

Epidermal Growth Factor Induces Insulin Receptor Substrate-2 in Breast Cancer Cells via c-Jun NH₂-Terminal Kinase/Activator Protein-1 Signaling to Regulate Cell Migration

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Abstract

The epidermal growth factor (EGF) and insulin-like growth factor (IGF) signaling pathways are critically involved in cancer development and progression. However, how these two signals cross-talk with each other to regulate cancer cell growth is not clearly understood. In this study, we found that EGF remarkably induced expression of major IGF signaling components, insulin receptor substrate (IRS)-1 and IRS-2, an effect that could be blocked by EGF receptor (EGFR) tyrosine kinase inhibitors. Although both extracellular signal-regulated kinase and c-Jun NH₂-terminal kinase (JNK) signaling pathways were involved in the EGF up-regulation of IRS-1, the IRS-2 induction by EGF was specifically mediated by JNK signaling. Consistent with this, EGF increased IRS-2 promoter activity, which was associated with recruitment of activator protein-1 (AP-1) transcription factors and was inhibited by blocking AP-1 activity. Moreover, EGF treatment enhanced IGF-I and integrin engagement-elicited tyrosine phosphorylation of IRS and their downstream signaling, such as binding to phosphatidylinositol 3'-kinase regulatory subunit p85. Finally, repressing the induction of IRS-2 levels abolished the EGF enhancement of cell motility, suggesting that increased IRS-2 is essential for the EGF regulation of breast cancer cell migration. Taken together, our results reveal a novel mechanism of cross-talk between the EGF and IGF signaling pathways, which could have implications in therapeutic applications of targeting EGFR in tumors. Because AP-1 activity is involved in breast cancer progression, our work may also suggest IRS-2 as a useful marker for aggressive breast cancer. (Cancer Res 2006; 66(10): 5304-13)

Introduction

The insulin-like growth factors (IGF) are potent polypeptide mitogens involved in cell proliferation, survival, and malignant transformation. The IGF signals are transduced from the IGF-I receptor (IGF-IR) to key intracellular signaling molecules, the insulin receptor substrates (IRS), which further coordinate activation of downstream signaling pathways resulting in specific biological functions of the IGFs. IRS constitute a family of related proteins, including at least four members, IRS-1, IRS-2, IRS-3, and IRS-4 (reviewed in ref. 1). Studies with IRS-1 and IRS-2 knockout mice have highlighted the pivotal roles of these two IRS in cell

growth and other physiologic functions (2–4), whereas IRS-3- and IRS-4-null mice display mild or no defects. Mice deficient in IRS-1 are growth retarded and moderately insulin resistant, whereas IRS-2-null mice develop diabetes early in life due to severe insulin resistance combined with a failure of the pancreatic β -cells to proliferate. As the major substrates of both IGF-IR and insulin receptor, IRS-1 and IRS-2 contain a conserved pleckstrin homology (PH) domain adjacent to a phosphotyrosine binding domain, which interacts with the phosphorylated NPEY motifs in the IGF-IR β -subunit. On activation of IGF-IR, IRS-1 and IRS-2 become rapidly phosphorylated on multiple tyrosine residues, which then serve as docking sites for Src homology 2 domain-containing proteins, such as growth factor receptor binding protein 2 (Grb2), Nck/Crk, SHP2, Syp, and the p85 regulatory subunit of phosphatidylinositol 3'-kinase (PI3K; ref. 1). These protein-protein interactions activate a series of signal transduction cascades, such as the Ras/extracellular signal-regulated kinase (ERK) and PI3K/Akt pathways, which ultimately affect different cellular processes.

In addition to playing critical roles in IGF signaling, IRS-1 and IRS-2 are also involved in insulin, interleukin, IFN, growth hormone, and integrin signaling. The IRS network of upstream and downstream signaling may place them in a central position to integrate and coordinate multiple signaling pathways. Interestingly, IRS-1 and IRS-2, despite their structural and functional similarities, are not completely interchangeable in terms of their mediation of IGF-stimulated gene expression and cell cycle progression (5), as reflected by the distinct phenotypes in respective knockout mice (2–4).

In parallel with their diverse functions in cell growth, the tyrosine phosphorylation and thus the ability of IRS-1 and IRS-2 to activate downstream signaling is regulated by a variety of mechanisms. It has been shown that protein tyrosine phosphatases, such as SH-PTP2, can dephosphorylate IRS-1 (6). In addition, phosphorylation of >20 potential serine phosphorylation sites in IRS can inhibit IRS tyrosine phosphorylation. This negative regulatory mechanism depends on many different kinases, such as Akt (7), casein kinase II (8), ERK1/2 (9), c-Jun NH₂-terminal kinase (JNK; ref. 10), mammalian target of rapamycin (11), PI3K (12), protein kinase C (PKC; ref. 13), and Rho-associated kinases (14). On the other hand, Akt can also positively modulate IRS-1 tyrosine phosphorylation and activity through different serine phosphorylation (15). Recently, IRS-1 was found to be an acetylated protein, and acetylation of IRS-1 is permissive for its tyrosine phosphorylation and facilitates insulin-stimulated signal transduction (16).

Another mechanism of controlling the duration and strength of IRS activity is via modulation of IRS levels. It has been shown that the IRS-1 and IRS-2 proteins can be ubiquitinated and consequently degraded by the 26S proteasome pathway, which is dependent on the PI3K pathway (17, 18). Moreover, IRS-1 can

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doi:10.1158/0008-5472.CAN-05-2858

undergo calpain- or caspase-mediated protein cleavage (19, 20). One common mechanism of regulating IRS levels is increase of their transcription by steroid hormones, such as estrogen and progesterone (21–23). In addition, integrin clustering can also alter IRS-1 expression in a FAK-dependent manner (24).

Like the IGFs, epidermal growth factor (EGF) is also important for cell growth and malignant transformation. Interestingly, previous work has shown that EGF and IGF-I synergistically promote DNA synthesis, cell cycle progression, and cell proliferation in mammary epithelial cells (25, 26). Similarly, these two growth factors cooperate to stimulate progression of fibroblast cells through G₁ into S phase (27). In prostate epithelial cells, IGF-mediated degradation of IRS-1 is antagonized by the presence of EGF (28). Based on this evidence suggesting that EGF and IGF signals interact with each other, the present study was designed to determine how EGF modulates IGF signaling in breast cancer cells. Here, we show that EGF dramatically up-regulates IRS-1 and IRS-2 in EGF receptor (EGFR)-positive breast cancer cells. We further characterize the molecular mechanism underlying the EGF effect on IRS-2, finding that EGF induction of IRS-2 plays a critical role in EGF-enhanced breast cancer cell migration. Thus, our studies have provided evidence that EGF may regulate breast cancer development and progression by influencing IRS signaling, which may have implications in developing therapeutic strategies to target IRS-2 in cancer treatment.

Materials and Methods

Materials. All general materials and chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. IGF-I was purchased from GroPep Ltd. (Adelaide, South Australia, Australia). The small-molecule inhibitors GF109203X, H-89, LY294002, SB203580, SP600125, and U0126 were purchased from Calbiochem (La Jolla, CA). All cell culture and transfection reagents were purchased from Invitrogen (Carlsbad, CA). The lentiviral construct pLKO-puro-expressing JNK2 short hairpin RNA (shRNA) was obtained from Sigma. Chambers for the cell migration assay were purchased from BD Biosciences Discovery (Bedford, MA).

Cell culture. Breast cancer cell lines were obtained from the Breast Center at Baylor College of Medicine (Houston, TX). Cells were routinely maintained in DMEM supplemented with 5% fetal bovine serum, 2 mmol/L glutamine, 50 IU/mL penicillin, 50 µg/mL streptomycin, and 10 µg/mL insulin. Serum-free medium (SFM) consists of DMEM without phenol red plus 10 mmol/L HEPES (pH 7.4), 1 µg/mL transferrin, 1 µg/mL fibronectin, 2 mmol/L glutamine, 50 IU/mL penicillin, 50 µg/mL streptomycin, and trace elements (BioSource, Camarillo, CA). Cells were kept at 37°C in a humidified incubator with 5% CO₂. For the growth factor stimulation experiment, cells were plated at a density of 1.5×10^6 per 6-cm dish (BD Biosciences, Lincoln Park, NJ) and allowed to grow for 48 hours. Then, the medium was changed to SFM, and 12 hours later or overnight, the cells in SFM were stimulated with 50 ng/mL EGF for different times. In particular, MCF-10A cells were stimulated with EGF in DMEM-F12 supplemented with 5% horse serum and 10 µg/mL insulin. For experiments using small-molecule inhibitors, cells were first preincubated separately with GF109203X (2 µmol/L), LY294002 (20 µmol/L), SB203580 (10 µmol/L), SP600125 (10 µmol/L), or U0126 (5 µmol/L) for 30 minutes before stimulation with EGF in the presence of an inhibitor as described previously (29). For the experiment with the translation inhibitor cycloheximide (10 µg/mL), the same procedure was followed. Control cells were incubated with the vehicle DMSO alone. After stimulation, cells were washed twice with ice-cold PBS and then lysed in 200 µL lysis buffer, which contained 50 mmol/L Tris-HCl (pH 7.4), 1% NP40, 2 mmol/L EDTA, 100 mmol/L NaCl, 10% glycerol, and a fresh protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN). Then, cells were left on ice for 20 minutes, and the cell lysates were clarified by centrifugation at $14,000 \times g$ for 15 minutes at 4°C and stored at -20°C. Protein concentration of the supernatant was

measured by BCA detection reagents in accordance with the manufacturer's instruction manual (Pierce, Rockford, IL).

Transfection. Cells were plated in six-well dishes. After reaching 80% confluency, the cells were transiently transfected with 100 ng activator protein-1 (AP-1)-luciferase reporter construct using LipofectAMINE 2000 (Invitrogen) according to the instruction manual. After 4 to 6 hours of transfection, the cells were washed with PBS and cultured with fresh DMEM for 12 hours. Then, the cells were starved in SFM and subsequently treated with EGF for 12 hours in the presence or absence of signaling inhibitors. For stable transfections of MDA-MB-468 cells with JNK mutants in pcDNA3.1, 600 µg/mL G418 was used as the selection marker. The dominant-negative JNK (DN-JNK) vector was provided by Dr. Tse-Hua Tan (Baylor College of Medicine). The constitutively active JNK (CA-JNK) expression vector was a gift from Dr. Ulf Rapp (University of Wurzburg, Wurzburg, Germany; ref. 30). For cotransfection experiments, 1 µg of a constitutively active mitogen-activated protein kinase (MAPK)/ERK kinase kinase (MEKK) 1 (a NH₂-terminally truncated activated version of MEKK1) construct pFC-MEKK1 (Stratagene, San Diego, CA), a dominant-negative c-Fos (A-Fos) vector (ref. 31; kindly provided by Dr. Charles Vinson, Laboratory of Biochemistry, National Cancer Institute, NIH), or a dominant-negative c-Jun (TAM67) plasmid was added along with the 2.4-kb human IRS-2 promoter-luciferase construct pGL3-IRS2-2399 (a generous gift from Dr. M. Hashimoto, Kobe University, Kobe, Japan). After 12 hours of transfection, cells were stimulated with EGF for an additional 12 hours. Then, cell lysates were harvested. The luciferase activity was measured and normalized by total input protein (Promega, Madison, WI). In the small interfering RNA (siRNA) transfection experiments, siRNA specific to IRS-1 and IRS-2 was obtained from Dharmacon (Lafayette, CO). Each siRNA with a final concentration of 100 nmol/L was used in the transfection. One day after transfection, 50 ng/mL EGF was added to the medium and cells were grown for an additional 12 hours before cells were lysed or trypsinized.

Reverse transcription-PCR. Cells were plated at a density of 5×10^6 per 10-cm dish and allowed to grow for 48 hours. Then, the medium was changed to SFM. After overnight starvation, the cells were stimulated with 50 ng/mL EGF for the indicated time. When the transcription inhibitor 5,6-dichlorobenzimidazole riboside (DRB, 50 µmol/L) was used, cells were first preincubated with the inhibitor for 30 minutes before EGF stimulation. Total RNA was isolated with RNeasy Midi kit (Qiagen, Valencia, CA) according to the instruction manual. RNA integrity was checked by separation on a formaldehyde agarose gel. Gene specific primers were designed as follows: IRS-1, 5'-GAGGAAGAGACTGGCACTGA-3' and 5'-TCTGACGGG-GACAACATCATC-3' and IRS-2, 5'-AGCTCCCCAAGTCTCCTAA-3' and 5'-AGCCATCTCGGTGTAGTAC-3'. Another pair of primers, 5'-GGCTCTC-CAGAACATCATCCCTGC-3' and 5'-GGGTTCGCTGTTGAAGTCAGAGG-3', was used in the amplification of a 299-bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene fragment, which was selected as a control. Reverse transcription-PCR (RT-PCR) experiments were done as described previously (29). Briefly, 2 µg total RNA was used to produce cDNA with SuperScript II reverse transcriptase (Invitrogen) in a 20-µL volume. Then, 1 µL cDNA synthesis reaction was added to the PCR mixture, and PCR was done using IRS-1, IRS-2, and GAPDH primers with an annealing temperature at 60°C and 28 to 35 cycles. PCR products were revealed by ethidium bromide staining under UV after agarose gel electrophoresis.

Immunoblotting and immunoprecipitation. Total protein (40 µg) was separated by 8% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane overnight at 4°C. The remaining steps were conducted according to a standard immunoblotting protocol. Briefly, the membrane was blocked with PBS plus 0.05% Tween 20 (PBST) containing 5% nonfat milk for 1 hour and followed by incubation with a 1:1,000 dilution of anti-IRS-1, anti-IRS-2 (Upstate Biotechnology, Lake Placid, NY), or anti-β-actin (Sigma) antibodies in blocking solution at 37°C for 1 to 2 hours. For phosphotyrosine detection, the blocked membrane was first washed thrice for 5 minutes each with PBST and then incubated with a 1:1,000 dilution of PY-20 antibody (BD Biosciences) in PBST. After the primary antibody incubation, the membrane was again washed thrice for 5 minutes each with PBST and then incubated with a horseradish peroxidase-linked secondary antibody (Amersham, Piscataway, NJ) at a dilution of 1:4,000 in blocking

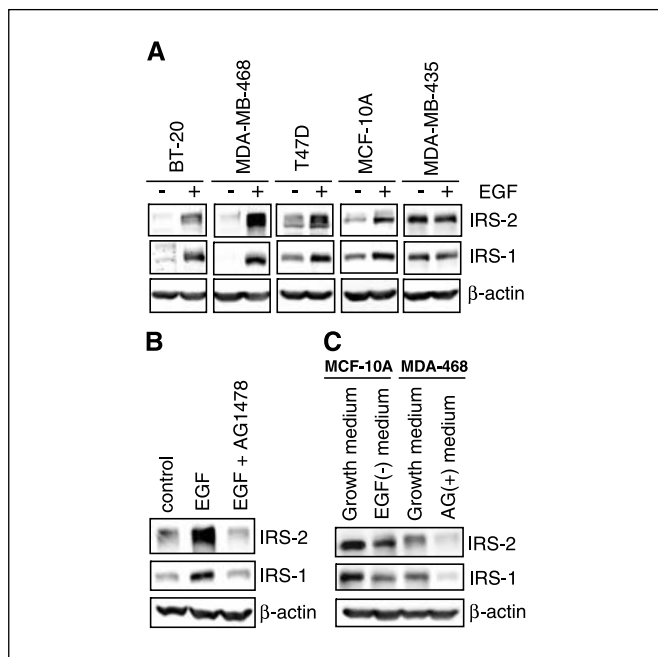


Figure 1. EGF induces IRS-1 and IRS-2 expression in breast cancer cells. **A**, regulation of IRS expression by EGF in a panel of EGFR-positive (+) or EGFR-negative (-) human breast cancer and immortalized mammary epithelial cell lines was examined by immunoblotting. Cells were treated with 50 ng/mL EGF for 24 hours. β -Actin was used as a loading control. **B**, MDA-MB-468 cells were treated with 50 ng/mL EGF for 24 hours in the presence or absence of the EGFR kinase inhibitor AG1478 (100 nmol/L). Cell lysates were subjected to the same immunoblot analysis as in (A). **C**, MCF-10A cells were cultured in their growth medium or under EGF withdrawal conditions. MDA-MB-468 cells were cultured in their growth medium in the absence or presence of the EGFR inhibitor AG1478. After 48 hours, cells were lysed and immunoblotting of IRS-1 and IRS-2 was conducted.

solution. After the membrane was stringently washed, bands were visualized by the Supersignal Chemiluminescence Assay kit according to the manufacturer's protocols (Pierce). For immunoprecipitation, cell lysates (500 μ g) were precleared by protein G agarose beads (Zymed, San Francisco, CA) and then incubated with specific antibodies at a 1:100 dilution overnight at 4°C. The beads were washed with the above lysis buffer thrice and resuspended in protein sample buffer before the immunoprecipitated protein was subjected to immunoblotting.

Chromatin immunoprecipitation assay. Sonicated soluble protein-chromatin fractions were prepared using 1% formaldehyde treated cell lysates. After chromatin DNA was sheared to 200- to 2,000-bp fragments, 5 μ g c-Fos antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and 60 μ L protein G agarose beads were used to immunoprecipitate 600 μ g DNA-protein complexes in a 1 mL volume. Primers (5'-CCATGCTTCCACTGCAAGG-3' and 5'-CAGCGGCTTGTTGGATGC-3') flanking the 5' end of a potential AP-1-binding site (-300) in the IRS-2 promoter were used in the PCR amplification of a 240-bp fragment. A 200-bp fragment of the IRS-2 coding region (the same as in the above RT-PCR), which is 3.5 kb downstream from the transcription initiation site, was used as a negative control. Chromatin immunoprecipitation assays for AP-1 binding to the matrix metalloproteinase (MMP)-1 promoter were used as a positive control. The primers used for the 240-bp MMP-1 promoter region (-242/-3) are 5'-TTGCAACACCAAGTGATTCCA-3' and 5'-CCCAGCCTCTGCTACTCCA-3' (32).

Cell migration assay. Cell migration was measured using the modified Boyden chamber assay (BD Biosciences). Briefly, 2×10^4 cells were plated on the top of the inserts, which were coated with 5 μ g/cm² fibronectin and contained an 8- μ m pore size. The inserts were then transferred into a 24-well plate. Each well contained DMEM with 5% serum as a chemo-attractant. To rule out the effect of cell proliferation, 2 μ g/mL mitomycin C was added to the cells. After 16-hour incubation, cells remaining on the

upper surface of the chambers were removed with cotton swabs. Cells on the lower surface of the inserts were fixed and stained with the HEMA3 kit (Fisher, Middletown, VA). The membrane was then mounted onto a microscope slide and the migrating cells were counted in five different areas using a light microscope. For detecting cell invasion *in vitro*, inserts were coated with a thin layer (~3 μ m thick) of Matrigel basement membrane matrix (BD Biosciences) and the same procedures were followed.

Results

EGF induces IRS-1 and IRS-2 expression. As IRS proteins are crucial IGF signaling elements, we investigated whether EGF regulates IRS levels. To this end, we used a panel of EGFR-positive breast cancer cell lines to first test EGF effects on the expression of IRS-1 and IRS-2. After starvation in SFM, the breast cancer cells were treated with 50 ng/mL EGF for 24 hours and Western blotting was conducted. As shown in Fig. 1A, EGF treatment markedly up-regulated IRS-1 and IRS-2 protein levels in EGFR-positive breast cancer cell lines BT-20, MDA-MB-468, and T47D as well as the immortalized human mammary epithelial cell line MCF-10A, which relies on EGF for growth. EGF did not significantly increase IGF-IR expression in any of these EGFR-positive breast cancer cells (data not shown). This IRS up-regulation was not observed in MDA-MB-435 breast cancer cells, which possess very low or no EGFR. Previously, it was reported that IRS-1 levels only showed a marginal change in response to EGF in MCF-7 breast cancer cells (33), which could be attributed to very low EGFR expression in these cells.

To confirm that EGFR mediated this EGF effect, we pretreated MDA-MB-468 cells, which possess relatively high EGFR levels, with the EGFR tyrosine kinase inhibitor AG1478 and then stimulated the cells with EGF in the presence of the inhibitor. As expected, AG1478 completely blocked the EGF induction of IRS-1 and IRS-2 (Fig. 1B). In agreement with this, endogenous IRS-1 and IRS-2 levels were decreased when MCF-10A and MDA-MB-468 cells were cultured under EGF withdrawal or EGFR blockade conditions (Fig. 1C).

To further characterize the EGF effect, we did dose-response and time course assays in MDA-MB-468 cells. As shown in Fig. 2, a maximal increase of IRS-1 and IRS-2 protein levels occurred with 50 ng/mL EGF after 24-hour exposure (Fig. 2A), and at this EGF concentration, IRS-1 and IRS-2 protein levels were detectably elevated after 2 hours and continued to increase with time during the 24-hour period (Fig. 2B). Taken together, these data indicate that EGF is a potent inducer of IRS-1 and IRS-2 expression in EGFR-positive breast cancer cells.

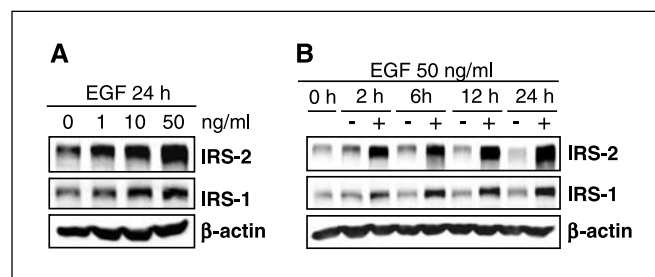


Figure 2. EGF induces IRS-1 and IRS-2 in a time- and dose-dependent manner in breast cancer cells. **A**, MDA-MB-468 cells were stimulated for 24 hours with increasing concentrations of EGF in a dose-response assay. Cell lysates were subjected to immunoblotting with anti-IRS-1, anti-IRS-2, and anti- β -actin antibodies. **B**, MDA-MB-468 cells were exposed to 50 ng/mL EGF for increasing times, and immunoblot analysis was done. Representative of three independent experiments.

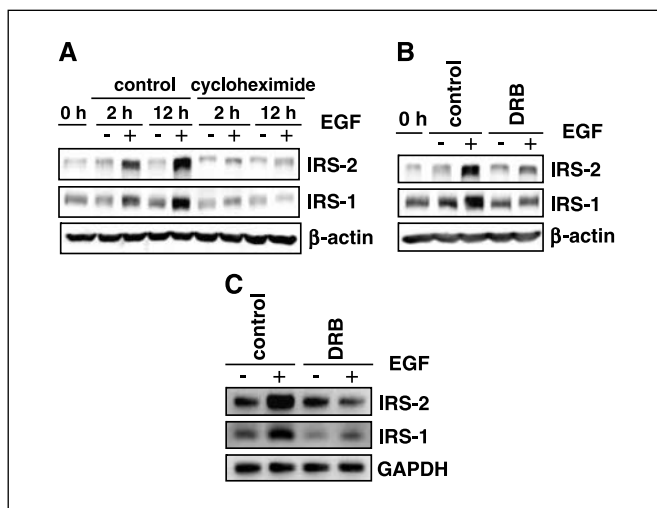


Figure 3. EGF induces IRS-1 and IRS-2 transcription in breast cancer cells. **A**, MDA-MB-468 cells were treated with 50 ng/mL EGF in the presence or absence of the translation inhibitor cycloheximide (10 μ g/mL) for the indicated times. Total protein was then isolated and immunoblotting was done for IRS-1, IRS-2, or β -actin. **B**, MDA-MB-468 cells were treated with 50 ng/mL EGF in the presence or absence of the transcription inhibitor DRB (50 μ mol/L) for 6 hours. Then, immunoblotting procedures were followed as in (A). **C**, MDA-MB-468 cells were treated with 50 ng/mL EGF in the presence or absence of the transcription inhibitor DRB (50 μ mol/L) for 1 hour. Total RNA was isolated and 100 ng RNA was used in RT-PCR of IRS-1 and IRS-2 as described in Materials and Methods. PCR products were revealed by ethidium bromide staining under UV after agarose gel electrophoresis. GAPDH was used as a control.

EGF induces IRS-1 and IRS-2 transcription. Next, we explored potential mechanisms of this EGF up-regulation of IRS-1 and IRS-2. It is well known that IGF-I induces the 26S proteasome-mediated degradation of IRS proteins (17, 18). To assess whether the EGF effect also involves an alteration of IRS protein stability, we treated MDA-MB-468 breast cancer cells with the protein translation inhibitor cycloheximide before stimulating the cells with EGF in the continued presence of the inhibitor. Total proteins were then harvested at different time points. As illustrated in Fig. 3A, cycloheximide treatment virtually abolished the EGF up-regulation of IRS-1 and IRS-2 protein levels, whereas both IRS proteins display a relatively high stability even without EGF stimulation, suggesting that the EGF effects on IRS-1 and IRS-2 expression in breast cancer cells require *de novo* protein synthesis and are not mediated by an increase of IRS protein stability. This result is also consistent with the previous report that IRS-1 stability is relatively constant in the presence or absence of EGF in prostate epithelial cells (28).

To determine whether RNA synthesis is required in the EGF induction of IRS-1 and IRS-2, we first treated MDA-MB-468 breast cancer cells with the transcription inhibitor DRB before stimulating the cells with EGF in the presence of the inhibitor. Western blotting showed that blocking RNA synthesis by DRB completely nullified the increase of IRS-1 and IRS-2 protein levels in response to EGF treatment (Fig. 3B). To confirm that a transcriptional mechanism is involved in this EGF function, we isolated total RNA from these treated cells and did RT-PCR. As expected, 1-hour treatment of EGF dramatically increased IRS-1 and IRS-2 mRNA levels (Fig. 3C). Consistent with the results shown in Fig. 3B, DRB diminished this EGF effect on IRS-1 and IRS-2 transcription. Moreover, using a 2.4-kb human IRS-2 promoter-luciferase reporter construct (IRS-2-luciferase), we also found that IRS-2 promoter activity was increased by EGF (Fig. 4). In summary, these data indicate that

the control of IRS-1 and IRS-2 expression by EGF in breast cancer cells is mediated solely by a transcriptional mechanism.

EGF regulates IRS-1 and IRS-2 expression via distinct signaling pathways. The transmission of growth factor signals in cells involves several well-characterized signaling cascades downstream of the growth factor receptors, including the Akt, ERK1/2, and JNK pathways. Indeed, immunoblot analysis showed that strong phosphorylation of Akt, ERK1/2, and JNK was elicited in MDA-MB-468 cells after exposure to EGF for 10 minutes (Fig. 5A). To identify the signaling pathway implicated in the EGF up-regulation of IRS-1 and IRS-2, we used a series of small-molecule signaling inhibitors, which have been applied in our previous studies (29). Cells were first preincubated with the inhibitors SP600125 (JNK), LY294002 (PI3K/Akt), or U0126 (ERK) and then stimulated with EGF in the presence of these inhibitors. Western blot analysis showed that the EGF induction of IRS-2 was specifically blocked by SP600125, whereas the IRS-1 increase by EGF was mostly inhibited by U0126 and partially impaired by SP600125 (Fig. 5B). In contrast, LY294002 did not block the EGF effect on either IRS-1 or IRS-2. In addition, the glycogen synthase kinase-3 inhibitor maleimide, the p38 MAPK inhibitor SB203580, the protein kinase A inhibitor H-89, and the PKC inhibitor GF109203X did not suppress this EGF effect (data not shown). Our finding that ERK activation is essential for the EGF regulation of IRS-1 is consistent with a previous study showing that activation of ERK1/2 by fibroblast growth factor treatment or constitutively active MEK1 expression increases IRS-1 levels in MCF-7 breast cancer cells (33).

To confirm that JNK signaling is critically involved in the IRS-2 up-regulation by EGF, we overexpressed a FLAG-tagged DN-JNK (T183A/Y185F) in MDA-MB-468 cells. The increase of IRS-1 and IRS-2 levels in response to EGF was compared in control and DN-JNK-expressing cells. As shown in Fig. 5C, ectopic expression of DN-JNK significantly impeded the EGF modulation of IRS-2 levels, whereas it only mildly affected the elevation of IRS-1 levels. Noticeably, the effect of DN-JNK expression was not as dramatic as that of SP600125, most probably because small-molecule inhibitors are much more efficient than dominant-negative proteins in suppressing kinase activities (data not shown). Thus, these results indicate that JNK activity is essential for the EGF induction of IRS-2 and is also partially required for the increase of IRS-1.

To further investigate whether activation of JNK signaling is sufficient to mimic the EGF up-regulation of IRS-1 and IRS-2, we treated MDA-MB-468 cells with anisomycin, a potent elicitor of JNK activation, for 1 hour. As this antibiotic is also a protein translation inhibitor, we examined changes of IRS-1 and IRS-2 transcription. To this end, total RNA was isolated and subjected to RT-PCR. As illustrated in Fig. 5D, 1-hour anisomycin treatment markedly raised IRS-1 and IRS-2 mRNA levels, although not to the same extent as EGF treatment. We speculated that this discrepancy may be attributed to the broad function of EGF on cellular gene expression and perhaps the inhibition of general protein synthesis by anisomycin. To consolidate the study of JNK signaling in IRS-1 and IRS-2 expression, we ectopically overexpressed a constitutively active Myc-tagged JNK-MKK7 fusion protein (CA-JNK) in MDA-MB-468 cells and compared their IRS-1 and IRS-2 levels with those in vector-transfected cells. Western blot analysis showed that expression of CA-JNK robustly lifted the basal IRS-2 levels and mildly increased the basal IRS-1 levels in EGF-untreated cells (Fig. 5E). There was no significant difference when comparing

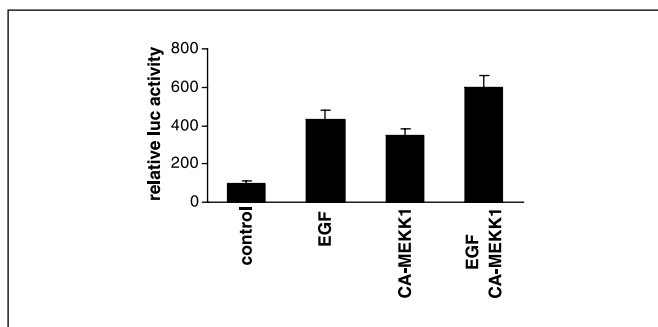


Figure 4. MEKK1 activates IRS-2 promoter activity. MDA-MB-468 cells were transiently cotransfected with the IRS-2 promoter reporter plasmid and either a vector expressing the constitutively active MEKK1 mutant CA-MEKK1 or the empty vector. Cells were grown for 12 hours and subsequently treated with 50 ng/mL EGF for an additional 12 hours. Cells were then lysed and luciferase assays were done. Columns, mean relative luciferase activity compared with the control; bars, SD. Representative of three independent experiments, each done in duplicate.

EGF-induced IRS-1 and IRS-2 levels in vector- and CA-JNK-transfected cells. As expected, CA-JNK was constitutively phosphorylated under unstimulated conditions and its phosphorylation was enhanced by EGF probably because CA-JNK expression driven by cytomegalovirus promoter was further enhanced by EGF (data not shown). The observation that expression of CA-JNK did not much raise basal IRS-1 levels may provide additional evidence that JNK is not the dominant signaling pathway controlling IRS-1 expression. Consistent with these data, the high levels of IRS-2 in 4T1 mouse mammary tumor cells were also dramatically down-regulated by shRNA knockdown of JNK2 (Fig. 5F). In conclusion, our data indicate that JNK signaling particularly mediates EGF-modulated IRS-2 expression in breast cancer cells.

It is well established that JNK can be activated by MEKK1 through MKK4 and/or MKK7. Hence, we assessed whether MEKK1

activation also regulates IRS-2 transcription. To this end, MDA-MB-468 cells were transiently cotransfected with a human IRS-2 promoter reporter construct and the plasmid pFC-MEKK1 containing a NH₂-terminally truncated constitutively active MEKK1 (CA-MEKK1). The cells were then treated with EGF for 12 hours. As illustrated in Fig. 4, EGF alone increased IRS-2 promoter activity by 4-fold, whereas overexpression of CA-MEKK1 raised IRS-2 promoter activity, in the absence of EGF, up to the same level detected in EGF-alone transfections. Not surprisingly, EGF combined with CA-MEKK1 further enhanced IRS-2 promoter activity compared with EGF-alone or CA-MEKK1-alone, which may be because EGF can activate endogenous MEKK1. Taken together, these data strengthen the conclusion that JNK signaling mediates the EGF control of IRS-2 expression in breast cancer cells.

AP-1 activity is required for the EGF up-regulation of IRS-2.

One of the best-studied targets of JNK is AP-1 family of transcription factors (34). We thus explored whether AP-1 proteins relay JNK signaling and thus are involved in the induction of IRS-2 transcription in response to EGF treatment. For this purpose, we first transiently transfected MDA-MB-468 cells with an AP-1-luciferase reporter construct and then treated the cells with EGF in the presence and absence of the inhibitors SP600125 (JNK) and U0126 (ERK). As illustrated in Fig. 6A, EGF dramatically increased the AP-1-driven luciferase activity. Inhibition of JNK with SP600125 significantly impaired the EGF-induced AP-1 activity, whereas blockade of ERK with U0126 did not impede the EGF-enhanced AP-1 activity. In addition, repressing other signaling pathways, such as PI3K, p38 MAPK, PKA, and PKC, also failed to impede EGF stimulation of AP-1 activity (data not shown). These data suggest that JNK is specifically involved in the activation of AP-1 transcriptional function induced by EGF in breast cancer cells.

Next, we examined whether AP-1 function was essential for the IRS-2 up-regulation by EGF. To this end, we used the IRS-2 promoter reporter construct in cotransfection assays. As shown in Fig. 6B, expression of either a A-Fos or a TAM67 (35), both of which

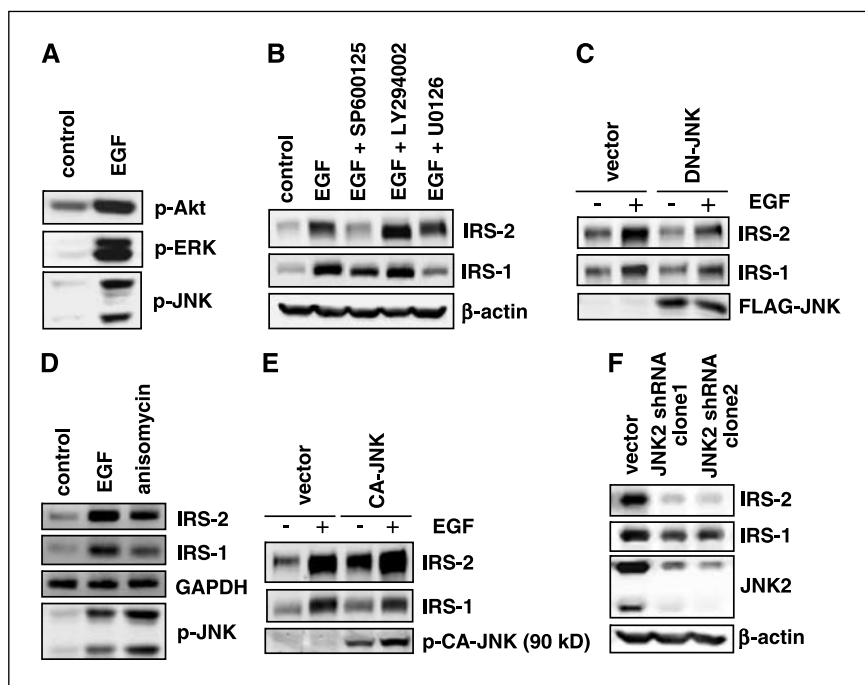


Figure 5. EGF induces IRS-1 and IRS-2 expression via distinct signaling pathways. A, MDA-MB-468 cells were stimulated with 50 ng/mL EGF for 10 minutes in SFM. Cell lysates were harvested and immunoblot analysis was done for phosphorylated Akt (p-Akt), phosphorylated ERK (p-ERK), and phosphorylated JNK (p-JNK). B, MDA-MB-468 cells were stimulated with 50 ng/mL EGF for 24 hours in the continued presence of the signaling inhibitors after preincubation for 30 minutes with 10 μmol/L SP600125 (JNK), 20 μmol/L LY294002 (PI3K), or 5 μmol/L U0126 (ERK). Cell lysates were immunoblotted for IRS-1 and IRS-2. C, control MDA-MB-468 cells and cells overexpressing DN-JNK were treated with 50 ng/mL EGF for 24 hours. Cell lysates were immunoblotted for IRS-1 and IRS-2. D, MDA-MB-468 cells were stimulated with 50 ng/mL EGF or anisomycin for 1 hour. Total RNA was isolated and 100 ng RNA was used in RT-PCR of IRS-1 and IRS-2. PCR products were revealed by ethidium bromide staining under UV after agarose gel electrophoresis. GAPDH was used as a control. E, control MDA-MB-468 cells and cells overexpressing the CA-JNK were treated with 50 ng/mL EGF for 24 hours. Cell lysates were immunoblotted for IRS-1 and IRS-2. F, 4T1 mouse breast cancer cells were stably transduced by a lentiviral construct pLKO-puro-expressing JNK2 shRNA. Cell lysates of control and stable clones were immunoblotted for IRS-1, IRS-2, and JNK2.

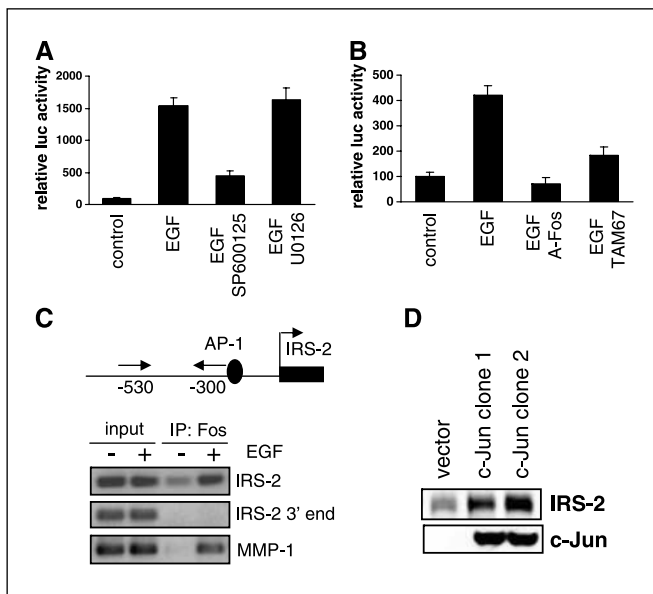


Figure 6. AP-1 activity is required for the EGF induction of IRS-2. **A**, MDA-MB-468 cells were transiently transfected with the IRS-2 promoter reporter plasmid and treated with EGF for 12 hours in the presence of 10 μ mol/L SP600125 or 5 μ mol/L U0126 after preincubation with the inhibitors for 30 minutes. Cells were then lysed and luciferase assays were done. *Columns*, mean relative luciferase activity compared with the control; *bars*, SD. **B**, MDA-MB-468 cells were transiently cotransfected with the IRS-2-luciferase reporter plasmid and AP-1 dominant-negative mutants A-Fos or TAM67. Cells were grown for 12 hours and subsequently treated with 50 ng/mL EGF for an additional 12 hours. Cells were then lysed and luciferase assays were done. **C**, MDA-MB-468 cells were stimulated with 50 ng/mL EGF for 6 hours and chromatin immunoprecipitation assays were done as described in Materials and Methods. Antibodies against Fos proteins were used to immunoprecipitate AP-1-DNA complexes. The amplified IRS-2 promoter region is -545/-300. The IRS-2 region as a negative control is 3.5 kb from the transcription initiation site. The MMP-1 promoter fragment (-242/-3) was used as a positive control. The input control was 1% of the protein-chromatin supernatant subjected to chromatin immunoprecipitation assays. **D**, a pcDNA3.1 construct expressing c-Jun was stably transfected into MCF-7 cells. Cell lysates were subjected to immunoblotting.

inhibit AP-1 transactivation of gene transcription, suppressed the EGF-stimulated IRS-2 promoter activity. This indicates that AP-1 function contributes to the activation of IRS-2 expression in response to EGF. Not surprisingly, because A-Fos has been shown to inhibit AP-1 activity more potently than other forms of AP-1 loss-of-function mutants (31), A-Fos displayed a stronger inhibition of the EGF-induced IRS-2 promoter activity than TAM67 (Fig. 6B). Interestingly, we also found that EGF markedly elevated c-Jun and c-Fos levels in MDA-MB-468 cells (data not shown), which may further enhance the activation of IRS-2 transcription by AP-1.

There are several potential AP-1-binding sites in the 2.4-kb IRS-2 promoter compared with the consensus AP-1-binding site TGACTCA. To test whether AP-1 is recruited to the IRS-2 promoter when cells are exposed to EGF, we did chromatin immunoprecipitation assays. The amplified promoter region is near one potential AP-1 site, -300 bp from the transcription initiation site. As illustrated in Fig. 6C, EGF treatment of MDA-MB-468 cells caused a pronounced increase in AP-1 binding to the IRS-2 promoter. As expected, chromatin immunoprecipitation assays also showed that AP-1 binding to the MMP-1 promoter is enhanced by EGF stimulation, which is consistent with previous findings that MMP-1 is a typical AP-1-regulated gene (32, 36). Moreover, the barely

detectable levels of IRS-2 in MCF-7 breast cancer cells were markedly increased by stable overexpression of c-Jun (Fig. 6D). Taken together, our data suggest that the MEKK1/JNK/AP-1 signaling pathway mediates the IRS-2 induction by EGF in breast cancer cells. Although ERK has been shown to activate AP-1 in some studies (37), we have shown that EGF-elicited AP-1 activity in breast cancer cells is dependent on JNK signaling. Thus, transcription factors other than the AP-1 proteins may mediate the increase of IRS-1 by ERK activation.

EGF potentiates IGF- and integrin-induced IRS signaling. As IRS are signaling adaptors to integrate and coordinate IGF and other extracellular signals, we evaluated whether EGF treatment of breast cancer cells and the consequent up-regulation of IRS-2 could affect the activation of IRS-2 by IGF-I. With this in view, we pretreated MDA-MB-468 cells with EGF for 12 hours and then stimulated the cells with IGF-I for 10 minutes. Immunoprecipitation of IRS-2 followed by immunoblotting showed that exposure to EGF increased levels of total cellular IRS-2 and its tyrosine phosphorylation induced by IGF-I (Fig. 7A). A well-documented feature of the activated IRS proteins is their association with the PI3K regulatory subunits p85 and Grb2 (1), leading to activation of the PI3K/Akt and Ras/ERK pathways. As shown in Fig. 7A, EGF indeed enhanced the IGF-I-elicited binding of p85 and Grb2 to IRS-2. This is most probably due to higher levels of tyrosine-phosphorylated IRS-2 in cells, because total cellular p85 and Grb2 levels were not altered by EGF. As for IRS-1, the identical procedure

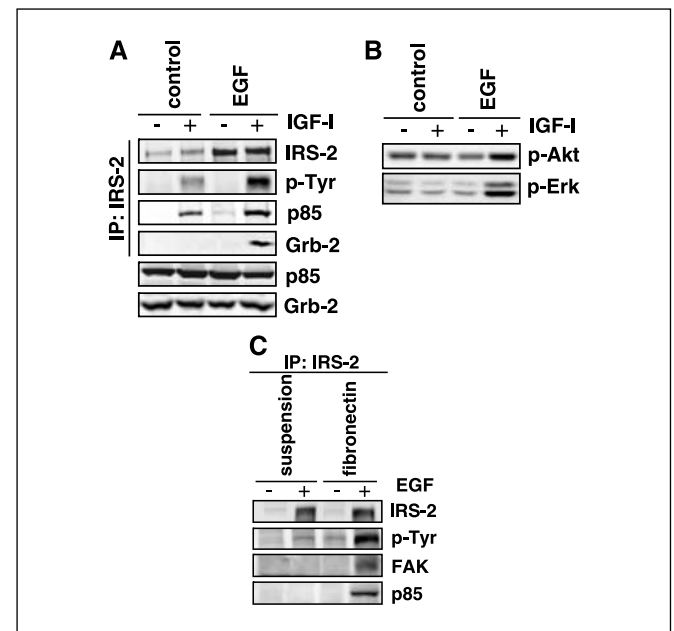


Figure 7. EGF potentiates IGF- and integrin-induced IRS signaling. **A**, MDA-MB-468 cells were first treated with 50 ng/mL EGF for 12 hours and then stimulated with 100 ng/mL IGF-I for 10 minutes. Cell lysates (1 mg) were immunoprecipitated with an anti-IRS-2 antibody. Aliquots of the immunoprecipitates were subjected to immunoblot analysis for the PI3K subunit p85 and for Grb2. Tyrosine phosphorylation of IRS-2 was detected with the antibody PY20 against phosphotyrosines. Total cellular p85 and Grb2 levels were also examined. **B**, same treatment as in (A). Activation of Akt and ERK were determined by Western blot analysis with their corresponding phosphorylation-specific antibodies. **C**, MDA-MB-468 cells were first treated with 50 ng/mL EGF for 12 hours, trypsinized, and plated on 5 μ g/cm² fibronectin for 40 minutes. Cell lysates were immunoprecipitated with an anti-IRS-2 antibody. Aliquots of the immunoprecipitates were subjected to immunoblot analysis for p85, FAK, and tyrosine phosphorylation of IRS-2.

was also conducted and the result was the same as observed for IRS-2 (data not shown). Furthermore, as hallmarks of signaling activation downstream of IRS, IGF-I-induced Akt and ERK activation was enhanced by EGF pretreatment (Fig. 7B). This was attributed to the increase of both IRS, because knockdown of both proteins with the respective siRNAs abolished this effect (data not shown). Thus, these data suggest that EGF potentiates IGF-induced IRS signaling via up-regulation of IRS-1 and IRS-2 levels in breast cancer cells.

Interestingly, IRS-1 has been shown to interact with integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ (38, 39), and clustering of the laminin receptor $\alpha_6\beta_4$ can tyrosine phosphorylate both IRS to activate PI3K signaling (40). Furthermore, IRS-1 was found to directly associate with FAK (41). To address the question whether EGF-increased IRS-2 can also be engaged in integrin signaling, we first treated MDA-MB-468 cells with EGF and then plated them on fibronectin. Immunoblotting after immunoprecipitation by anti-IRS-2 antibodies showed that EGF dramatically enhanced IRS-2 tyrosine phosphorylation levels elicited by fibronectin-mediated integrin engagement (Fig. 7C). In correlation, the p85 binding to IRS-2 was also increased. Moreover, association of FAK with IRS-2 after integrin engagement was detected only under EGF pretreatment conditions, suggesting that the dramatically elevated IRS-2 levels are involved in integrin/FAK signaling. Thus, these data suggest that exposure to EGF also potentiates integrin-elicited IRS-2 signaling in breast cancer cells.

IRS-2 is required for EGF-enhanced cell migration. It is well documented that overexpression of IRS-1 promotes cell proliferation in breast cancer cells (42). Interestingly, high levels of IRS-2 and its tyrosine phosphorylation have been reported to be associated with IGF-I-induced breast cancer cell motility (43, 44). In correlation, invasive breast cancer cell lines (by the invasion chamber assay), such as MDA-MB-231 and MDA-MB-435, possess much higher IRS-2 than noninvasive breast cancer cell lines, such as MCF-7 and T47D. On the other hand, IRS-1 frequently exhibits higher levels in noninvasive breast cancer cells (40, 43). In MDA-MB-468 cells, EGF treatment induces epithelial-mesenchymal transition (EMT) and associated migratory behavior as well as decreased cell adhesion to extracellular matrix proteins, although it does not markedly alter cell proliferation. As EGF dramatically up-regulates IRS-2 in MDA-MB-468 cells and this increased IRS-2 level is comparable with those observed in invasive breast cancer cell lines, whereas IRS-1 levels are still lower than those in MCF-7 cells, we speculated that induced IRS-2 may play an important role in EGF-elicited cell migration. To address this question, we used specific siRNA to knockdown IRS-1 and IRS-2 and thus block their induction by EGF in MDA-MB-468 cells. As indicated in Fig. 8A, the presence of IRS-1 siRNA specifically prevented the EGF induction of IRS-1, whereas IRS-2 siRNA exclusively inhibited the increase of IRS-2 levels. These siRNAs did not significantly decrease the basal target levels most probably due to the very low IRS expression under non-EGF conditions. The *in vitro* cell migration assay was then done. As presented in Fig. 8B, pretreatment with EGF markedly enhanced cell migration and blockade of IRS-2 elevation abolished this EGF effect, whereas inhibition of IRS-1 increase did not exhibit any effect. Use of control siRNA also did not cause any change of cell migration after EGF stimulation. In agreement with the results from the cell migration assay, siRNA suppression of IRS-2 up-regulation also significantly impeded EGF-triggered cell invasion when Matrigel matrix-coated invasion chambers were used (Fig. 8C). It is speculative that this IRS-2 role in cell invasion

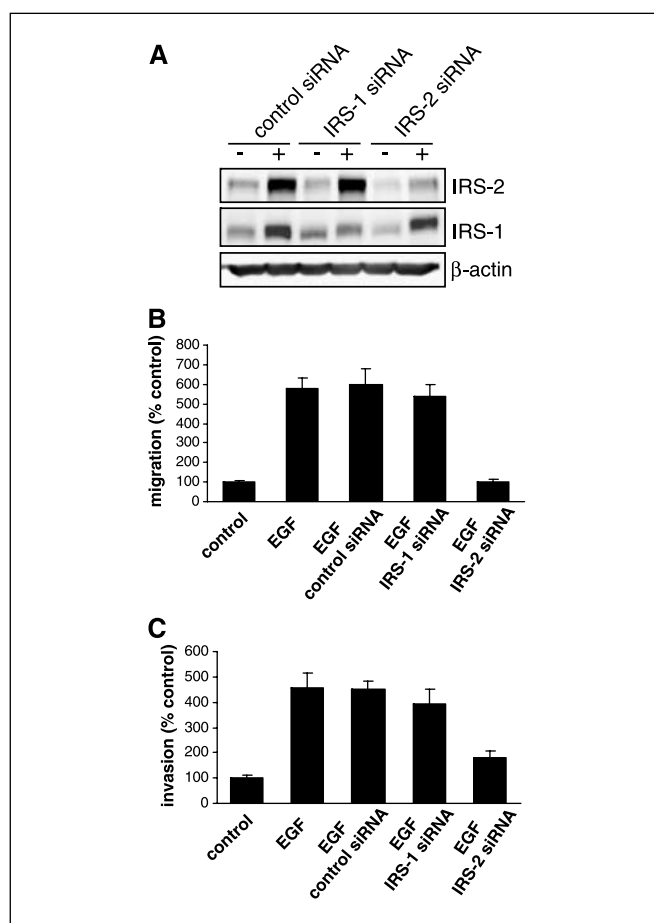


Figure 8. IRS-2 is essential for EGF-enhanced cell migration. *A*, MDA-MB-468 cells were transfected with control siRNA or siRNA against IRS-1 or IRS-2 for 24 hours. Then, the cells were stimulated with 50 ng/mL EGF for 12 hours, lysed, and immunoblotted for IRS-1 and IRS-2 to assess their reduction by siRNA knockdown. *B*, same treatment as in (*A*). Cells were trypsinized and subjected to the *in vitro* migration chamber assay with 5% serum medium as chemoattractant. The migration ability is presented as percent increase of migrating cells over the non-EGF-treated control. Columns, mean of samples measured in triplicate; bars, SD. Representative of three independent experiments. *C*, same treatment as in (*A*). Cells were assayed for their ability to invade through Matrigel coated on the chamber upper surface.

may be attributed to its requirement for EGF-elicited cell migration. In summary, our data indicate that IRS-2 is essential for EGF-induced and EMT-associated cell migration and invasion in breast cancer cells.

Discussion

In this study, we have revealed a novel mechanism of regulation of IRS-2 in breast cancer cells. Previously, we and others have shown that steroid hormones, such as estrogen and progesterone, transcriptionally up-regulate IRS-1 and IRS-2 levels, whereas IGF-I down-regulates IRS-1 and IRS-2 through a different mechanism (i.e., a proteasome-mediated protein degradation pathway). In addition, insulin has been found to inhibit IRS-2 transcription most likely through a PI3K/Akt-dependent repression of the binding of forkhead family transcription factors to an insulin response element in the IRS-2 promoter (45). Our studies show that through a well-defined signal transduction cascade, MEKK1/JNK/AP-1, IRS-2 transcription can also be dramatically induced in response to

EGF. This finding, together with other reports showing that IRS-2 is regulated by an array of extracellular stimuli in the mammary gland, further implicates the role of IRS-2 in breast cancer development and progression.

By showing that EGF has a profound effect on IRS-1 and IRS-2 expression and their downstream signaling, we also provide new implications to the long-held observation that EGF and the IGFs cross-talk with each other to regulate cellular functions. Mammary epithelial cells, for example, display an absolute requirement for exogenous EGF and IGF-I for growth (25, 26). Blockade of IGF signaling by IGFBP-3 not only inhibits the effects on mammary epithelial cell growth by the IGFs in combination with EGF but also impedes EGF-alone-stimulated growth (46), suggesting that the maximal EGF effect on cell growth relies on the presence of IGF signals. We propose three possibilities that may account for this growth factor synergism. First, EGF and IGF-I preferentially activate different signaling pathways (i.e., EGF strongly activates ERK signaling, whereas IGF-I potently provokes Akt signaling in mammary epithelial cells).¹ It is conceivable that these two signaling pathways may cooperatively regulate cell cycle and antiapoptosis proteins, because they target different proteins in these processes (26, 47, 48). A second explanation may be that EGFR and IGF-IR transactivate each other. EGF induces activation of IGF-IR in mouse embryo fibroblasts and rat hepatocytes potentially via a Src-mediated signaling pathway (49, 50). On the other hand, IGF-I has also been shown to cause activation of EGFR and consequently induction of the ERK pathway in mammary epithelial and breast cancer cells (51, 52). It was proposed that a MMP-dependent release of heparin-binding EGF may be involved in IGF-triggered EGFR phosphorylation (52). In most of these studies, IGF-IR and EGFR were found to form a complex. A third explanation may reside in the EGF modulation of IGF signaling transduction elements. As we present in this study, EGF considerably increases IRS-1 and IRS-2 and consequently increases their downstream signaling. It is well documented that levels of IRS, crucial components of IGF signal transduction, dictate the strength of the mitogenic response to IGF-I (53, 54). It should also be noted that regulation of IRS-1 and IRS-2 is a common mechanism used by many mitogens to fine-tune cell response to the IGFs (55).

Recently, emerging evidence suggests that IRS-2 is involved in IGF-I-induced breast cancer cell migration and invasion (43, 44, 56). It has been found that high levels of IRS-2 and its tyrosine phosphorylation are associated with IGF-I-induced breast cancer cell motility (43, 44). IRS-2 has also been implicated in integrin-mediated cell migration and invasion (40). Consistent with these findings, a recent report has shown that the latency and incidence of metastases in mice with overexpression of polyoma virus middle T antigen in the mammary gland were significantly decreased in the absence of IRS-2 expression (56). Interestingly, the loss of IRS-1, unlike that of IRS-2, did not suppress metastasis of mouse mammary tumors, suggesting that IRS-2 possesses a unique role in cancer metastasis. It is well known that EGF promotes tumor cell motility and invasion. Here, we have shown that IRS-2 is essential for EGF-potentiated cell migration and invasion in breast cancer cells. In addition, EGF can also cause EMT in EGFR-overexpressing cancer cells, such as MDA-MB-468

cells. Increase of cell motility represents one of the hallmarks of EMT. Our data suggest that EGF increases IRS-2 to facilitate EMT-associated breast cancer migration, further consolidating the notion that IRS-2 may serve in the general protein machinery for cell migration.

Although IRS-1 and IRS-2 clearly have diverse roles in cell proliferation and metabolism, the ability of IRS-2 but not IRS-1 to affect migration and invasion in multiple cell types is a strong indication that IRS-2 is able to activate unique signaling pathways involved in cell migration and that these pathways are not induced by IRS-1. However, the underlying mechanism distinguishing IRS-2 from IRS-1 in this respect is still not understood. To make matters more complicated, both IRS-1 and IRS-2 have been shown to associate with proteins regulating cell movement (e.g., integrins, FAK, and PI3K; refs. 38–40). One explanation for the distinct function of IRS-1 and IRS-2 in cell motility may reside in their subtly different downstream signaling cascades on binding to integrins. Previously, it has been shown that IRS-1 and IRS-2 PH domains bind preferentially to phosphatidylinositol lipids PI(3,4,5)P₃ and PI(3,4)P₂, respectively (57). Whether this promotes IRS-2 interaction with PIP2-binding proteins, such as TAPP1 (58), which is involved in cytoskeleton reorganization, remains to be examined. Another intriguing hypothesis is related to the IRS-2 role in metabolism. It has been shown that IRS-2 is linked to lipid biosynthesis (59). Numerous reports have indicated a critical role of lipids in cell migration and invasion (60), but further studies will be required to determine whether the IRS-2 effect on cell migration is mediated, at least in part, through lipid turnover.

In mouse fibroblasts, cell adhesion regulates IRS-1 transcription, and JNK is partially responsible for this integrin effect (24). In agreement with these studies, we found that JNK is to some extent involved in the EGF up-regulation of IRS-1 in breast cancer cells, whereas it is essential for the EGF-induced increase of IRS-2. The JNK pathway has been implicated previously in cell migration and invasion. It has been shown that JNK activation is correlated with an increase in cell migration elicited by different signals (61). In addition, the JNK/AP-1 signaling pathway is involved in constitutive MMP expression in various carcinoma cells (36, 62). Association between increased JNK/c-Jun activation and distant metastasis has also been reported in breast cancer (63). Given the facts that IRS-2 is overexpressed in highly invasive breast cancer cell lines and that IRS-2-null mouse mammary tumors have low metastatic capability, we speculate that IRS-2 induction may play a role in cancer progression characterized by high JNK/AP-1 activity. Furthermore, it has been reported that activated JNK/AP-1 is associated with tamoxifen resistance in a ER-positive breast cancer xenograft model (64). Because IRS-mediated ERK and Akt activation is involved in antiapoptosis, it is conceivable that JNK/AP-1 induction of IRS-2 may also contribute to the tamoxifen resistance feature in aggressive breast cancer types.

In summary, our findings identify a novel mechanism of cross-talk between the EGF and IGF signaling pathways. Because IRS are critical elements of IGF signaling, explorations of interactions between the IGFs and other growth factors have naturally centered on IRS proteins. The EGFR inhibitor Iressa has been used in clinical cancer treatment, and our studies may implicate the potential disruption of IRS-1 and IRS-2, and thus IGF functions, in the efficacy of targeting EGFR. As IRS are also involved in non-IGF-initiated cell responses, it is conceivable that increased IRS will

¹ X. Cui and A.V. Lee, unpublished data.

exert fully IGF-independent functions. IRS, especially IRS-2, may represent as useful markers for aggressive tumor types and may also be exploited as novel therapeutic targets.

Acknowledgments

Received 8/16/2005; revised 2/24/2006; accepted 3/14/2006.

Grant support: Department of Defense Breast Cancer Research Program postdoctoral fellowship award DAMD 17-01-1-0133 and NIH/National Cancer Institute

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