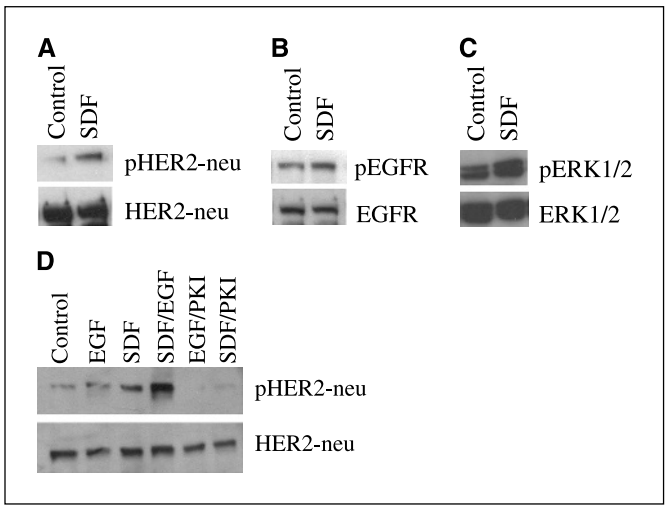

Figure 3. Immunoblot analysis of lysates from SKBR3 cells showing the SDF-1α-induced increases in phosphorylation of HER2-neu (*A*), by a factor of 6.4; EGFR by a factor of 3.4 (*B*), ERK1/2 by a factor of 2.8 (*C*). *D,* EGF and SDF-1α increased phosphorylation of HER2-neu by a factor of 4 and 3, respectively, and the combination of the ligands results in increased phosphorylation by a factor of 13. Addition of PKI 166 (0.5 μmol/L) inhibited phosphorylation induced by either EGF or SDF-1α.

SDF-1α (Fig. 3*D*). In two additional breast cancer cell lines, T47D and MCF-7, which express moderate levels of CXCR4 and relatively less HER2-neu, stimulation with SDF-1α did not induce HER2-neu phosphorylation (data not shown).

A biological response stimulated by SDF-1α was shown in migration assays. SDF-1α promoted the migration of MDA-MB-361 in a dose-dependent manner (Fig. 4*A*). The SDF-1α-induced increase in migration was completely inhibited by the Src kinase inhibitor PP2, or the HER2-neu/EGFR tyrosine kinase inhibitor PKI 166 (Fig. 4*B*), using concentrations of the inhibitors that blocked SDF-1α-induced HER2-neu phosphorylation. Infection of cells with a kinase-inactive Src adenovirus construct substantially reduced the baseline migration of cells and abrogated SDF-1α-induced migration, whereas the migration of cells infected with control adenovirus was significantly increased by the addition of SDF-1α (Fig. 4*C*).

Discussion

This study shows the expression of CXCR4 and HER2-neu in breast cancer specimens and cell lines, and describes a novel mechanism of HER2-neu transactivation induced by SDF-1α/CXCR4 interactions in HER2-neu- and CXCR4-expressing breast cancer cells through a pertussis toxin–sensitive, G protein–dependent signal transduction mechanism. Previous reports (11, 12) similarly showed that stimulation of chemokine receptors resulted in phosphorylation of certain tyrosine kinase receptors, triggered by $G_{i\alpha}$ protein–dependent and -independent signaling pathways, despite the lack of intrinsic tyrosine kinase activity in the chemokine receptors.

Transactivation of EGFR, unlike that of HER2-neu, is a well-documented pathway (3). A recent report suggested crosstalk between EGFR and CXCR4 based on the observation that an inhibitor of the EGFR kinase (AG1478) blocked both SDF-1α-dependent proliferation and ERK1/2 activation (13). Consistent with this report, our results show EGFR transactivation in SKBR3 cells, which express EGFR and HER2-neu, following stimulation with SDF-1α. The few studies addressing HER2-neu receptor kinase transactivation by G protein–coupled receptor used normal prostate stromal cells, or head and neck cancer cells (14, 15). Most previous studies have focused on the mechanisms underlying EGFR transactivation, and members of the Src family of cytoplasmic tyrosine kinases were examined as potential mediators. Src has been reported to induce EGFR tyrosine phosphorylation after stimulation by various different ligands (4). In contrast to EGFR, less is known about the relationship between HER2-neu and c-Src. HER2-neu has been shown to associate with the SH_2 domain of c-Src in a tyrosine phosphorylation–dependent manner, raising the possibility that phosphorylation of HER2-neu increases Src kinase activity as a downstream signaling pathway (10). Transgenic mouse

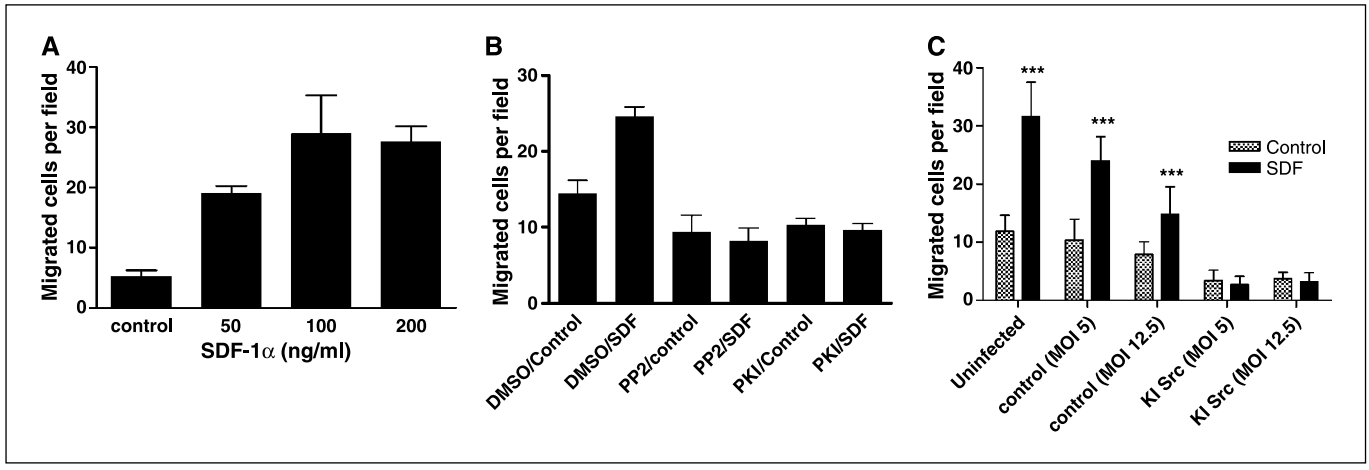

Figure 4. *A,* migration of MDA-MB-361 was stimulated by SDF-1α (50-200 ng/mL) in medium with 0.1% BSA. Cells were plated in the upper chamber of culture well inserts with membranes with 8 μm pores, and with SDF-1α in the lower chamber. After 24 hours of incubation, the membranes were fixed and stained, and cells counted on the underside of the membranes. *B,* the addition of PP2 (2.5 μmol/L) or PKI 166 (0.5 μmol/L) significantly inhibited the migration of cells towards SDF-1α ($P < 0.0001$, Student's *t* test), compared with the control condition, cells treated with 0.01% DMSO. The results shown are the mean and SD from 10 fields counted in triplicate filters, and are representative of repeated experiments. *C,* infection of MDA-MB-361 with adenovirus expressing kinase-inactive Src (KI) abrogated the SDF-1α-induced migration. The cells were incubated for 48 hours with 5 or 12.5 multiplicity of infection of the kinase inactive virus or control adenovirus before plating in culture well inserts. ***, significant increase ($P < 0.0001$, Student's *t* test) in numbers of cells migrating to the underside of the filter in the presence of SDF-1α (100 ng/mL), seen only in uninfected, or control virus–infected cells, but not those expressing kinase-inactive Src.

mammary tumors expressing mutant-activated Neu exhibit a correlative increase in c-Src activity (16). Our results are evidence that Src kinase activation is a component of HER2-neu activation. We showed that SDF-1α/CXCR4 signaling activated Src kinase in breast cancer cells, findings similar to those reported recently with oral squamous cell carcinoma cells (17). As in previous reports, we found that the increase in Src kinase phosphorylation at Tyr^{416} and Tyr^{215} residues correlated with SDF-1α-induced HER2-neu phosphorylation. Even though the biological significance of induction of Src Tyr^{215} is not well understood, ligands such as platelet-derived growth factor and heregulin were shown to induce phosphorylation of this residue (18). Inhibition of Src kinase abolished the SDF-1α-induced HER2-neu phosphorylation, as well as the increased motility of SDF-1α-stimulated MDA-MB-361 cells; the latter was shown using a kinase-inactive Src construct and the PP2 kinase inhibitor. Therefore, the induction of Src kinase by SDF-1α might be important for activating downstream signaling pathways involved in the migration and invasion of cancer cells. SDF-1α has been shown to promote migration and chemoinvasion of breast cancer cells by additional mechanisms. Fernandis et al. showed SDF-1α-induced activation of phosphatidylinositol-3-kinase and focal adhesion complex components (19). Lee et al. showed the involvement of phosphoinositide-3-kinase/Akt and calcium-mediated signaling in SDF-1α-induced breast cancer migration through endothelial cell monolayers (20). Both of these studies used CXCR4-expressing breast cancer cell lines (MDA-MB-231 and DU4475) that do not express high levels of HER2-neu. Thus, different signaling pathways may be activated in breast cancer cells that express CXCR4, but differ in ErbB receptor expression.

We showed SDF-1α-induced HER2-neu phosphorylation in breast cancer cells that express relatively high levels of HER2-neu and either high or moderate CXCR4 expression (MDA-MB-361 and SKBR3, respectively), but not in breast cancer cells with moderate levels of expression, such as T47D and MCF-7. This may suggest that the abundance of HER2-neu may be more critical than that of CXCR4 for SDF-1α-induced transactivation. Confocal microscopy and coimmunoprecipitation of lysates from MDA-MB-361 cells (data not shown) did not show a physical interaction between the two proteins, implying key roles for other intracellular mediators. However, further studies are needed to understand the molecular mechanisms involved in SDF-1α-induced HER2-neu transactivation, including whether the conserved three–amino acid cytoplasmic domain required for ligand-dependent HER2-neu transactivation (21) is essential for chemokine-stimulated transactivation.

In conclusion, our data suggest a novel mechanism of HER2-neu transactivation, through SDF-1α stimulation of the CXCR4 chemokine receptor that involves Src kinase activation. SDF-1α is expressed in organs that are some of the major sites of breast cancer metastasis (6), and signaling through CXCR4 may contribute to critical pathways that determine the survival, invasion, or growth of disseminated HER2-neu-expressing breast cancer cells. Another observation with significance for breast cancer progression is the finding that HER2-neu could enhance the expression of CXCR4, which was required for metastatic colonization by HER2-neu expressing cells (22). Whether the inhibition of CXCR4-mediated signaling with novel CXCR4 targeting drugs can improve HER2-neu targeted therapies merits further study.

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