Down-regulation of Type I Insulin-like Growth Factor Receptor Increases Sensitivity of Breast Cancer Cells to Insulin

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Abstract

The type I insulin-like growth factor receptor (IGF1R) and insulin receptor (IR) are structurally and functionally related heterotetrameric receptors. Activation of IGF1R has been shown to regulate breast cancer cell biology, and it has become an attractive therapeutic target. Most strategies have focused on targeting IGF1R alone without affecting IR levels given the known physiologic functions of IR. Human breast cancer cell lines and tissues revealed mRNA expression of both IGF1R and IR. Because αβ chains of IGF1R and IR form hybrid receptors, we hypothesized that agents solely targeting IGF1R may affect tumor biology mediated by IGF1R/IR hybrids and IR. We used small interfering RNA (siRNA) technology to specifically down-regulate IGF1R by 60% to 80% in the MDA-435/LCC6 cell line, which was sufficient to diminish activation of IGF1R by IGF-I. IGF1R down-regulation by siRNA did not affect IR levels but, interestingly, sensitized cells to insulin activation of downstream signaling pathways in several breast cancer cell lines. IGF1R siRNA treatment diminished hybrid receptor formation, suggesting that specific down-regulation of IGF1R resulted in enhanced holo-IR formation. In addition, IGF1R down-regulation increased insulin binding consistent with the formation of an increased number of holo-IR on the cell surface. Accordingly, insulin-stimulated glucose uptake was enhanced on IGF1R down-regulation. In conclusion, our data suggest that specific siRNA targeting of IGF1R alone in breast cancer increases insulin sensitivity. Because IR also activates signaling pathways similar to IGF1R in breast cancer cells, agents targeting both receptors may be necessary to disrupt the malignant phenotype regulated by this growth factor system. [Cancer Res 2007;67(1):391–7]

Introduction

Multiple lines of evidence have indicated a critical role of the type I insulin-like growth factor (IGF) receptor (IGF1R) in many types of cancer, including breast, prostate, colon, and melanoma (1). This receptor is composed of extracellular α subunits covalently linked to the β subunits, which contain the transmembrane and intracellular tyrosine kinase domains. αβ dimers are covalently linked to form a heterotetramer (2). Activation of IGF1R is initiated through its binding to ligands, IGF-I and IGF-II, which leads to a conformational change resulting in tyrosine kinase activity and autophosphorylation of the β subunits. Subsequent recruitment and activation of adaptor molecules, such as insulin receptor (IR) substrate-1 (IRS-1) and IRS-2, couple the initial ligand binding event to multiple downstream signaling pathways, including the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K) pathways (3).

IR is structurally and functionally related to IGF1R. On insulin binding to IR, IRS proteins are phosphorylated and activated, which lead to activation of downstream pathways, also including MAPK and PI3K pathways (4). Although similar pathways are activated, the physiologic effects mediated by these two receptors differ. IGF1R activation normally leads to cell proliferation, motility, and metastasis, whereas IR activation normally leads to glucose metabolism and cell proliferation (5).

In addition, IGF1R and IR can form hybrid receptor by heterotetramerization (6). The hybrid receptor binds IGF-I and IGF-II with high affinity but has much lower affinity for insulin (7). Recent evidence supports the idea that IR and IGF1R/IR hybrid receptor may also be involved in cancer biology (8).

IGF1R has received attention as a potential target for cancer therapy. Because the metabolic effects of IR are well known, there has been an effort to specifically target IGF1R without affecting IR signaling. However, it has been difficult to design specific IGF1R antagonists. The kinase domains of IGF1R and IR are highly homologous; therefore, specific small-molecule inhibitors of IGF1R are difficult to develop. Major efforts have been to develop monoclonal antibodies immunoreactive with IGF1R but not IR. However, recent findings from our laboratory have indicated that IGF1R antibodies also down-regulate IR. Coexpression of IGF1R and IR is necessary for IGF1R antibody-mediated down-regulation of IR (9). Thus, monoclonal antibodies directed against IGF1R have functional effects on IR expression and signaling as well.

In this study, we found that IGF1R and IR are commonly coexpressed in primary breast cancers and cell lines. We used small interfering RNA (siRNA) to specifically target down-regulation of IGF1R and found that IGF1R was efficiently suppressed without affecting IR expression. However, IGF1R down-regulation by siRNA sensitized cells to insulin. Our results suggest that specific targeting of IGF1R alone enhances insulin signaling, which may be an undesirable effect in breast cancer cells.

Materials and Methods

Reagents. All chemical reagents were purchased from Sigma unless otherwise indicated. IGF-I and insulin were purchased from GroPep and Eli Lilly respectively. Antibodies used are IGF1Rβ polyclonal antibody (C-20; Santa Cruz Biotechnology), R-phycoerythrin (R-PE)–conjugated mouse anti-human IGF1Rα antibody (BD Biosciences), IRβ polyclonal antibody (C-19; Santa Cruz Biotechnology), IRα monoclonal antibody (83-7; Chemicon International) actin monoclonal antibody (Sigma),

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phosphorylated Akt (Ser^{473}) antibody (Cell Signaling), PY20-horseradish peroxidase (HRP)–conjugated antibody (BD Transduction Laboratories), and HRP-conjugated secondary antibodies (Pierce). Control antibodies are R-PE mouse IgG1κ (BD Biosciences) and normal mouse IgG (Santa Cruz Biotechnology).

RNase protection assay. Excess breast cancer tissues were frozen in liquid nitrogen after steroid hormone receptor analysis. These specimens were not linked to clinical data or patient identifiers. RNA from breast tumor samples and cell lines was extracted using TRI Reagent. An IR-B cDNA fragment of 636 bp (2,230–2,865 bp) was inserted into pCR 2.1 vector. The primer sequences to reverse transcription-PCR this 636-bp fragment from mRNA were adopted from Pandini et al. (7). The vectors containing the IGF1R cDNA fragment of 293 bp (2,737–3,030 bp) or the 36B4 cDNA fragment were constructed previously (10). Vectors were linearized by restriction enzyme digestion and transcribed *in vitro* using [^{32}P]UTP and other cold ribonucleotides to generate riboprobes for IGF1R, IR, and 36B4. To measure the levels of IGF1R, IR-A, and IR-B, 30 μg RNA from each sample was hybridized with ^{32}P-labeled anticomplementary probes in a solution containing 80% formamide. The products were then digested with 40 μg/mL RNase A. Protected fragments were visualized by 6% polyacrylamide gel, and the intensity of the hybridization bands was quantified by phosphorimaging (STORM system from Molecular Dynamics).

Cell lines and culture. LCC6 cells, MDA-MB231-BO, and MDA-MB231 were cultured in DMEM with 10% fetal bovine serum (FBS), 11.25 nmol/L human insulin, 50 units/mL penicillin, and 50 μg/mL streptomycin. MCF-7 cells, MCF-10A cells, and T47D were cultured according to literature.

siRNA sequences. The following oligonucleotides were synthesized from Dharmacon control siRNA, 5′-AUGAACGUGAAUUGCUCAAUU and complement sequence; IGF1R siRNA-R2, 5′-GCCGAUGUGUGAGAAGACCUU and complement sequence; and IGF1R siRNA-5, 5′-CAACGAAGCUUCUGUGAUGUU and complement sequence. The sequence of siRNA-R2 was according to Bohula et al. (11).

Flow cytometry. LCC6 cells were incubated with PE-conjugated mouse IgG or PE-conjugated IGF1R antibody in PBS/1% bovine serum albumin (BSA)/0.1% sodium azide [fluorescence-activated cell sorting (FACS) buffer] for 1 h at 4°C. Cells were washed twice and resuspended with 400 μL FACS buffer. IGF1R levels on cell surface were measured using a FACSCalibur flow cytometer with FL2 channel (585/42 nm band pass).

siRNA transfection and cell stimulation. Cells were grown in regular growth medium. Cells with 50% confluency were transfected with 100 nmol/L siRNA using the TransIT-siQUEST transfection reagent (Mirus) according to the manufacturer's protocol. Twenty-four hours later, cells were washed twice with PBS and serum deprived for another 24 h in serum-free medium as described previously. Medium was replaced with serum-free medium containing IGF-I or insulin for times as indicated in the figure legends.

Cell lysates. Cells were washed twice with ice-cold PBS on ice and lysed as described previously (12). Protein concentration of cell lysates was determined using the bicinchoninic acid protein assay reagent kit (Pierce).

Immunoprecipitation. Equal amounts of protein lysates (1 mg) were first precleared with 25 μL of protein A agarose (Santa Cruz Biotechnology) for 30 min and then incubated with the indicated antibody for 3 to 5 h. Protein A agarose (25 μL) was then added overnight followed by three washes with TNESV buffer. Beads were resuspended in 2× Laemmli loading buffer with 30 mg/mL DTT.

Gel electrophoresis and Western blotting. Proteins from lysates and immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were immunoblotted using IGF1Rβ antibody (1:2,000 dilution), IRβ antibody (1:1,000 dilution), phosphorylated Akt antibody (1:2,000 dilution), or actin antibody (1:5,000 dilution) followed by the appropriate HRP-conjugated secondary antibody (Pharmacia). For detecting phosphorylated IRS proteins, membranes were immunoblotted with PY20-HRP anti-phosphotyrosine antibody. Western blots were developed by the chemiluminescence method.

^{125}I-insulin binding assay. LCC6 cells were transfected with siRNA for 24 h and then serum starved for 16 h. Cells were washed with PBS twice and then incubated in binding buffer (DMEM, 50 mmol/L HEPES, 1% BSA, penicillin/streptomycin) in the presence of increasing concentrations of unlabeled ligands and 10 pmol/L ^{125}I-insulin. After 2 h of incubation at 16°C, cells were washed twice with ice-cold PBS and lysed using 0.1 mol/L NaOH. The bound ^{125}I-insulin was measured by a gamma counter. The radioactivity bound in the presence of excess unlabeled insulin (10^{-6} mol/L) was designated as nonspecific binding and subtracted from total binding to obtain specific binding (13).

Glucose uptake assay. Cells were seeded in six-well plates and allowed to attach for 24 h. Then, cells were transfected with siRNA. The next day, complete medium was replaced with DMEM containing 0.1% FBS for another 24 h. Cells were then washed thrice with KRH buffer [20 mmol/L HEPES (pH 7.4), 120 mmol/L NaCl, 1.2 mmol/L $MgSO_4$, 2 mmol/L $CaCl_2$, 2.5 mmol/L KCl] and incubated in 1.8 mL KRH buffer/0.1% BSA for 3 h. Insulin (20 nmol/L) was added for 15 min. Then, 200 μL of KRH buffer/0.1% BSA with 2-deoxy-D-[1-^{3}H]glucose (0.5 μCi/mL final concentration) were added, and incubation was continued for 5 min. Cells were washed twice with ice-cold PBS and then solubilized with 0.1 mol/L NaOH. The amount of radioactivity incorporated into the cells was measured by liquid scintillation spectroscopy.

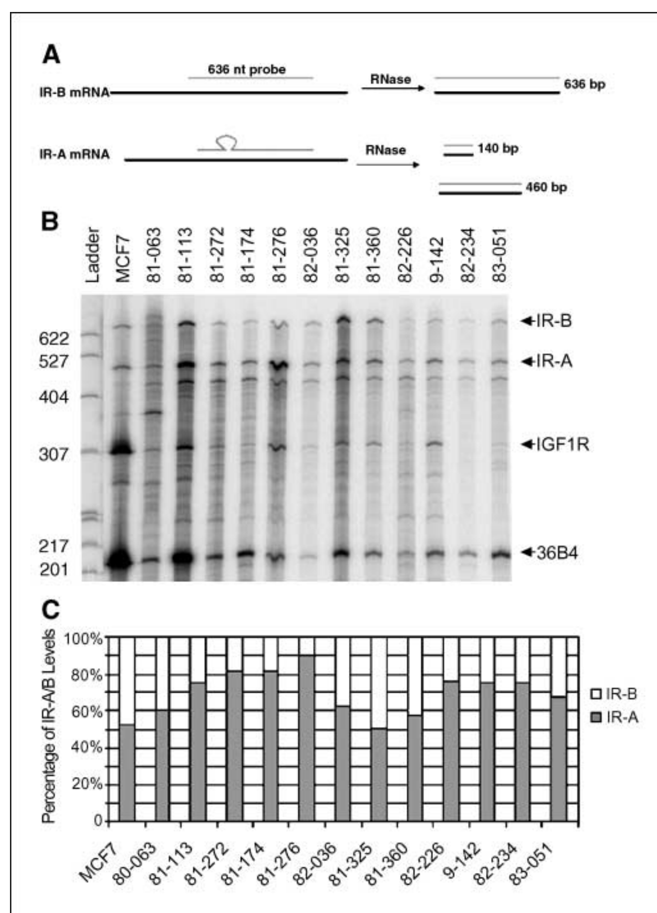

Figure 1. IR and IGF1R mRNA levels in breast cancer primary tumors. *A,* schematic of the IR mRNA probes used in RNase protection assay. When IR-B mRNA hybridizes with the 636-nucleotide probe, a fragment of 636 bp is protected. When IR-A mRNA hybridizes with the 636-nucleotide probe, two fragments are protected after RNase cleavage, with 460 and 140 bp, respectively. *B,* mRNA of 27 tumors was isolated and analyzed by RNase protection assay. Representative assay from 12 tumors is shown. *Left, numbers,* size of fragments in base pairs; *right, arrows,* protected fragments by different probes. *C,* mean percentage of IR-A versus IR-B mRNA levels in selected breast tumors from two independent experiments. Densitometry of the radioactive bands representing both isoforms was quantified by phosphorimaging and normalized to 36B4 mRNA levels.

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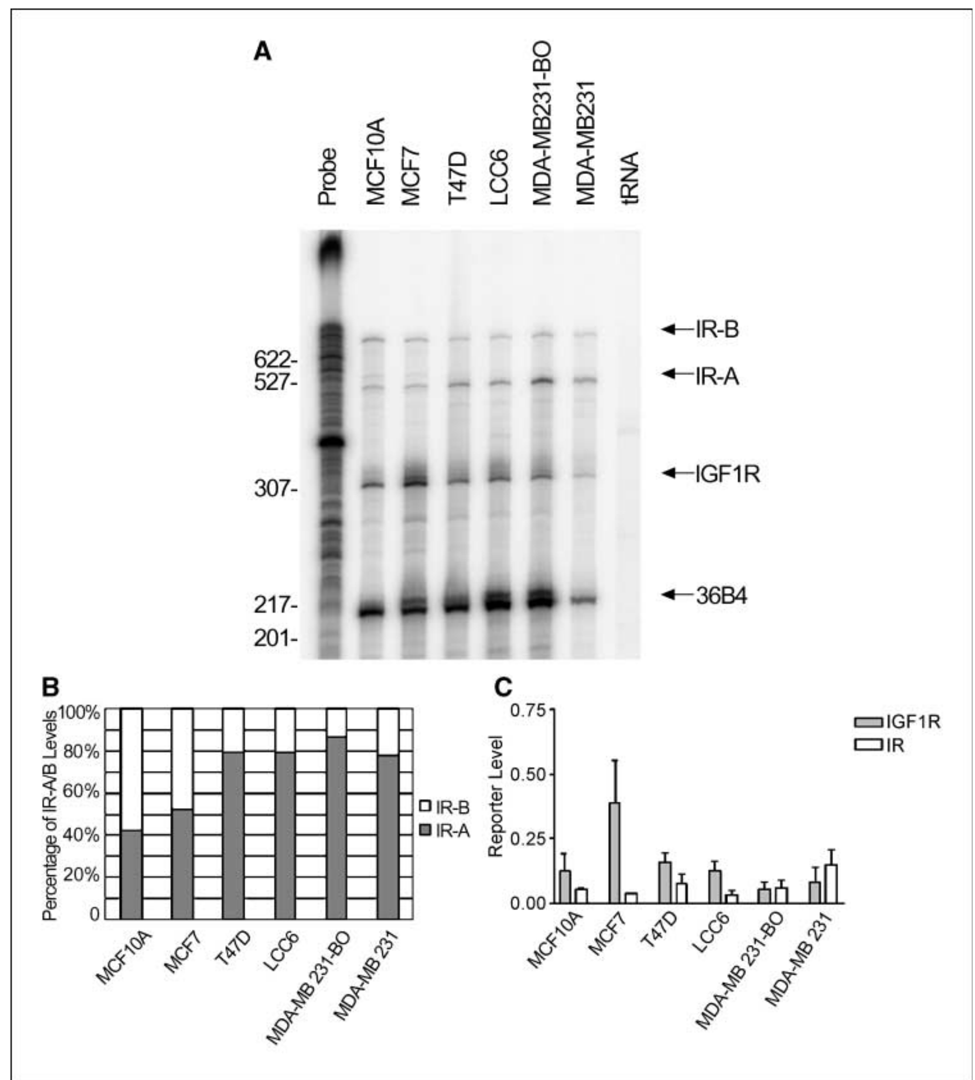

Figure 2. IR and IGF1R mRNA levels in breast cancer cell lines. *A,* mRNA of seven breast epithelial cell lines was isolated and subjected to RNase protection assay. Yeast tRNA was used as a negative control. *Left, numbers,* size of fragments in base pairs; *right, arrows,* fragments protected by different probes. *B,* mean of percentage of IR-A versus IR-B mRNA levels in breast cancer cell lines from three independent experiments. Data were quantified by phosphorimaging and normalized to 36B4 mRNA levels. *C, columns,* mean of receptor levels from three independent experiments; *bars,* SE. IR levels represent both IR-A and IR-B. Data were quantified by phosphorimaging.

Results

IR isoforms and IGF1R coexpression in breast cancer primary tumors and cell lines. To determine if IGF1R down-regulation could affect IR signaling, we first assessed whether IR and IGF1R are coexpressed in primary breast cancers. Total RNA was extracted from 27 primary breast cancers. The mRNA expression of IR and IGF1R was measured by RNase protection assay. Due to alternative splicing, IR exists in two isoforms: IR-A and IR-B (14). To measure the ratio of IR-A to IR-B, we designated an IR probe that recognized both isoforms (Fig. 1*A*). This probe protected an IR-A fragment of 460 bp and an IR-B fragment of 636 bp. The protected fragment for IGF1R is 293 bp. mRNA from MCF-7 cells was used as a control. Figure 1*B* shows a representative experiment. All tumors expressed IR or IGF1R mRNA, and most samples expressed both receptors. In addition, we observed that IR-A was the predominant IR isoform (Fig. 1*C*). The presence of IGF1R and IR implies that both receptors could have physiologic relevance in breast cancer.

We next measured the expression of IR and IGF1R in several breast epithelial cell lines. As shown in Fig. 2, all cell lines coexpressed IGF1R and IR isoforms. Similar to primary breast cancer specimens, we found that IR-A was the predominant IR isoform in all breast cancer cells but not in the diploid, nontransformed MCF-10A cells (Fig. 2*B*). In addition, we compared the total levels of IR and IGF1R mRNA (Fig. 2*C*). In MCF-7 cells, IGF1R mRNA was expressed ~10-fold higher than IR. In other breast cancer cell lines, IR and IGF1R were expressed at comparable levels.

IGF1R down-regulation by siRNA diminished signaling response to IGF–I. To examine the effects of IGF1R down-regulation on IGF-I–mediated signaling, we chose LCC6 cells for further studies. These cells have equivalent levels of IGF1R and IR mRNA, have constitutively activated MAPK pathways, and do not proliferate in response to IGF-I or insulin (15). Data from our laboratory have shown that IGF1R and IRS-2 in LCC6 are critical for cell motility and metastasis (16, 17). Thus, down-regulation of IGF1R may not affect the viability of these cells, although the biochemical activity of the receptor could be measured. We examined four pairs of siRNA and found that one of these (siRNA-5) efficiently down-regulated IGF1R expression. In addition, Bohula et al. (11) previously published a siRNA sequence (siRNA-R2) that down-regulated IGF1R levels. To quantitatively measure the down-regulation of IGF1R by siRNA, surface IGF1R was measured by flow cytometry in LCC6 cells. As shown in Fig. 3*A*, siRNA-5 down-regulated IGF1R level to 20% of control levels, whereas siRNA-R2 down-regulated IGF1R level to 40%. Therefore, in the following

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studies, we used siRNA-5 to show the effect of IGF1R down-regulation.

We then assessed whether down-regulation of IGF1R by siRNA abolished IGF-I signaling in LCC6 cells by measuring phosphorylation of adaptor proteins and Akt. As shown in Fig. 3*B*, down-regulation of IGF1R by siRNA-5 diminished the phosphorylation of a 185-kDa band consistent with IRS-1/IRS-2 (17) and Akt in response to IGF-I. Therefore, down-regulation of IGF1R by siRNA was sufficient to diminish IGF-1–stimulated activation of downstream signaling pathways.

IGF1R down-regulation enhanced insulin sensitivity in LCC6 cells. To determine if IGF1R down-regulation affected insulin signaling, LCC6 cells were pretreated with control or IGF1R siRNA-5 for 24 h and then starved for an additional 24 h. Cells were treated with increasing concentrations of insulin for 5 min, and the phosphorylation of IRS-1/IRS-2 and Akt was assessed by Western blot. As shown in Fig. 4*A*, although the level of IR remained the same, insulin signaling was greater when the cells were pretreated with IGF1R siRNA at all doses of insulin. In addition, we assessed whether IGF1R down-regulation caused enhanced insulin signaling over time. After treatment with 20 nmol/L insulin, cells were harvested at several time points and Akt phosphorylation was measured. As shown in Fig. 4*B* and *C*, insulin signaling was enhanced during all times measured. Our data suggest that down-regulation of IGF1R increased insulin signaling.

IGF1R down-regulation enhanced insulin signaling in other breast cancer cell lines. As shown in Fig. 2*A*, IR and IGF1R are

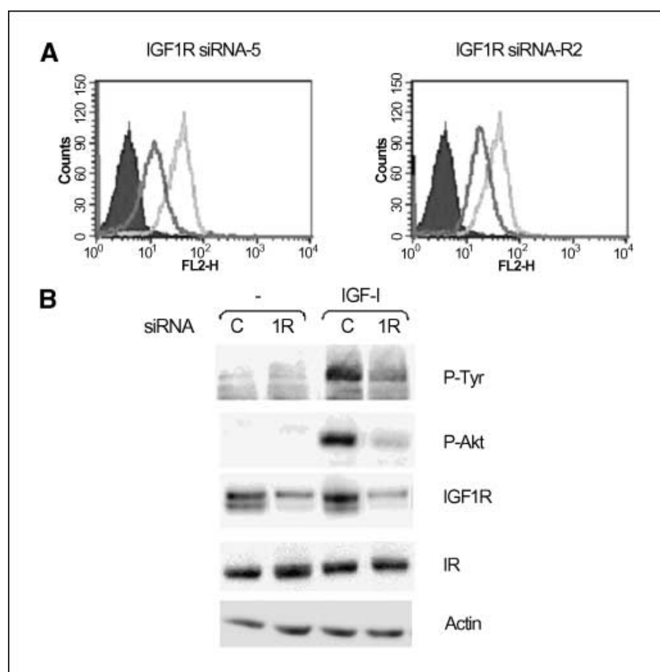

Figure 3. IGF1R down-regulation by siRNA diminished response to IGF-I treatment. *A,* LCC6 cells were transfected with control siRNA, siRNA-5, or siRNA-R2 for 48 h. After cells were stained with R-PE–conjugated IGF1R mouse antibody or control antibody, the level of IGF1R was measured by flow cytometry. *Shaded peak,* negative controls after cells were stained with control antibody; *middle peak,* IGF1R levels after IGF1R siRNA down-regulation; *right peak,* IGF1R levels after control siRNA treatment. *B,* LCC6 cells were transfected with control siRNA (*C*) or IGF1R siRNA-5 (*1R*) for 24 h and then incubated in serum-free medium. Twenty-four hours later, cells were treated with or without IGF-I for 5 min. Cell lysates were analyzed by SDS-PAGE and Western blotting. Total or phosphorylated levels of the indicated proteins are shown. Actin was used as a control.

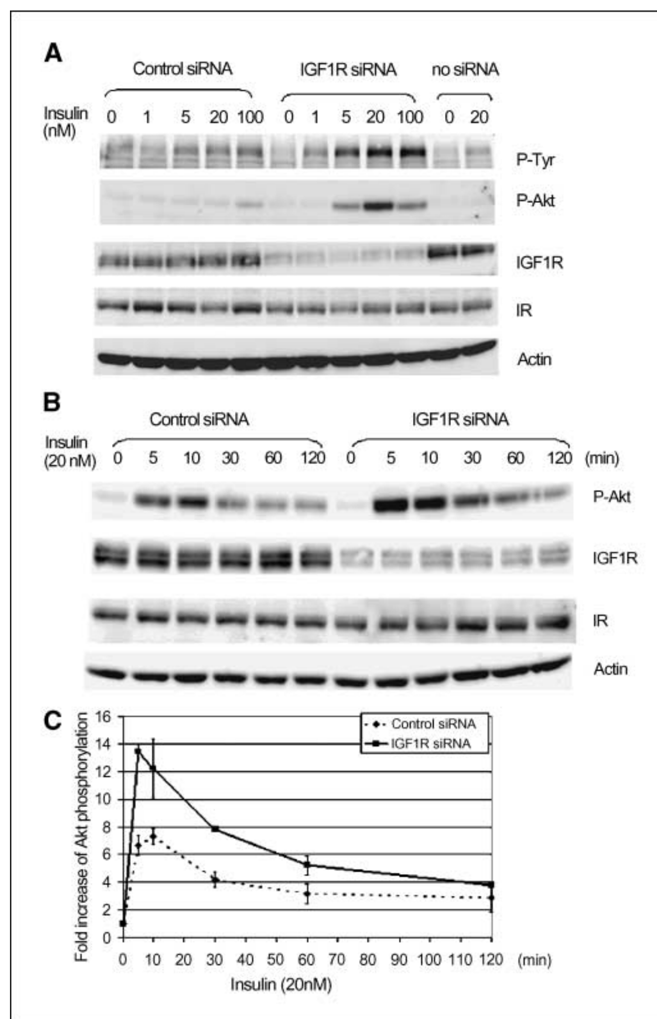

Figure 4. Enhancement of insulin signaling on IGF1R down-regulation by siRNA. LCC6 cells were transfected with control siRNA or IGF1R siRNA-5 for 24 h and then starved with serum-free medium for 24 h. *A,* cells were treated with increasing amounts of insulin and lysed after 5 min of treatment. *B,* cells were treated with 20 nmol/L insulin and lysed at different time points. Cellular lysates were separated by SDS-PAGE, and the phosphorylation and protein levels were assessed using specific antibodies by Western blotting. *C, points,* mean of fold increase of Akt phosphorylation on 20 nmol/L insulin treatment; *bars,* SE.

coexpressed in all breast cancer cell lines. We next determined if down-regulation of IGF1R affected insulin signaling in other cell lines beyond LCC6 cells. MDA-MB231-BO (18), MDA-MB231, and MCF-7 cells were pretreated with IGF1R siRNA or control siRNA and then treated with insulin for 5 min. As shown in Fig. 5, insulin-mediated phosphorylation of Akt was greatly enhanced when IGF1R was down-regulated in all three breast cancer cell lines. As in LCC6 cells, IGF-I effects were diminished after IGF1R siRNA treatment (data not shown). Our data suggested that the increase of insulin signaling on IGF1R down-regulation was a common phenomenon among breast cancer cells.

IGF1R down-regulation disrupted hybrid receptor formation and increased insulin binding. To determine the mechanisms by which down-regulation of IGF1R enhanced insulin signaling, we sought to examine whether down-regulation of IGF1R affected the IGF1R/IR hybrid receptor formation. Cells were pretreated with control siRNA or IGF1R siRNA-5. IR was

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immunoprecipitated with an IRα subunit antibody (83-7) that recognizes both holo-IR and hybrid IR/IGF1R. The presence or absence of IGF1R in the immunoprecipitate was measured by IGF1R Western blotting. As shown in Fig. 6*A*, IGF1R and IR coimmunoprecipitated in LCC6 cells and down-regulation of IGF1R by siRNA disrupted the hybrid receptor formation.

Because the level of IR was unaffected by IGF1R siRNA, disruption of IGF1R/IR hybrid receptor may force the balance to formation of more holo-IR. This, coupled with the fact that insulin binds holo-IR with high affinity compared with the IGF1R/IR hybrid receptor, led us to hypothesize that IGF1R down-regulation may enhance insulin binding in LCC6 cells. Indeed, as shown in Fig. 6*B*, IGF1R siRNA–treated cells had increased binding of ^{125}I-insulin compared with control siRNA–treated cells. This binding was specific as unlabeled insulin competed for binding with ^{125}I-insulin.

IGF1R down-regulation increased glucose uptake in LCC6 cells. Because Akt activation is required for insulin-induced glucose uptake (4), we assessed the effect of IGF1R siRNA on this process in LCC6 cells. LCC6 cells were incubated with or without insulin, and the glucose uptake was measured by incorporation of [^{3}H]2-deoxyglucose. As shown in Fig. 6*C*, insulin did not induce a significant increase of glucose transport in control siRNA–treated cells, yet we observed a weak Akt activation (Fig. 4). In contrast, insulin-induced glucose uptake was significantly enhanced when IGF1R was down-regulated. Thus, down-regulation of IGF1R increased insulin-induced glucose uptake in LCC6 cells.

Discussion

The IGF system has been implicated in cancer progression and metastasis. Therefore, as the major receptor that mediates IGF effects, IGF1R has been the primary target for anti-IGF strategies.

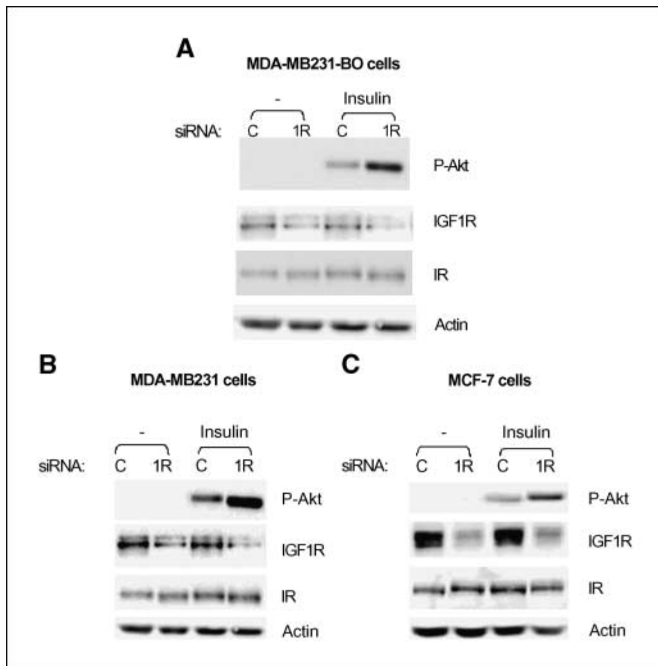

Figure 5. Insulin signaling in three breast cancer cell lines upon IGF1R down-regulation. *A* to *C,* three cell lines, as indicated in the figure, were transfected with control siRNA (*C*) or IGF1R siRNA-5 (*1R*) for 24 h and then starved with serum-free medium for 24 h. Cells were treated with or without 20 nmol/L insulin for 5 min and lysed. Cellular lysates were separated by SDS-PAGE, and the phosphorylation level of Akt and protein levels were assessed using specific antibodies by Western blotting.

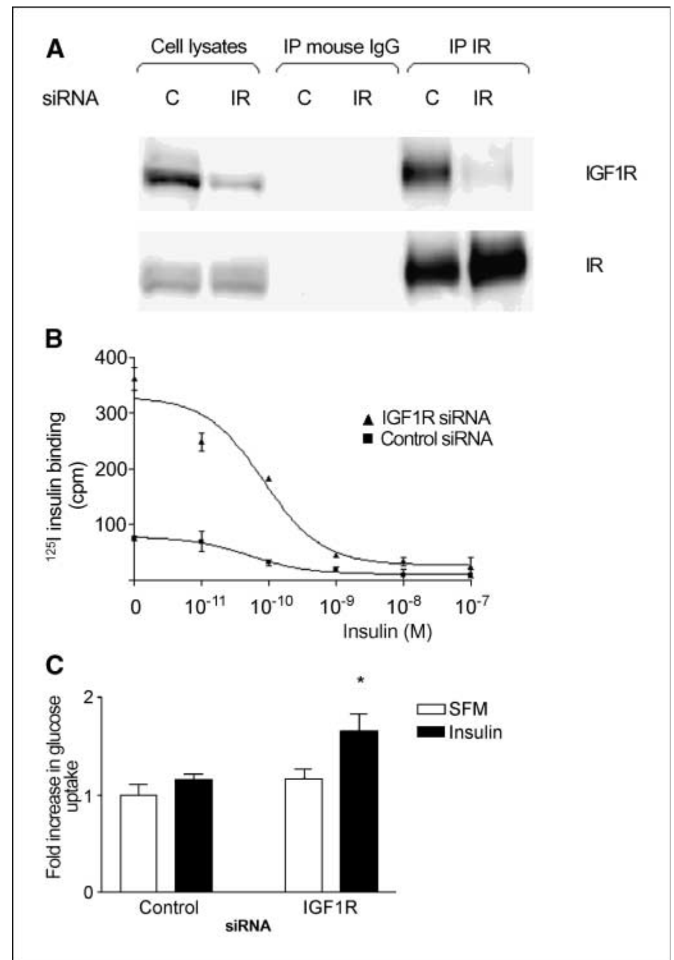

Figure 6. IGF1R down-regulation by siRNA disrupted hybrid receptor formation and increased insulin binding and glucose uptake. *A,* after LCC6 cells were transfected with siRNA for 48 h, cells were lysed and immunoprecipitated (*IP*) with an anti-IR antibody (83-7) or a control mouse IgG. *Top,* IGF1R cellular levels (*left two lanes*) or the presence or absence of bound IGF1R (*right four lanes*) was assessed using IGF1R antibody by Western blotting; *bottom,* IR cellular levels (*left two lanes*) or the presence or absence of IR (*right four lanes*) was assessed using IR antibody by Western blotting. *B,* LCC6 cells were transfected with siRNA for 24 h and then starved for 16 h. ^{125}I-insulin binding assay was done as described in Materials and Methods. *X axis,* concentration of unlabeled insulin; *Y axis,* ^{125}I-insulin binding. *Points,* mean of duplicate samples; *bars,* SE. This experiment was repeated twice and representative data are shown. *C,* LCC6 cells were transfected with control siRNA or IGF1R siRNA-5 for 24 h and then starved with DMEM containing 0.1% FBS for another 24 h. The next day, glucose uptake assay was done as described in Materials and Methods. The radioactivity incorporated into cells was measured by liquid scintillation spectroscopy. *Columns,* mean of fold increase in glucose uptake from three independent experiments; *bars,* SE. ANOVA was used to compare the statistical significance among the data. *, $P < 0.05$, only the IGF1R siRNA/insulin was significantly different from the other groups.

Because the consequences of IR inhibition are well known, drugs designed to specifically inhibit IGF1R have been deemed desirable. However, there are several factors to consider when targeting this system. First, the kinase domains of IR and IGF1R are highly homologous, which makes specific small-molecule inhibitors difficult to develop. Indeed, several small-molecule inhibitors developed thus far (19–22) are not completely specific for IGF1R. Second, IR is in close physical proximity to IGF1R in lipid rafts on cell membrane. When IGF1R-specific antibodies bind to IGF1R, we found that IR was also down-regulated by these antibodies via endocytosis (9). Third, IGF1R and IR form hybrid receptors. The relative contribution of IGF1R versus IGF1R/IR hybrid receptors to cancer biology is

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not well understood. Finally, nanomolar concentrations of insulin have long been known to stimulate breast cancer cell proliferation (23). Thus, the potential that multiple ligands and receptors affect breast cancer biology needs to be considered when designing drugs targeting this system. In this study, we found that specific inhibition of IGF1R by siRNA can influence the IR signaling system.

Of course, for IGF1R inhibition to influence IR signaling, both receptors need to be expressed. Here, we show that IGF1R and IR coexpression is common in breast cancer. Unlike immunoblotting or immunohistochemistry, where the need to use different antibodies for each receptor makes it difficult to compare levels of receptor expression because of varied antibody affinities for the proteins, the affinity of different RNA probes binding to their cognate mRNA is essentially the same, and the RNase protection assay is capable of comparing mRNA levels of IGF1R and IR within tissues and cell lines. Screening of 27 breast tumors revealed that most tumors expressed both IGF1R and IR. However, we did not observe a correlation between the level of IGF1R and IR (data not shown), suggesting that the expression of IGF1R and IR may be regulated by distinct mechanisms. Data from cell line studies showed that MCF-7 cells had much higher levels of IGF1R mRNA than IR mRNA, whereas other cell lines express relatively similar amounts of each mRNA species. Our data are in accord with previously published report by Pandini et al. (24) using ELISA methods, which measured the protein levels of holoreceptor IGF1R and IR in breast cancer cell lines. Because IGF1R and IR are frequently coexpressed in breast cancer, understanding the effect of IGF1R disruption on IR signaling has physiologic and clinical relevance.

Although immunoblotting showed that both siRNA-5 and siRNA-R2 treatment diminished IGF1R expression, flow cytometry analysis revealed that siRNA-5 was a more potent inhibitor of IGF1R than siRNA-R2. Regardless of the down-regulation efficiency, we observed that both siRNA sequences diminished IGF-I–mediated signaling events (data on siRNA-R2 not shown).

Because the total level of IR was unchanged with IGF1R siRNA, coupled with our finding that down-regulation of IGF1R affected IGF1R/IR hybrid receptor formation, one can imagine that the remaining IR proteins would be forced into IR holoreceptors. However, immunoblotting is unable to distinguish between the IGF1R/IR hybrids and holo-IR. MA-20, an IR antibody that has been reported only to recognize holo-IR (24), also recognized IGF1R/IR hybrid receptor in our studies. IGF1R could be detected by immunoblot after immunoprecipitation with MA-20 (data not shown). To address IR function, we did insulin binding studies and showed that IGF1R down-regulation caused increased insulin binding on the cell surface and enhanced insulin signaling as measured by the phosphorylation of IRS-1/IRS-2 and Akt. In addition, IGF1R down-regulation did not lead to prolonged insulin signaling activation, suggesting that attenuation of insulin signals was mediated by other mechanisms other than the levels of IR holoreceptor.

PI3K activation is required for insulin-induced glucose transport and metabolism (4). Accordingly, our data showed that IGF1R down-regulation enhanced insulin-induced Akt phosphorylation and increased insulin-induced glucose transport significantly. Glucose transport is the first rate-limiting step for glucose metabolism. Enhanced glucose metabolism has long been associated with the malignant phenotype of cancer cells (25). In clinical practice, positron emission tomography (PET) scanning was developed to measure glucose uptake in tumor cells. Interestingly, enhanced glucose uptake correlates with tumor progression; the lack of a response in glucose uptake determined by PET scan correlates with a poor response to therapy (26, 27). In addition, inhibition of glucose metabolism by 2-deoxy-glucose functions to prevent and treat cancer, showing the importance of enhanced glucose transport in the malignant phenotype (28, 29). Our findings suggest that down-regulation of IGF1R increases insulin-induced glucose uptake; therefore, in long term, this increase in metabolism could enhance the malignant phenotype. Thus, further studies are needed to evaluate the long-term effect of IGF1R down-regulation on cancer cell metabolism to determine if enhanced IR signaling can overcome specific IGF1R inhibition.

Our studies show that low levels of Akt activation were not sufficient to stimulate glucose uptake. Phosphorylation of Akt was observed after insulin exposure in parental LCC6 cells, yet glucose uptake was not significantly enhanced. It has been shown that activation of signaling, particularly through PI3K, does not completely link IR activation to glucose uptake (30). In addition, Akt2, but not Akt1, more efficiently stimulates glucose transporter translocation to the plasma membrane (31, 32). These data show an incomplete correlation between biochemical signaling and physiologic function. Although detection of phosphoproteins downstream of growth factor receptors may play an increasingly important role in stratifying tumors into responsive and unresponsive categories, there are additional qualitative and quantitative measurements that need to be considered.

Taken together, our studies have shown that down-regulation of IGF1R by siRNA enhances insulin signaling, which suggests that agents targeting IGF1R alone may have undesirable effects on cancer cells. This raises an important question: is cotargeting IGF1R and IR more appropriate in cancer therapy? Several lines of evidence support this idea. Recently, a tyrosine kinase inhibitor (BMS-554417) has been developed as a dual IGF1R and IR inhibitor. BMS-554417 blocks both IGF-I–mediated and insulin-mediated signaling events and inhibits xenograft tumor growth *in vivo* (20). In addition, although many anti-IGF1R antibodies were designed specifically against IGF1R, recent findings from our laboratory suggest that these antibodies, including EM164 and scFv-Fc, disrupt both IGF and insulin signaling (9). The cyclolignan picropodophyllin (PPP) has been shown to specifically inhibit IGF1R in an *in vitro* kinase assay (33); however, the effects in cell lines expressing both receptors are not clear. It is notable that PPP inhibition of multiple myeloma cells cannot be overcome by insulin (34), although these cells have been shown to express functional IR (35), suggesting that PPP may block both IGF1R and IR action. Therefore, the antiproliferative and antimetastatic function of these strategies may require inhibition or down-regulation of both IGF1R and IR. To date, the *in vivo* effects of solely down-regulating IGF1R are not known.

In conclusion, our studies, coupled with the findings of others, indicate that IGF1R and IR are an intimately related and highly interactive system. The development of anti-IGF1R agents should move forward with consideration of targeting both receptors. Certainly, specific down-regulation of IGF1R expression enhances IR function, which may lead to undesirable effects.

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