Transactivation of CXCR4 by the Insulin-like Growth Factor-1 Receptor (IGF-1R) in Human MDA-MB-231 Breast Cancer Epithelial Cells*

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In the multimolecular environment in tissues and organs, cross-talk between growth factor and G protein-coupled receptors is likely to play an important role in both normal and pathological responses. In this report, we demonstrate transactivation of the chemokine receptor CXCR4 by the growth factor insulin-like growth factor (IGF)-1 by IGF-1-induced cell migration in metastatic MDA-MB-231 cells. The induction of chemotaxis in MDA-MB-231 cells by IGF-1 was inhibited by pretreatment of the cells with pertussis toxin (PTX) and by RNAi-mediated knockdown of CXCR4. Transactivation of the CXCR4 pathway by IGF-1 occurred independently of CXCL12, the chemokine ligand of CXCR4. Neither CXCR4 knockdown nor PTX had any effect on the ability of IGF-1 to activate IGF-1R, suggesting that CXCR4 and G proteins are activated subsequent to, or independently of, phosphorylation of IGF-1 by IGF-1R. Coprecipitation studies revealed the presence of a constitutive complex containing IGF-1R, CXCR4, and the G protein subunits, Gα13 and Gβ, and stimulation of MDA-MB-231 cells with IGF-1 led to the release of Gα13 and Gβ from CXCR4. Based on our findings, we propose that CXCR4 constitutively forms a complex with IGF-1R in MDA-MB-231 cells, and that this interaction allows IGF-1 to activate migrational signaling pathways through CXCR4, Gα13, and Gβ.

The G protein-coupled receptor (GPCR)2 CXCR4 is the receptor for the chemokine CXCL12. Both molecules are essential for life, with genetic deletion in mice of either CXCR4 or CXCL12 resulting in a lethal phenotype (1–3). Activation of CXCR4 by CXCL12 has been implicated in the homeostasis and activation of the immune system, and influences a range of other biological systems under both normal and pathological conditions (4–6). These include angiogenesis (7–9), cell survival (10, 11), and more recently, tumor growth and metastasis (12–14). Indeed, it has recently been shown that CXCR4 is expressed in breast cancer tissues and cell lines, and that CXCL12 is expressed in several target organs of breast cancer metastasis (13). Additionally, treatment of mice with neutralizing Abs against CXCR4 inhibits metastasis in a mouse model of breast cancer, as does RNAi-mediated knockdown of CXCR4 on orthotopically transplanted breast carcinoma cells (12, 13). These data point to an important role for CXCR4 in cancer.

The cellular signal transduction pathways induced by CXCL12 have been well characterized in leukocytes. Interaction of CXCL12 with CXCR4 leads to the release of the G protein subunits Gα and Gβ from intracellular domains of CXCR4. These subunits then bind and activate downstream enzyme systems including phospholipase C, which leads to a transient increase in the level of intracellular Ca2+, and sphingosine 1-kinase (PI3K), which results in activation of Akt and subsequently, cell migration (15–17). In contrast, the role of CXCR4, including characterization of signal transduction mechanisms in cell types other than leukocytes is less well established despite the fact that CXCR4 is expressed in most tissues and organs.

Cross-talk between GPCRs and growth factor receptor tyrosine kinase (RTKs) induced signaling pathways has become increasingly well documented in different cellular systems. For example, EGFR is tyrosine-phosphorylated in response to CCL11, a ligand for the GPCR CCR3, leading to MAP kinase activation and IL-8 production in bronchial epithelial cells (18). In rat aortic vascular smooth muscle cells, both PDGFR and EGFR are phosphorylated by sphingosine 1-phosphate (S1P), a lipid mediator that is a ligand for the S1PR family of GPCRs, leading to activation of effectors downstream of PDGFR and EGFR including Shc, and the p85 regulatory subunit of the class IA PI3K (19).

In contrast, examples of transactivation of GPCRs by RTKs are less abundant, although recently it has been shown that IGF-1 stimulated phosphorylation of CCR5 in MCF-7 cells. Chemotaxis induced by IGF-1 was inhibited by a neutralizing anti-CCL5 antibody, which indicates that transactivation of CCR5 by IGF-1 is indirect, requiring production of a CCR5 ligand (20).

Because investigating interactions between different receptor classes is essential for our understanding of the mechanisms by which cells process multiple signaling inputs, we have examined potential cross-talk in the signal transduction pathways induced following ligation of CXCR4 and IGF-1R. Our data demonstrate the existence of a physical association between IGF-1R, CXCR4, and the G protein subunits, Gα and Gβ in the breast cancer epithelial cell lines, MDA-MB-231. This interaction drives a unidirectional transactivation of CXCR4 and G proteins by IGF-1 leading to cell migration in MDA-MB-231 cells, which is independent of the CXCR4 chemokine ligand, CXCL12. These data indicate the existence of a novel form of transactivation between these two important receptors.

**MATERIALS AND METHODS**

**Cell Lines and Cell Culture Conditions**—Breast cancer cell lines, MCF-7 and MDA-MB-231, were obtained from the American Type Culture Collection. P6 cells (BALB/c3T3 cells overexpressing human IGF-1R) were kindly provided by Professor R. Baserga (Philadelphia, PA). MCF-7 and P6 cells were cultured in Dulbecco’s modified Eagle’s...
medium supplemented with 10% fetal bovine serum whereas MDA-MB-231 cells were in RPMI 1640 with 10% fetal bovine serum, at 37 °C in a 5% CO₂ atmosphere.

Reagents—A hybridoma supernatant containing anti-IGF-1R (7C2 clone) was produced in the Monoclonal Antibody Facility in the School of Molecular & Biomedical Science, The University of Adelaide as described.³ A monoclonal anti-IGF-1R 24-31 (21) was a gift from Dr. Leah Cosgrove (CSIRO, Human Nutrition, Adelaide, South Australia). Monoclonal anti-human CXCR4 antibodies (clone 12G5) were purchased from R&D systems (Minneapolis, MN), and polyclonal CXCR4 antibodies were purchased from Chemicon International Inc. Monoclonal anti-IGF-1R antibodies (clone 2C8), antibodies to Gαs (T-19) and Gβ (M-14) and monoclonal control antibodies IgG (anti-hemagglutinin clone F-7) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescein isothiocyanate-conjugated anti-CXCR4 was from R&D Systems whereas PE-conjugated anti-mouse IgG and horseradish peroxidase-labeled donkey anti-rabbit IgG were purchased from Rockland (Gilbertsville, PA). DELFIA Eu-labeling kit reagents composed of europium-labeled anti-phosphotyrosine PY20 Abs and DELFIA enhancement solution were purchased from PerkinElmer Life Sciences. IGF-1 was obtained from GroPep Pty Ltd (Adelaide, South Australia). CXCL12 was kindly provided by Professor Ian Clark-Lewis (UBC, Vancouver). Pertussis toxin (PTX) was purchased from Sapphire Bioscience, NSW, Australia.

Retroviral-mediated RNAi Knockdown of CXCR4—The shRNA retroviral expression vector was constructed by subcloning the human H1 gene promoter into the self-inactivating pMSCV plasmid. The resultant vector was digested with BglII and HindIII, and the annealed oligos 5'-gagctgTGTGCTATATGTGGCGCTGTtaacaAGACAGACGC-CAACATAGACCCTtttttta-3' and 5'-agctgtaaaaAGTGGTGGCTAT- GTGGCGCTTCGTctaacaagacgacaccc-3' were inserted to produce CXCR4 shRNA-expressing construct. The 21-nucleotide CXCR4 target sites at position 470–490 of human CXCR4 cDNA are indicated in capitals in the oligonucleotide sequences. Previously described oligonucleotides containing specific target sequences for Renilla luciferase were used to produce the expression vector for the negative control (22).

To produce retroviral supernatants, 293T packaging cells were transfected with 10 μg of specific or control expression vectors, 8 μg of pVPack-VSV-G, 8 μg of pVPack-GP (Stratagene), and 60 μl of Lipofectamine 2000 reagent (Invitrogen, Life Technologies, Inc.) in 100-mm tissue culture dishes in Opti-MEM medium (Invitrogen, Life Technologies, Inc.) without fetal calf serum and without antibiotics, essentially as recommended by the supplier. The medium was replaced 16 h later, and virus-containing supernatants were harvested at 48 h post-transfection. Supernatants were filtered through a 0.45-μm Minisart syringe filter (Sartorius AG, Gottingen, Germany), and polybrene (Sigma) was added to a final concentration of 8 μg/ml. MDA-MB-231 cells were plated in a 60-mm tissue culture dish at ~40% confluency, and 24 h later the cell medium was removed before 5 ml of specific or control viral supernatants were added. The supernatant was replaced by cell growth medium after 6 h of infection. The infected cells were then incubated for an additional 24 h at 37 °C before being plated at 1:20 dilution for the selection of individual clones in puromycin (5 ng/ml) -containing media. After 1 week, individual clones were picked and expanded for further analysis.

Immunofluorescent Staining and Flow Cytometric Analysis—Cells were trypsinized and suspended to 5 × 10⁶ cells/ml in staining buffer (phosphate-buffered saline containing 1% BSA and 0.04% sodium azide). After the cells were fixed with 3.7% paraformaldehyde (BDH Laboratory Supplies, Poole, UK) in PBS at room temperature for 10 min, Fc receptors were blocked with purified human IgG (Sigma) (10 μg per 10⁶ of cells) at room temperature for 30 min. The blocked cell suspension (50 μl) was aliquoted to each round bottom tube and incubated each with tested or isotype control Abs at 4 °C for 30 min. For IGF-1R detection, the cells were labeled with hybridoma supernatants and washed with staining buffer, followed by staining with PE-conjugated anti-mouse detection Abs. For CXCR4 detection, the cells were stained with fluorescein isothiocyanate-conjugated anti-CXCR4. The labeled cells were washed with staining buffer followed by phosphate-buffered saline and then detected on a FACScan (BD Australia).

Chemoattract Assay—Chemoattract was measured in a Modified Boyden chamber using polycarbonate filters (8 μm for MDA-MB-231 cells and 12 μm for MCF-7 cells, Neuroprobe, Gaithersburg, MD) coated with 25 μg/ml collagen type I (Sigma) in 10 mM acetic acid. Cell suspensions in serum-free medium (RPMI 1640) containing 0.5% BSA were preincubated with calcine-AM (1 μg/ml of final concentration, Molecular Probes (Eugene, OR) for 30 min before being loaded in the upper chamber (5 × 10⁵ viable cells/well) whereas the lower chamber contained various concentrations of CXCL12 and/or IGF-1. After the chamber was incubated at 37 °C (4 h for MDA-MB-231 cells and 6 h for MCF-7 cells), membranes were taken out, and cells on the upper surface were removed. The transmigrated cells on the lower surface were measured by their fluorescent intensity using Molecular Imager® Fx (Bio-Rad) and expressed as a migration index, representing the fluorescent signals of stimulated cells compared with those of non-stimulated cells.

Kinase Receptor Activation Assay (KIRA)—The KIRA assay was performed with modifications to a previously described protocol (23–25). Cells (2.5 × 10⁵ cells/well) were cultured in 24-well flat bottom culture plates overnight then placed in serum-free medium (RPMI 1640 with 0.5% BSA) for 4 h before being incubated with various concentrations of CXCL12 or IGF-1. After 10 min of stimulation, cell lysates were prepared by addition of lysis buffer (20 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 10 mM EGTA, 10% glycerol, and 1% Triton X-100) containing 2 mM Na₃VO₄ and protease inhibitor solution (Sigma) and then transferred to 96-well white polystyrene plates (Greiner Bioone, Germany) which were precoated with anti-IGF-1R antibodies (mAb 24-31) diluted in 50 mM NaHCO₃/Na₂CO₃, pH 9.6 (0.25 μg/well), and blocked with 0.5% BSA in TBST. After overnight incubation, the plates

³ M. Keyhanfar, B. Forbe, L. Cosgrove, G. Booker and J. Wallace, manuscript in preparation.
were washed with TBST, and the activated receptor complex formed was detected by incubating with europium-labeled anti-phosphotyrosine PY20 (10 ng/well) for 2 h at room temperature. After washing with distilled water, the plates were added with DELFIA enhancement solution (100 μl/well). Time-resolved fluorescence was then measured using 340-nm excitation and 610-nm emission filters on a BMG Lab Technologies Polarstar™ Fluorometer.

**Immunoprecipitations and Western Blot Analyses**—Cells were lysed at 4 °C for 20 min in Triton-lysis buffer (20 mM HEPES, 150 mM NaCl, 1.5 MgCl₂, 1 mM EGTA, 10% glycerol, and 1% Triton X-100) containing 2 mM Na₃VO₄, 50 mM NaF, 10 mM phenylmethylsulfonyl fluoride, and protease inhibitor (1:100, Sigma-Aldrich). The lysates were centrifuged at 1,400 rpm at 4 °C for 10 min to remove insoluble materials, and the supernatants were collected. Total protein was determined using the BCA assay (Pierce). For immunoprecipitation, the lysates (1 mg of total proteins) were incubated with 1 μg of either anti-IGF-1R 2C8, anti-IGF-1R 2C8, or anti-CXCR4/300. The activated receptor complex formed was detected by incubating with europium-labeled anti-phosphotyrosine PY20 (10 ng/well) for 2 h at room temperature. After washing with distilled water, the plates were added with DELFIA enhancement solution (100 μl/well). Time-resolved fluorescence was then measured using 340-nm excitation and 610-nm emission filters on a BMG Lab Technologies Polarstar™ Fluorometer.

**TABLE ONE**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% Cells positive</th>
<th>Geometric mean</th>
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</thead>
<tbody>
<tr>
<td>CXCR4 expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B300⁷</td>
<td>49.63 ± 23.24⁷</td>
<td>38.03 ± 6.59</td>
</tr>
<tr>
<td>MCF-7</td>
<td>94.74 ± 4.01</td>
<td>65.28 ± 17.08</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>95.71 ± 1.10</td>
<td>77.32 ± 18.77</td>
</tr>
<tr>
<td>IGF-1R expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P6⁶</td>
<td>98.05 ± 1.38</td>
<td>154.72 ± 35.96</td>
</tr>
<tr>
<td>huIRB⁺</td>
<td>1.41 ± 0.36</td>
<td>16.11 ± 4.15</td>
</tr>
<tr>
<td>MCF-7</td>
<td>98.85 ± 0.69</td>
<td>26.75 ± 7.12</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>22.65 ± 9.34</td>
<td>13.10 ± 3.47</td>
</tr>
</tbody>
</table>

⁷ B300: positive control cell line for huCXCR4.
⁶ P6: positive control cell line for huIGF-1R.
⁺ huIRB: positive control cell line for human insulin receptor B.

**FIGURE 2.** The effect of CXCL12 and IGF-1 on chemotaxis of MCF-7 and MDA-MB-231 cell lines. The ability of the cells to migrate in response to CXCL12 and IGF-1 was tested using a modified Boyden chamber as described under “Materials and Methods.” The migration index represents the fluorescent signals of stimulated cells compared with those of non-stimulated cells. A, the response of MCF-7 and MDA-MB-231 cells to CXCL12 or IGF-1. B, the response of MDA-MB-231 cells to combinations of CXCL12 and IGF-1. All panels are expressed mean ± S.E. of migration index from at least three separate experiments each performed in triplicate. Asterisks indicate statistically significantly different from control values (Student’s unpaired t test) at *, p < 0.05; **, p < 0.005; #, p < 0.0001.
anti-human CXCR4 12G5, or IgG control antibodies, monoclonal anti-HA, at 4 °C overnight. Immunocomplexes were precipitated with protein G-coated microbeads at 4 °C for 1 h and purified on magnetic microcolumns (Miltenyi Biotec). The bound proteins were eluted from the column in preheated sample buffer (50 mM Tris-HCl pH 6.8, 50 mM dithiothreitol, 1% SDS, 0.005% bromphenol blue, and 10% glycerol). For whole lysate sample preparation, the lysates (50 µg of total proteins/well) were denatured by boiling for 5 min in sample buffer. The immunoprecipitates and whole lysates were then subjected to 15% SDS-PAGE, transferred to nitrocellulose membrane (Hybond™ P, Amersham Biosciences), and analyzed by Western blotting. The transferred membranes were blocked with 1% casein (Roche Applied Science) and incubated with primary Abs (1:1000 of polyclonal anti-CXCR4, 1:500 of anti-Gα and -Gβ) followed by horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:1000). Membranes were visualized by enhanced chemiluminescence (Amersham Biosciences).

RESULTS

Expression of CXCR4 and IGF-1R on the MCF-7 and MDA-MB-231 Breast Cancer Cell Lines—Two breast cancer cell lines, the non-metastatic MCF-7 and metastatic MDA-MB-231, were characterized in terms of the expression and function of CXCR4 and IGF-1R. Flow cytometric analysis showed expression of both CXCR4 and IGF-1R on both cell types (Fig. 1 and TABLE ONE). MCF-7 cells expressed both receptors at high levels (94.74 ± 4.01% of positive cells with a geometric mean of 65.28 ± 17.08% for CXCR4 and 98.85 ± 0.69% of positive cells with a geometric mean of 26.75 ± 7.12% for IGF-1R) whereas MDA-MB-231 cells showed a high level of CXCR4 (95.71 ± 1.10% positive cells with a geometric mean of 77.32 ± 18.77%) and a lower level of IGF-1R expression (22.65 ± 9.34% of positive cells with a geometric mean of 13.10 ± 3.47%). Western blot analysis also confirmed the expression of CXCR4 and IGF-1R in both cell lines (data not shown).

Chemotactic Response of MCF-7 and MDA-MB-231 Cells to CXCL12 and IGF-1—The function of CXCR4 and IGF-1R on MCF-7 and MDA-MB-231 cells was examined by testing the migrational response of the cells to respective ligands, CXCL12 and IGF-1, using a modified Boyden chamber chemotaxis assay. Interestingly, even though CXCR4 expression was similar on MDA-MB-231 and MCF-7 cells, only the former responded to CXCL12 (Fig. 2A). In contrast, both cell lines migrated in response to IGF-1. However, in keeping with the lower level of IGF-1R expression, IGF-1-induced chemotaxis of MDA-MB-231 cells was lower than that observed in MCF-7 cells. Additional experimentation was conducted in MDA-MB-231 cells to determine the effect of combined stimulation with CXCL12 and IGF-1. The results of these experiments indicated an additive effect of those ligands on chemotaxis of MDA-MB-231 cells (Fig. 2B).

CXCL12 Does Not Transactivate IGF-1R on MDA-MB-231 Cells—To investigate potential cross-talk between CXCR4 and IGF-1R-induced signal transduction pathways, we initially determined whether there is cross-activation of IGF-1R by CXCL12 on MDA-MB-231 cells. Because activation of IGF-1R by IGF-1 leads to the rapid formation of a tyrosine-phosphorylated receptor complex, a KIRA assay was performed to compare the levels of IGF-1R activation induced by CXCL12 and IGF-1. Preliminary experiments indicated that in P6 (positive control), MCF-7 and MDA-MB-231 cells, maximal levels of activated IGF-1R complex formed after stimulation with 10 nM IGF-1 at 10 min (data not shown). Therefore, in subsequent experiments, the cells were stimulated with various concentrations of IGF-1 and CXCL12 for 10 min. The results of these experiments indicate that IGF-1 dose-dependently induced the activation of IGF-1R in all three cell lines (Fig. 3A) whereas CXCL12 failed to do so at any of the concentrations tested (Fig. 3B).

Pertussis Toxin Inhibits CXCL12- and IGF-1-induced Chemotaxis but Does Not Affect the Activation of IGF-1R Induced by IGF-1 in MDA-MB-231 Cells—To investigate the involvement of Gαi in IGF-1-induced chemotaxis of MDA-MB-231 cells, the cells were treated with various concentrations of PTX, a specific inhibitor of Gαi subunits. The cells were then tested for their chemotactic response to various concentrations of both CXCL12 and IGF-1. As shown in Fig. 4, A and B, PTX at a concentration of 10 ng/ml completely blocked the response to CXCL12 and partially inhibited that to IGF-1 in MDA-MB-231 cells. Similar levels of inhibition were observed when the cells were pretreated with 100 and 1,000 ng/ml PTX (data not shown). These data indicate a contribution of Gαi to IGF-1-induced chemotaxis of MDA-MB-231 cells. Pretreatment of MCF-7 cells with PTX had no effect on IGF-1-induced chemotaxis at any of the three doses tested (Fig. 4C).

To test the possibility that blocking Gαi with PTX inhibits the activation of IGF-1R by IGF-1, the lysates of cells untreated or treated with PTX were assayed for the level of tyrosine-phosphorylated IGF-1R complex formed in response to IGF-1 using the KIRA assay. Two different doses of PTX (10 and 100 ng/ml) failed to alter the level of IGF-1R activation in either MDA-MB-231 or MCF-7 cells (Fig. 5, A and B) indicating that Gαi is not involved in IGF-1-induced formation of the activated IGF-1R complex.

RNAi of CXCR4 Inhibits Both CXCL12- and IGF-1-induced Chemotaxis but Has No Effect on the Activation of IGF-1R in MDA-MB-231 Cells—The involvement of CXCR4 in IGF-1-induced chemotaxis of MDA-MB-231 cells was examined using CXCR4-deficient cells. MDA-MB-231 cells were infected with a retrovirus expressing either RNAi to transactivation of CXCR4 by IGF-1

![Image](355x26 to 383x38)

**FIGURE 3.** IGF-1 but not CXCL12 induces activation of IGF-1R in MCF-7 and MDA-MB-231 cells. A KIRA assay was performed to measure the level of tyrosine-phosphorylated IGF-1R complex formed after incubation with CXCL12 or IGF-1. Fold-increase represents the level of receptor complex formed in stimulated compared with non-stimulated cells. A and B show the level of IGF-1R activation induced by different doses of IGF-1 and CXCL12, respectively. The cell line P6 that overexpresses human IGF-1R was used as a positive control. Data are presented as the mean ± S.E. from at least three independent experiments each performed in triplicate.
knockdown CXCR4 or a retrovirus expressing specific target sequences for Renilla luciferase as a negative control. Individual clones were isolated and characterized for CXCR4 surface expression by flow cytometry and CXCR4 function was determined by assessing calcium mobilization and chemotaxis in response to CXCL12. Compared with wild-type MDA-MB-231 cells and the negative control clone, RNAi clones 11, 21, and 27 demonstrated a significant reduction of surface CXCR4 expression (Fig. 6A, shown only for clone 11) and of calcium mobilization in response to CXCL12 (data not shown). The surface expression of IGF-1R was not affected by RNAi CXCR4 knockdown in any of the clones (Fig. 6A shown only for clone 11). Compared with wild-type cells and the negative control clone, RNAi clones 11, 21, and 27 displayed a significant reduction in chemotaxis in response to CXCL12 (Fig. 6B) and IGF-1 (Fig. 6C). In contrast, knockdown of CXCR4 did not have any effect on IGF-1-induced IGF-1R activation as determined in the KIRA assay (Fig. 6D).

**IGF-1R Is Physically Associated with CXCR4 and G Protein Subunits in Both MDA-MB-231 and MCF-7 Cells, but IGF-1 Activates the CXCR4 Signaling Pathway through G Proteins Only in MDA-MB-231 Cells**—The nature of the interaction between IGF-1R, CXCR4, and G proteins in MCF-7 and MDA-MB-231 cells was investigated. Immunoprecipitations were performed on cell lysates using an anti-IGF-1R mAb, anti-human CXCR4, or control IgG, followed by Western blots for either CXCR4, Gαi, or Gβ. The immunoprecipitations with control IgG failed to coprecipitate CXCR4, Gαi, or Gβ. The immunoprecipitations with CXCR4 failed to coprecipitate CXCR4, Gαi, or Gβ, despite the fact that these three proteins were readily detectable in whole cell lysates subjected directly to Western blot (Fig. 7A). In contrast, immunoprecipitation of IGF-1R and CXCR4 in both MCF-7 and MDA-MB-231 cells led to coprecipitation of all three proteins, indicating the existence of a constitutive complex between IGF-1R, CXCR4, Gαi, and Gβ. Of note, the levels of Gαi and Gβ in immunoprecipitates of the two receptors consistently appeared to be higher in MDA-MB-231 cells than in MCF-7 cells.
The ability of IGF-1 to transactivate CXCR4 was investigated by examining the effect of stimulation with IGF-1 on the level of association of G\(_{\alpha}2\) and G\(_{\beta}\) with CXCR4 (Fig. 7B). The results of these experiments showed that stimulation of MCF-7 cells with IGF-1 failed to release either G\(_{\alpha}2\) or G\(_{\beta}\) from the CXCR4/IGF-1R complex, whereas, in contrast, both G\(_{\alpha}2\) and G\(_{\beta}\) were released from the complex in MDA-MB-231 cells.

DISCUSSION

Here we present evidence of cross-talk between CXCR4 and IGF-1R in the human epithelial breast cancer cell line, MDA-MB-231. The basis of this cross-talk appears to depend on a physical association between CXCR4 and IGF-1R. It is unidirectional, involving activation of G protein subunits by IGF-1 that is dependent on the presence of a functional pool of CXCR4, but independent of the CXCR4 ligand CXCL12. These observations potentially have major implications for our understanding of the intracellular signaling of these two important receptors in both normal and pathological situations.

In this study, two breast cancer cell lines, the non-metastatic MCF-7 and the highly metastatic MDA-MB-231 were characterized in terms of expression and function of CXCR4 and IGF-1R. MCF-7 cells exhibited a high level of IGF-1R expression and a strong chemotactic response to IGF-1 whereas MDA-MB-231 cells expressed a lower level of the receptor and a lower response to IGF-1. The lower level of IGF-1R expression in the metastatic MDA-MB-231 cells compared with the non-metastatic MCF-7 cells correlates well with the results of a recent study demonstrating that reduced expression of IGF-1R in MCF-7 cells leads to a more metastatic phenotype in those cells (26).

Although the two cell lines expressed high levels of CXCR4, only MDA-MB-231 cells responded functionally to CXCL12, indicating uncoupling of receptor expression and function in the MCF-7 cells. This phenomenon has been observed previously with respect to CXCR4 in the human hepatoma cell line HepG2 (27), and other chemokine receptors in a range of cell types (28, 29), although the molecular basis for this non-functional phenotype, at least with respect to cell migration, was not defined in those studies. However, the results of our studies suggest at least two mechanisms: differences in the level of expression of G\(_{\alpha}2\) and G\(_{\beta}\) in MB-MDA-231 cells and MCF-7 cells, which results in different levels of association of G\(_{\alpha}2\) with CXCR4 in those cells, and the failure of G\(_{\alpha}2\) and/or G\(_{\beta}\) to uncouple from CXCR4 upon activation of the receptor (data not shown).

Three forms of cross-talk between GPCR and RTK systems have been demonstrated in different cellular systems. First, RTKs can be transactivated by GPCRs. For example, EGF-R is phosphorylated in response to...
CCL11, a ligand for the GPCR CCR3, leading to the MAP kinase activation and IL-8 production in bronchial epithelial cells (18). This appears to depend on activation of CCR3 by CCL11. Second, GPCRs can be transactivated by RTKs. For example, it has been shown that IGF-1 stimulates phosphorylation of CCR5 in MCF-7 cells. Chemotaxis induced by IGF-1 was inhibited by a neutralizing anti-CCL5 antibody. Transactivation of CCR5 by IGF-1 was therefore indirect, requiring the activity of the ligand (CCL5) for the second receptor (20). Finally, bidirectional transactivation between the same two receptor systems has not previously been reported. Whereas our data are consistent with those demonstrating transactivation of CCR5 in MCF-7 cells by IGF-1 (20), the mechanism is different. In that system, transactivation of CCR5 by IGF-1 was dependent on production of the CCR5 ligand, CCL5. In contrast, the transactivation of CXCR4 by IGF-1 we observed in MDA-MB-231 cells is direct, because the cells do not synthesize or release CXCL12 either constitutively, or in response to IGF-1, as demonstrated by reverse transcriptase-PCR and ELISA (data not shown).

We found that formation of the tyrosine-phosphorylated IGF-1R complex is independent of CXCR4 and G proteins. This suggests the signal transduction pathway leading to activation of CXCR4 bifurcates either before or following tyrosine phosphorylation of IGF-1R. Of note, the inhibition of IGF-1-induced chemotaxis by PTX and knockdown of CXCR4 was only partial even though CXCL12-induced migration of MDA-MB-231 cells was completely inhibited. The partial inhibition of IGF-1-induced cell migration indicates CXCR4- and G protein-independent induction of chemotaxis through IGF-1R. Cell migration in eukaryotic cells is known to depend on activation of Class IA and IB PI3Ks (31, 32). In general, Class IA PI3Ks link RTKs to cell migration, whereas Class IB PI3Ks mediate cell migration in response to ligation of GPCRs (33, 34). IGF-1 is known to induce activation of PI3K and this is dependent on tyrosine phosphorylation. In contrast, ligation of CXCR4 activates Class IB PI3K, and this is inhibited by PTX. Therefore, the residual cell migration observed in response to IGF-1 in PTX-treated cells is likely because of tyrosine phosphorylation-dependent Class IA PI3K activation. A physical interaction between IGF-1R and Gα and Gβ has previously been demonstrated (35, 36). Moreover, in those studies, PTX was shown to inhibit IGF-1-induced activation of MAPK in neuronal cells (36), and IGF-1-induced mitogenesis of HIRcB cells and 3T3L1 adipocytes (35). In contrast to the conclusion from that study that IGF-1R functions as a G protein-coupled receptor (35), our findings suggest that the association of the G protein subunits with IGF-1R is indirect, and requires the presence of CXCR4. Certainly, our data indicate that the presence of functional CXCR4 is required for G protein-dependent cell migration in response to IGF-1.

Interestingly, CXCR4 and IGF-1R could be coprecipitated in both MCF-7 and MDA-MB-231 cells, indicating that the lack of involvement of CXCR4 in IGF-1-induced chemotaxis of MCF-7 cells was not because of a lack of association of CXCR4 and IGF-1R in those cells. Rather, our data indicate that the cross-talk in MDA-MB-231 cells is mediated at the level of G protein activity; both Gα and Gβ are associated with the complex in both cell lines; however, activation of the complex, as determined by release of Gα and Gβ from CXCR4, only occurs in MDA-MB-231 cells. This is consistent with our observation that MCF-7 cells do not respond to CXCL12, at least in terms of the migratory response.

In summary, our data provide evidence of a novel transactivation between RTK and GPCR signal transduction pathways. We have observed the coprecipitation of IGF-1R, CXCR4, and the G protein subunits, Gα and Gβ, indicating a constitutive physical association between these molecules. Based on our data, we propose that this IGF-1R/CXCR4 complex allows CXCR4 and G proteins to act partially in IGF-1-induced chemotaxis of MDA-MB-231 cells probably through activation of class IB PI3K activity. Our data also demonstrate that CXCR4 and G proteins operate independently of the activation of IGF-1R because neither PTX pretreatment nor CXCR4 knockdown affected the levels of tyrosine-phosphorylated IGF-1R complex formed.
after IGF-1 stimulation. The fact that this pathway does not appear to be active in the non-metastatic MCF-7 cells suggests that CXCR4/IGF-1R receptor integration may play an important role in cancer metastasis. In addition, both IGF-1/IGF-1R and CXCL12/CXCR4 are essential for life (3, 37–39) raising the possibility that transactivation between IGF-1R and CXCR4 may be involved in development. Further experimentation comparing IGF-1R signaling complexes in both MCF-7 and MDA-MB-231 cells may provide further insights.

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REFERENCES