Interaction between nuclear insulin receptor substrate-2 and NF-κB in IGF-1 induces response in breast cancer cells

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Abstract. Despite significant homology between IRS-1 and IRS-2, recent studies have revealed distinct functions for these adaptor proteins in regulating breast cancer progression. Thus far, most of the studies on breast cancer have focused upon IRS-1, the biological pattern of IRS-2 is limited. We demonstrated that depletion of endogenous IRS-2 by antisense strategies impaired cell proliferation after serum withdrawal, blunted PI3K/Akt and NF-κB activation in IGF-1 induced response in MCF-7 and BT-20 breast cancer cells. In addition, IGF-1 promote nuclear translocation of IRS-2 and NF-κB in MCF-7 and BT-20 cells. Nuclear IRS-2 interaction with NF-κB-p65 and PI3K binding tyrosine residues of IRS-2 are crucial for the NF-κB activities. Moreover, nuclear IRS-2 is recruited to the cyclin D1 promoter both in MCF-7 and BT-20 cells. The selective inhibition of NF-κB-65 abolished the occupancy of IRS-2 to the cyclin D1 promoters. Our studies suggest that IRS-2 plays a significant role by activating, at least in part, NF-κB via PI3K/Akt pathway in IGF-1-induced responses in breast cancer cells and the crosstalk between nuclear IRS-2 and NF-κB might be responsible for transcriptional progression of the breast cancer cells.

Introduction

The insulin receptor substrate (IRS) proteins, IRS-1 and IRS-2, are important adaptor molecules that organize signaling complexes downstream of IGF-1 activated cell surface receptors in breast cancer cells (1,2). IRS-1 and IRS-2 are expressed in normal mammary epithelial cells and in breast carcinoma cells, where they have been implicated in mediating signals to promote tumor cell survival, growth and motility (3). Despite significant homology between IRS-1 and IRS-2, recent studies have revealed distinct functions for these adaptor proteins in regulating breast cancer progression (4,5).

Thus far, most of the studies on breast cancer have focused upon IRS-1, the biological pattern of IRS-2 is limited. Aberrant expression of IRS-2 has been associated with pathogenesis of many diseases, including diabetes and cancer. Activated IRS-2 transmits signals from IGF-1R by sequestering multiple effectors and stimulating different signaling pathways, including the PI3K/Akt pathways (6). While IRS-1 expression is regulated by estrogen, IRS-2 expression is regulated by progesterone and cyclic AMP-mediated activation of CREB. In ER-breast carcinoma cell lines, which lack or have decreased IRS-1expression but retain IRS-2 expression, IGF-1R is still required for metastasis but no longer promotes mitogenesis (7,8).

It was also shown that IRS-2 is an essential intermediate in the activation of phosphatidylinositol 3-kinase (PI3K) and promotion of breast carcinoma invasion by the α6ß4-integrin receptor (9,10). IRS-2 also mediates IGF-1-dependent breast carcinoma cell migration and metastasis (11). Mammary tumor cells that lack IRS-2 expression are less invasive, whereas IRS-1$^{-/-}$ cells that express only IRS-2 are more invasive and aggressive, than WT cells that express both isoforms. IRS-2-null tumors express IRS-1, but this isoform is not sufficient to support the metastasis of mammary tumor cells. Moreover, IRS-2, but not IRS-1, promotes tumor cell invasion and survival *in vitro* (12,13). These results suggest that IRS-1 and IRS-2 are not functionally redundant in breast cancer. IRS-2 is capable of activating a unique signaling pathway that is essential for tumor progression and that this pathway is not activated sufficiently, or at all, through IRS-1.

In addition to its conventional role as a cytoplasmic signaling molecule, nuclear translocation of IRS-2 has been described in mouse embryo fibroblasts stimulated with IGF-1. IRS-2 accumulated in the nucleoli and interacted with the upstream binding factor 1 (UBF1), a regulator of RNA polymerase I. In this cell model, the presence of nucleolar IRS-2 coincided with increased RNA synthesis

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Abbreviations: IGF-1R, IGF-1 receptor; IRS, insulin receptor substrate; MEF, mouse embryo fibroblast; PH, pleckstrin; PI3-K, phosphatidylinositol 3-kinase; SFM, serum free medium; NF-κB, the nuclear factor-κB; UBF, upstream binding factor

Key words: IRS-2, NF-κB, IGF-1 induced response, breast cancer cells, cyclin D1 promoters

(14,15). The mechanism by which IRS-2 is targeted to the nucleus is still unknown. Lines of evidences in a number of cell types suggest a functional interaction between IGF-1 and NF-κB. For example, insulin-like growth factor-1 prevented apoptosis by reciprocal NF-κB activation via PI3K-dependent pathway, and the activation of NF-κB is required for the upregulation of intracellular anti-apoptotic proteins via the IGF-1/Akt signaling (16-19).

Mammalian NF-κB is a group of transcription factors and upon activation by a wide variety of stimuli (growth factors, inflammatory cytokines and viral proteins), NF-κB translocates to the nucleus, where it modulates the expression of target genes involved in cell growth, survival, adhesion, and death (20). Constitutive NF-κB activity has been found in breast, prostate, colorectal, and ovarian cancers and in certain forms of leukemia and lymphoma (21,22). Recent studies have suggested that NF-κB regulates the expression of multiple genes involved in the metastasis of breast cancer cells. For instance, NF-κB promotes breast cancer cell migration and metastasis by inducing the expression of the chemokine receptor CXCR4, and the cooperation of Ras- and TGF-ß-dependent signaling pathways in late-stage tumorigenesis depends critically on NF-κB activity (23-26).

Since the existence of nuclear IRS-2 in breast cancer cells and its possible role in the regulation of gene expression have never been addressed (27). In the present study, we provide the requirement of IRS-2 involved in serum-free proliferation and PI3K/Akt activation and NF-κB activation of IGF-1 induced responses in MCF-7 and BT-20 breast cancer cells, the evidence for the functional significance of nuclear IRS-2 interaction with NF-κB and occupancy in the cyclin D promoter. These facts support the hypothesis that IRS-2 may play a significant role in the establishment of breast cancer cells.

Materials and methods

Cell lines and gene silencing of IRS-2. MCF-7 cells were grown in DMEM: F12 containing 5% calf serum (CS). BT-20 cells were grown in DMEM: F12 with 10% fetal bovine serum (FBS). In the experiments requiring IGF-1 and serum-free conditions, the cells were cultured in phenol red-free SFM MCF-7 cell lines. The mutant IRS-2 and 32D-derived cells as described previously in detail in previous studies (14).

Gene silencing of IRS-2 was obtained by RNA interference using small interfering RNA (siRNA) (28). Cells were transfected with vehicle (diethylpyrocarbonate-treated water), control siRNA (scrambled), or siRNA directed against IRS-2 (siRNA IRS-2: 5'-AAUAGCUGCAAGAGC GAUGAC-3') for each well of a 12-well plate, or control scrambled siRNA using the Gene porter system according to the manufacturer's instructions (Gene Therapy System, San Diego, CA). Cells were transfected with a siRNA targeted for rat NF-κB p65 (Santa Cruz Biotechnology, catalog no. sc-61876) using Lipofectamine 2000 (Invitrogen). An siRNA consisting of a scrambled sequence of similar length was similarly transfected as control siRNA. One day before transfection, cells were plated in 500 μl of growth medium without antibiotics such that they were 30-50% confluent at

the time of transfection. The transfected cells were cultured in DMEM containing 10% fetal calf serum for 72 h after transfection.

PI3 kinase and MAPK assay. PI3 kinase activity was determined by an *in vitro* kinase assay (Cell Signaling Technology Inc., MA). Cells were seeded into 96-well plates and incubated with IGF-I for 24 h. Cells were then fixed with 4% formaldehyde for 20 min at room temperature to preserve phosphorylation. Two primary antibodies are included in the kit. One antibody recognized only the activated (phosphorylated) form of the specific target protein (phospho-protein specific antibody), while another recognized the specific target protein regardless of its activation state (pan-protein specific antibody). Following incubation with primary and secondary antibodies, the amount of bound antibody in each well was determined using a developing solution and an ELISA plate reader. The absorbance readings were then normalized to relative cell number as determined by a cell staining solution. The relative extent of target protein phosphorylation was determined by normalizing absorbance reading of the phospho-protein specific antibody to the pan-protein specific antibody for the same experimental condition. Experiments were performed more than three times and data are expressed as percentage of control.

Confocal microscopy, subcellular fractionation and Western blotting. After fixation with 3% paraformaldehyde for 25 min and washing three times with PBS at room temperature, cells on cover slips were permeabilized with 0.2% Triton X-100 in PBS for 5 min, blocked with 10% normal donkey serum (sc-2044, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 20 min, and incubated for 1 h with the appropriate primary and secondary antibodies. Confocal analysis was carried out on an MRC-600 Ar/Kr laser scanning confocal microscope (Bio-Rad Laboratories, Inc., Hercules, CA) using a 40 objective (Carl Zeiss, Thornwood, NY) chromatin.

For subcellular fractionation, Western blotting, and immunoprecipitation: the nuclear pellet was passed through 5 ml sucrose buffer (10 mM Tris-HCl, pH 7.4, 15 mM NaCl, 60 mM KCl, 10% sucrose), washed three times with wash buffer (10 mM Tris-HCl, pH 7.4, 15 mM NaCl, 60 mM KCl), and resuspended in the lysis buffer containing 0.5 M NaCl to extract nuclear protein. The extract was centrifuged at 10,000 rpm for 10 min at 4˚C, and the supernatant was termed the 'nuclear fraction'. Cytoplasmic and nuclear fractions (50 μg) were separated on a 4-15% gradient gel (Bio-Rad Laboratories, Inc.) and transferred to a nitrocellulose membrane. In one experiment, the proteins were separated on a 7.5% gel. For immunoprecipitation, 200 μg of nuclear or cytoplasmic lysate were incubated for 2 h at 4˚C with the corresponding antibodies coupled to 20 μl of packed protein G-sepharose beads (Oncogene Science, Inc.). Immunocomplexes were resolved by means of SDS-PAGE and immunoblotted with the indicated antibodies.

GST pull-down analysis. All GST fusion proteins with various regions of IRS-2 were expressed in BL-21 bacterial cells (Invitrogen, Carlsbad, CA) and purified onto glutathione-agarose beads using standard techniques. Lysates were

prepared by lysis for 30 min on ice in 50 mm HEPES (pH 7.5), 1% Nonidet P-40, 1 mm EGTA, 10 mm NaF, 20 mm sodium pyrophosphate, 10 g/ml aproptinin, and 10 μg/ml leupeptin. The resulting supernatants were incubated with the immuno-immobilized GST proteins overnight and after extensive washing with 50 mM HEPES (pH 7.5), 150 mm NaCl, and 0.1% Triton X-100, the proteins that bound to IRS-2 or control GST proteins were analyzed by SDS-PAGE followed by immunoblotting with the appropriate antibodies.

Chromatin immunoprecipitation assays (ChIPs). Chromatin immunoprecipitation assays were carried out according to the manufacturer's instructions (Upstate, Lake Placid, NY) and the methods described (29). The primers we used were as follows: cyclin D1 forward, 5'-CGGACTAAGGGGA GTTTTGTTG-3'; reverse, 5'-TCCAGCATCAGGTGGCA CGAT-3'. Glyceraldehyde-3-phosphate dehydrogenase GAPDH) forward, 5'-TACAGGGTTTTACGGGCG-3'; reverse, 5'-TCCAGCATCCAGT GCACGAT-3'. Confluent cultures (90%) were shifted to SFM for 24 h and then treated with IGF-1, or left untreated in SFM. Following treatment, the cells were washed twice with PBS and crosslinked with 1% formaldehyde at 37˚C for 10 min. Next, the cells were washed twice with PBS at 41˚C, collected and resuspended in 200 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) and left on ice for 10 min. Then, the cells were sonicated four times for 10 sec at 30% of maximal power. The supernatants were collected and diluted in 1.3 ml of IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 16.7 mM NaCl) followed by immunoclearing with 80 ml of sonicated salmon sperm DNA/protein A agarose (UBI) for 1 h at 41˚C. The pre-cleared chromatin was immunoprecipitated for 12 h with specific antibodies. Precipitates were washed sequentially for 5 min with the following buffers: Wash A (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl); Wash B (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl); and Wash C (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and then twice with TE buffer (10 mM Tris, 1 mM EDTA). Epicentre Fail Safe PCR System (Epicentre, Madison, WI) was used for PCR. The amplification products were analyzed in a 2% agarose gel and visualized by SYBR Gold (Molecular Probes, Eugene, OR) staining. Gels stained with SYBR Gold were scanned by using Typhoon Laser scanner (Typhoon 9400 Variable Mode Imager, Amersham Biosciences, and Piscataway, NJ).

NF-κB binding activity and luciferase analysis. NF-κB binding activity was studied by using double-stranded oligo-nucleotides [5'-AGT TGA GGG GAC TTT CCC AGG C-3' (Promega), corresponding to the consensus NF-κB binding site]. The oligonucleotide probe was prepared by phos-phorylation with T4 polynucleotide kinase (Promega) in the presence of [γ-^{32}P]-ATP (Amersham), followed by inactivation of the kinase by adding 1 μl of 0.5 M EDTA. Nuclear proteins (10 μg) were preincubated for 10 min in NF-κB binding buffer (Promega). Radioactively labeled oligonucleotide was added and incubated for 30 min at room temperature. The complexes were then subjected to 6% non-denaturing acrylamide gel, electrophoresis and analyzed by autoradiography.

The cyclin D1 promoter driving luciferase was tested in transient. Luciferase determination was carried out by standard procedures (29). Efficiency of transfection was monitored as usual. Each cell line was transfected in a single batch, subdivided into aliquots measured at different intervals after transfection and IGF-1 (50 ng/ml) stimulation. Mock-transfected cells gave us a measure of the back-ground.

Statistical analysis. Experiments were performed in triplicate and repeated at least three times. Results are expressed as mean ± SD. All statistical analyses were performed with SigmaStat for Windows version 3.10 (Systat Software, Inc., Port Richmond, CA). Results were compared by using the two sided Student's t-test. Differences were considered statistically significant at $P<0.05$.

Results

Endogenous IRS-2 is required for cell proliferation after serum withdrawal, PI3K/Akt activation and NF-κB activation of IGF-1 induced responses in MCF-7 and BT-20 breast cancer cells. In consideration of the distinct functions of IRS-1 versus IRS-2 in breast cancer cells, we need to clarify the role of endogenous IRS-2 in IGF-1 induced response in MCF-7 and BT-20 breast cancer cells (BT-20 cells do not express endogenous IRS-1, so that the effect of IRS-1 is negligible) (2). Using siRNA strategies, we achieved almost complete depletion of endogenous IRS-2 levels, as compared with vehicle-treated or control-treated cells without any influences of both IRS-1 and internal control, GRB-2 (Fig. 1, top). Preliminary test showed that the same concentration of these siRNAs had no toxic effect on cells growing in serum. In Fig. 1, bottom, IRS-2 depletion induced a considerable growth reduction of MCF-7 and BT-20 cells in serum-deprived condition, suggesting that endogenous IRS-2 contributes to the ability of MCF-7 and BT-20 cells to grow in the absence of serum.

Previous study suggested that IRS-1 is not required for IGF-1-induced activation of PI3K in MCF-7 cells; however, the biological relevance of IRS-2 versus IRS-1 during this process still remains elusive (30). We determined PI3K/Akt activity in MCF-7 and BT-20 cells treated by IGF-1 in the presence or absence of IRS-2 siRNA. IGF-1 stimulated PI3K/Akt dramatically both in MCF-7 and BT-20 cells, with this stimulation being neutralized by the addition of IRS-2 siRNA. IRS-2 siRNA markedly abolished PI3K/Akt activities in the presence of IGF-1. In contrast, the activities of MAPK remained almost unchanged with the addition of IRS-2 siRNA. Fig. 2A shows IRS-2 siRNA can significantly blunt the PI3-K activities after IGF-1 stimulation; however, it had little effects on the MAPK activities. These results suggest that unlike IRS-1, IRS-2 seems to be at least in part required for activation of PI3K-dependent kinases by IGF-1 in MCF-7 and BT-20 breast cancer cells.

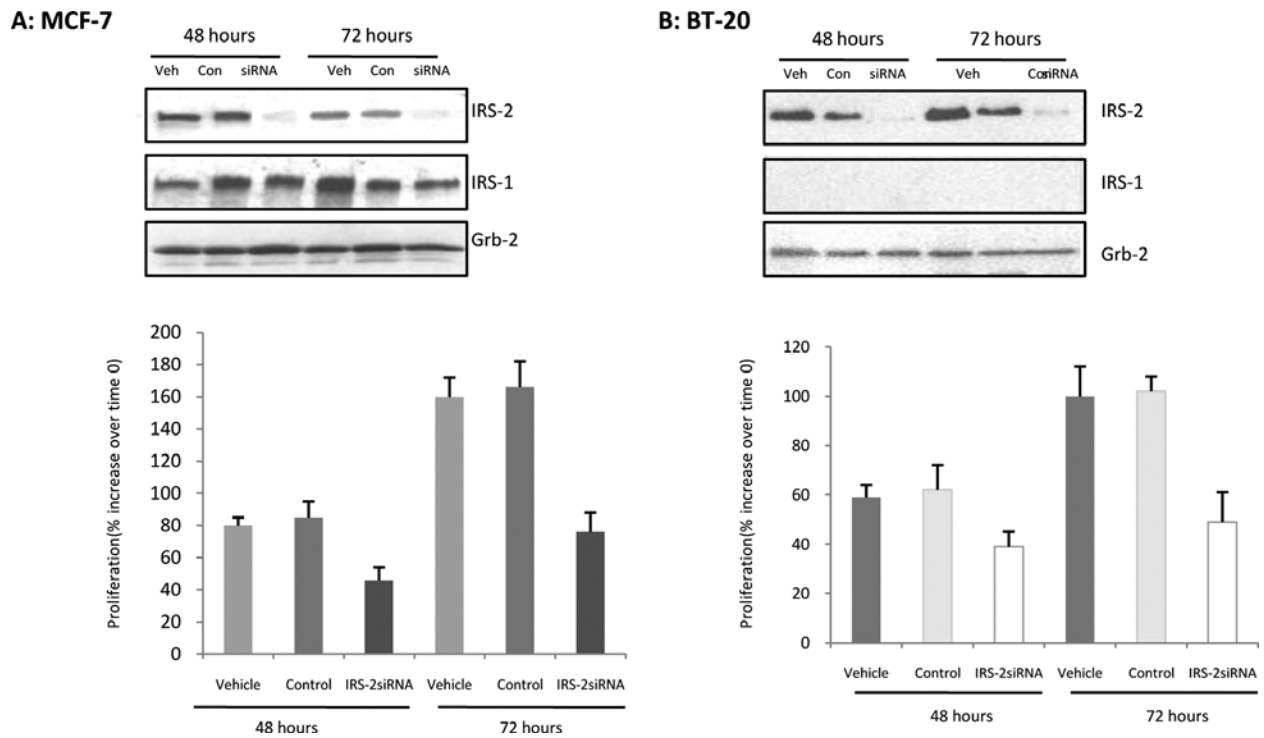Since NF-κB are the crucial downstream effectors depen-dent on PI3K/Akt in various cell backgrounds (31,32). We

Figure 1. IRS-2 is required for cell proliferation after serum withdrawal in MCF-7 and BT-20 breast cancer cells. Depletion of endogenous IRS-2 proteins effectively reduces proliferation of MCF-7 cells (A) and BT-20 cells (B) in serum-free condition (SFM). Gene knockdown for IRS-2 was achieved by siRNA. Cells at the indicated times for 48 and 72 h were processed and analyzed for growth. Values are expressed as percent increase over SFM. *P<0.05 compared with vehicle-treated control cells. The level of endogenous IRS-2 (one representative blot is shown) was detected by immunoblotting. Protein loading was normalized using anti-Grb2 antibodies.
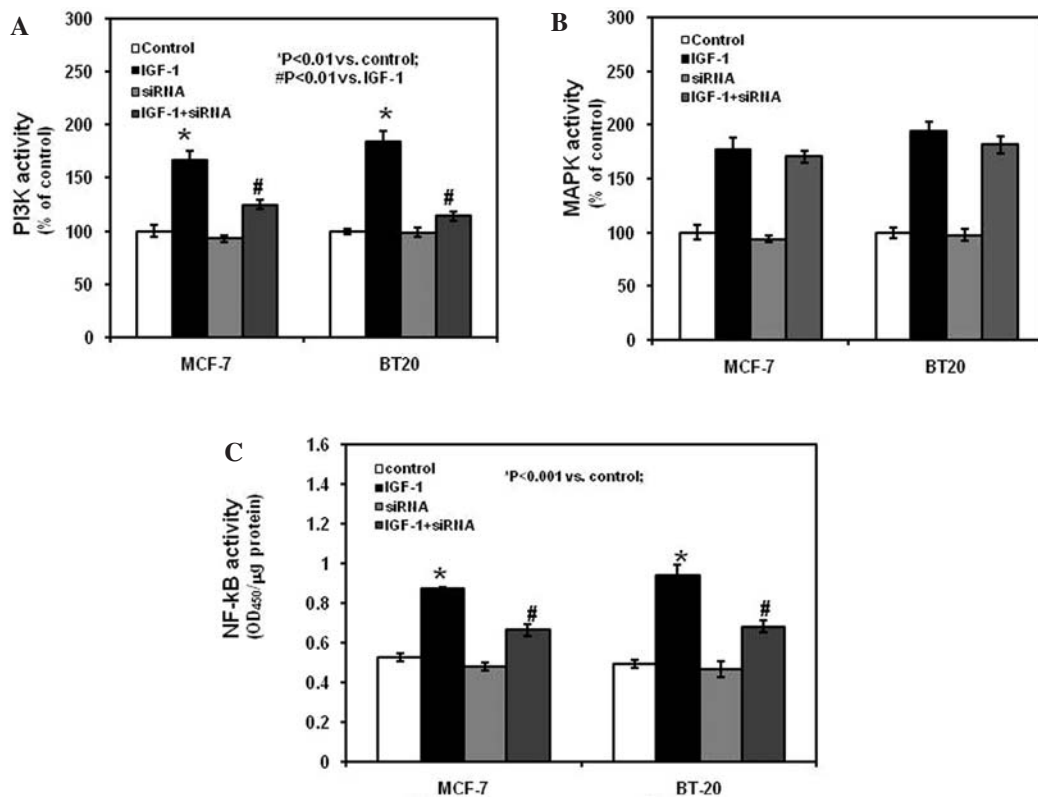

Figure 2. The effects of IRS-2 depletion on PI3K/Akt activation and NF-κB activation of IGF-1 induced responses in MCF-7 and BT-20 breast cancer cells. (A) Effects of IRS-2 siRNA on PI3K and MAPK activity. Total protein was extracted from cultured cells without or with 50 ng/ml IGF-1 for 24 h. PI3 Kinase activity was determined by ELISA kit (for details, see Materials and methods). (B) Effects of IRS-2 siRNA on MAPK activity. Experiments were performed more than three times and results are expressed as percentage of untreated control cells. (C) Effects of IRS-2 siRNA on NF-κB-DNA binding activity. Cells were cultured in the absence or presence of 50 ng/ml IGF-1. NF-κB-p65 transcription factor activity was determined by an enzyme-linked immunosorbent assay, according to the manufacturer's instructions. Data relative to the effects of 50 ng/ml IGF-1 are presented.
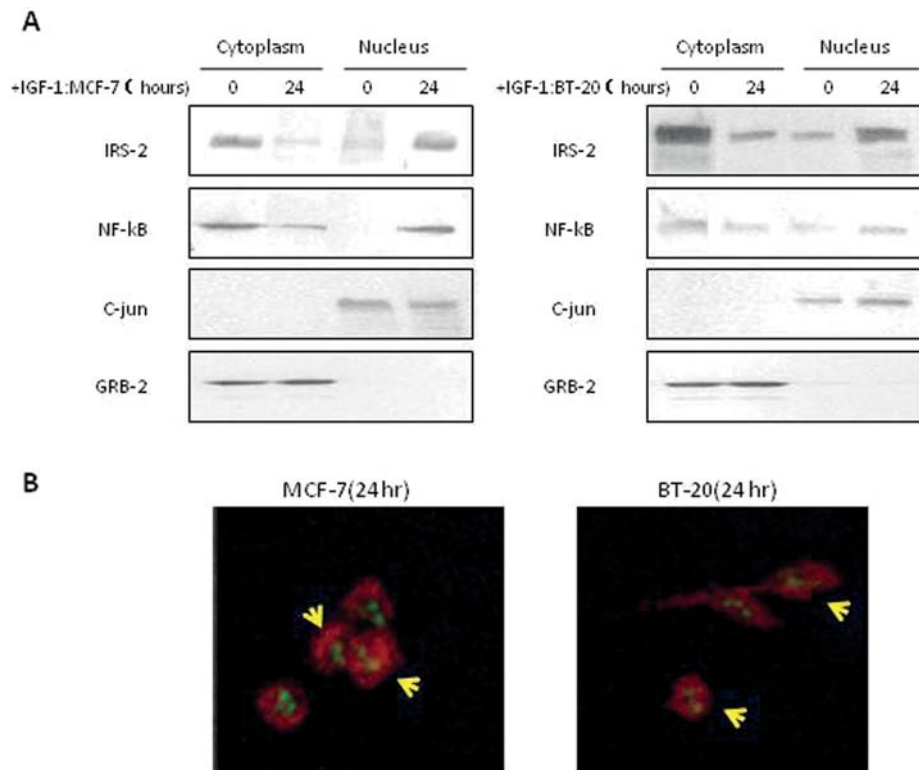
Figure 3. Subcellular localization of NF-κB and IRS-2 in IGF-1-treated MCF-7 and BT-20 cells. MCF-7 and BT-20 cells synchronized in SFM were treated with 50 ng/ml IGF-1 for 24 h or were left untreated. (A) The expression of IRS-2 (~160 kDa), NF-κB-p65 (~65 kDa), c-jun (~39 kDa), and GRB-2 (~25 kDa) was assessed by Western blot analysis in 100 μg cytoplasmic protein lysates or nuclear protein lysates. The expression of GAPDH and nucleolin were used as controls of cytoplasmic and nuclear protein loading, respectively. (B) The localization of NF-κB and IRS-2 was studied by confocal microscopy. The merged images of IRS-2 (red fluorescence), NF-κB (green fluorescence), merged IRS-2 and NF-κB (yellow fluorescence) are shown.

next investigated whether IRS-2 mediates the effects of IGF-1 on NF-κB-p65 activity in MCF-7 and BT-20 breast cancer cells. We cultured MCF-7 and BT-20 cells in the presence of IGF-1, with or without specific IRS-2 siRNA. In MCF-7 breast cancer cells. In Fig. 2B, IGF-1 stimulated NF-κB-p65 DNA binding activity dramatically and the addition of IRS-2 siRNA significantly reversed the stimulatory effects of IGF-1 on NF-κB-p65 DNA binding activity. Similar to this finding, IRS-2 siRNA also blocked NF-κB activity in BT-20 breast cancer cells.

Collectively, these findings revealed IRS-2 as essential for MCF-7 and BT-20 breast cancer cells in the functional regulation of serum-deprived growth, one key property of the aggressive cancer phenotype.

IGF-1 promotes nuclear translocation of insulin receptor substrates 2 and NF-κB in MCF-7 and BT-20 breast cancer cells. We have previously observed efficient nuclear translocation of IRS-2 in response to IGF-1 treatment in mouse embryo fibroblasts (1). Since IGF-1 is known to activate NF-κB in a ligand-independent manner (16), we assessed whether IGF-1 can stimulate NF-κB and IRS-2 nuclear translocation in MCF-7 and BT-20 breast cancer cells. We analyzed NF-κB protein abundance in cytoplasmic and nuclear protein lysates obtained from MCF-7 and BT-20 cells stimulated with IGF-1 (Fig. 3A). In unstimulated cells (SFM), higher levels of NF-κB and IRS-2 were found in the cytoplasm than in the nucleus. As expected, IGF-1 treatment significantly increased nuclear abundance of NF-κB,

decreasing NF-κB cytoplasmic levels (Fig. 3A). This effect was apparent at 24 h of stimulation.

The nuclear translocation of NF-κB and IRS-2 in MCF-7 and BT-20 cells was confirmed by confocal microscopy. Fig. 3B shows merged pictures of MCF-7 and BT-20 cells stained with IRS-2 (red fluorescence) and NF-κB (green fluorescence) after stimulation with IGF-1 for 24 h. Evident nuclear colocalization of NF-κB and IRS-2 was observed in 60-70% of the cells (yellow fluorescence). NF-κB was expressed almost exclusively in the nucleus, while IRS-2 was abundant in both cellular compartments.

Nuclear IRS-2 interaction with NF-κB-p65 and PI3K binding tyrosine residues of IRS-2 are crucial for the NF-κB activities and the cyclin D1 promoter activities. Confocal microscopy results suggested nuclear colocalization of IRS-2 and NF-κB. To confirm this observation, we studied IRS-2 and NF-κB interactions by immunoprecipitation and Western blotting using nuclear protein fractions obtained from MCF-7 and BT-20 cells grown in serum-free medium or treated with IGF-1 for 16 h. NF-κB-p65 was found in IRS-2 immunoprecipitates in treated cells (Fig. 4A). Similarly, IRS-2 co-precipitated with nuclear NF-κB-p65 under IGF-1 treatment (Fig. 4B). These results indicate that NF-κB favor co-precipitation of IRS-2 and the interaction seems to be dependent on abundance of nuclear IRS-2.

To further elucidate the domain required for the interaction between IRS-2 and NF-κB, we then determined the presence in the GST pull-down of proteins. PI3K and Grb2
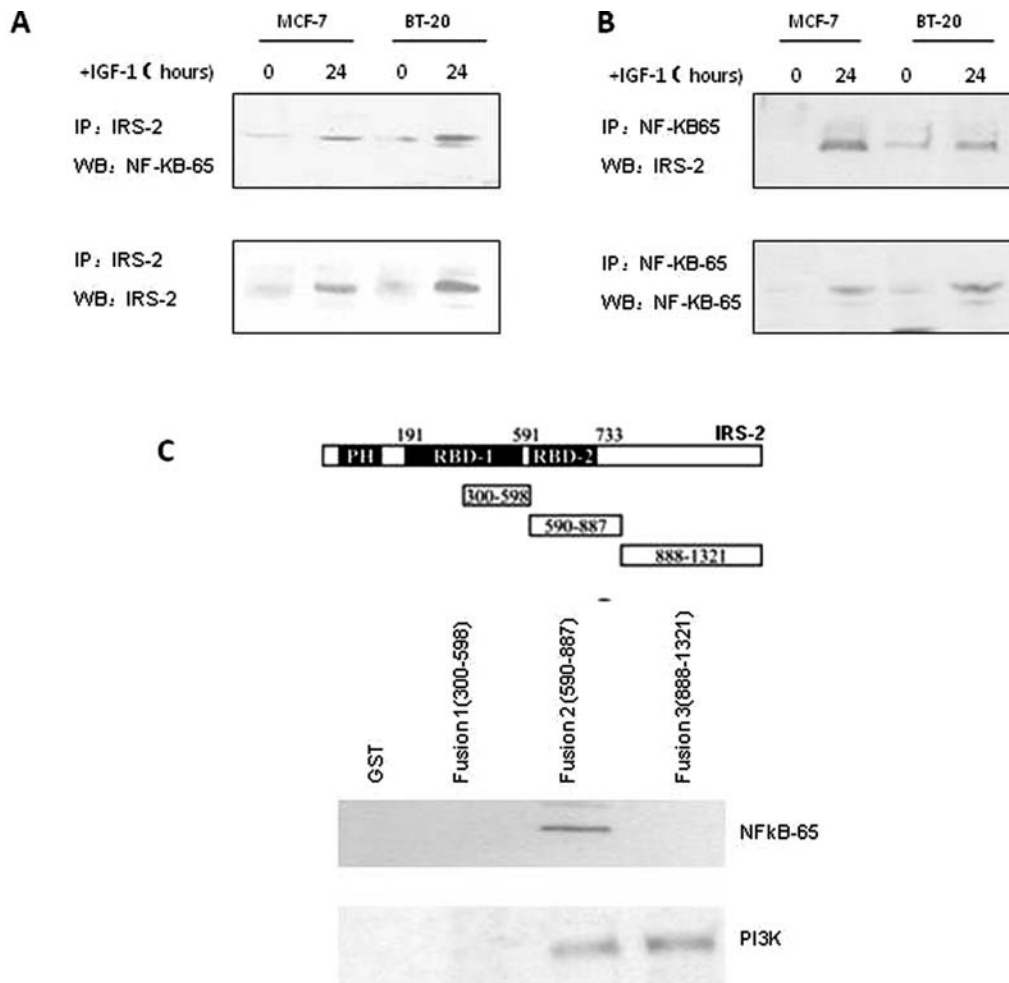
Figure 4. Interaction between IRS-2 and NF-κB-p65. (A) IP with an antibody to NF-κB-p65, Western blots with anti-IRS-2. (B) IP with an antibody to NF-κB-p65, Western blots with antibodies to IRS-2. (C) Diagram of IRS-2 GST constructs (RBD means the receptor binding domain) and pull-down experiments for PI3K, Grb2 and NF-κB-p65, which showed that the IRS-2 sequences 590-887 and 888-1321 interact with PI3K. The 590-887 sequence interacts with the NF-κB-p65.

serve as positive control because they are known to have binding sites on the IRS proteins. The results shown in Fig. 4C indicate that the sequences of IRS-2 between amino acids 590 and 1321 pull down the p85 subunit of PI3-K. The sequence between 590 and 888 also pulls down NF-κB, which indicate PI3-K binding tyrosine residues might be critical for the interaction between IRS-2 and NF-κB.

Based on the above results, we wished to further confirm whether PI3-K binding tyrosine residues are important for the IRS-2 mediated NF-κB activities involved in IGF-1 induced responses. Because 32D cell line does not express IRS-1 or IRS-2, it could be a useful model to examine the effect of mutant IRS-2 for the NF-κB activities (33). 32D-derived cell lines with IRS-2 mutant have been previously described in our publications. The mutant IRS-2 included a deletion of the Pleckstrin domain (dPH), and a mutation at the two tyrosines that bind PI3K. As expected, the results in Fig. 5A show that NF-κB-p65 DNA binding activity dramatically reduced in IRS-2 mutant at the PI3K binding sites, in contrast to the wild-type IRS-2, the mutant IRS-2 at the Pleckstrin domain (delta PH).

Because the transcriptional regulation of cyclin D1 is crucial for NF-κB function (34,35), we therefore, continued to investigate the cyclin D1 promoter activity in these 32D-derived cell lines. In these experiments, cyclin D1 promoters were transfected with luciferase reporter genes, and activity determined at the times indicated after stimulation with IGF-1. Fig. 5B, shows that the cyclin D1 promoters activity was slightly increased in parental 32DIGF-1R cells, and its activity was dramatically increased in wild-type IRS-2, the mutant IRS-2 at the Pleckstrin domain (delta PH). However, in IRS-2 mutant at the PI3K binding sites, cyclin D1 promoter activity was no better than in parental 32DIGF-1R cells. These results support the hypothesis that PI3K binding sites of IRS-2 might be important for IRS-2 mediated NF-κB activity.

Nuclear IRS-2 are recruited to the cyclin D1 promoter in MCF-7 and BT-20 breast cancer cells. The association between IRS-2 and cyclin D1 promoter activity suggests that the regulatory sequences of target genes such as cyclin D1 promoters, might be involved in the IRS-2 nuclear presence in MCF-7 and BT-20 cells. In Fig. 6A, a weak signal is already detectable in unstimulated cells, and after stimulation with IGF-1 for 24 and 48 h, IRS-2 is detectable in cyclin D1 promoters in MCF-7 cells. UBF serves as the positive control
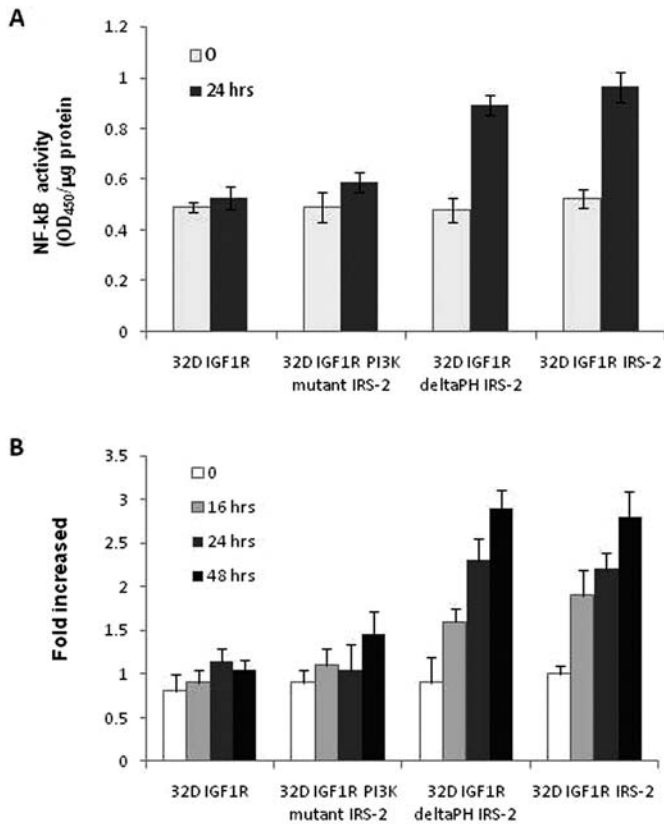
Figure 5. PI3K binding tyrosine residues of IRS-2 are crucial for the NF-κB activities. (A) Effects of IRS-2 siRNA on NF-κB-DNA binding activity. Cells were cultured in the absence or presence of 50 ng/ml IGF-1. NF-κB p65 transcription factor activity was determined by an enzyme-linked immune-sorbent assay, according to the manufacturer's instructions. Data relative to the effects of 50 ng/ml IGF-1 are presented. (B) Transcriptional activity of cyclin D1 promoter. After transient transfection, 32D derived cell lines were cultured with IGF-1 (50 ng/ml) as indicated times and then cells were harvested for luciferase assay. The data are the average (± SD) of three separate transfection.

because it is present in cyclin D1 promoters. To validate further this result, we also assessed the presence of IRS-2 in the cyclin D1 promoters of BT-20 cells; IRS-2 is still detectable in BT-20 cells after stimulation with IGF-1.

To determine the effects of NF-κB on the occupancy of IRS-2 to the cyclin D1 promoters in the breast cancer cells, we silenced NF-κB-65 activity via pyrrolidine dithio-carbamate (PDTC, a recognized and specific NF-κB inhibitor) and NF-κB-65 siRNA (16). To determine trans-fection efficiency, we transfected cells with fluorescein isothiocyanate-conjugated control siRNA in two separate experiments. Transfection efficiency was 42.5±2.9% (range of 30.8-51.4%). To determine whether p65 siRNA specifically silenced NF-κB p65 expression, we evaluated NF-κB-p65 mRNA and protein expression in transfected cells by RT-PCR and Western blot analysis, respectively (data not shown). Fig. 6B, lane showed the effects of pyrrolidine dithiocarbamate on nuclear IRS-2 association with cyclin D1 promoters. The selective inhibition of NF-κB-65 abolished the association of IRS-2 to the cyclin D1 promoters. In addition, NF-κB-65 siRNA also reversed presence of IRS-2 in the cyclin D1 promoters (Fig. 6B). These results suggested the absence of NF-κB blocks the recruitment of IRS-2 to cyclin D1 promoters.

Discussion

Insulin receptor substrates (IRSs) are adaptor proteins that link signaling from upstream activators to multiple down-stream effectors to modulate normal growth, metabolism, survival and differentiation (36). Breast cancer cells express both IRS-1 and IRS-2, while the studies support the notion that IRS-1 seems to be involved with cellular proliferation and recent evidence suggests that IRS-2 is overexpressed in
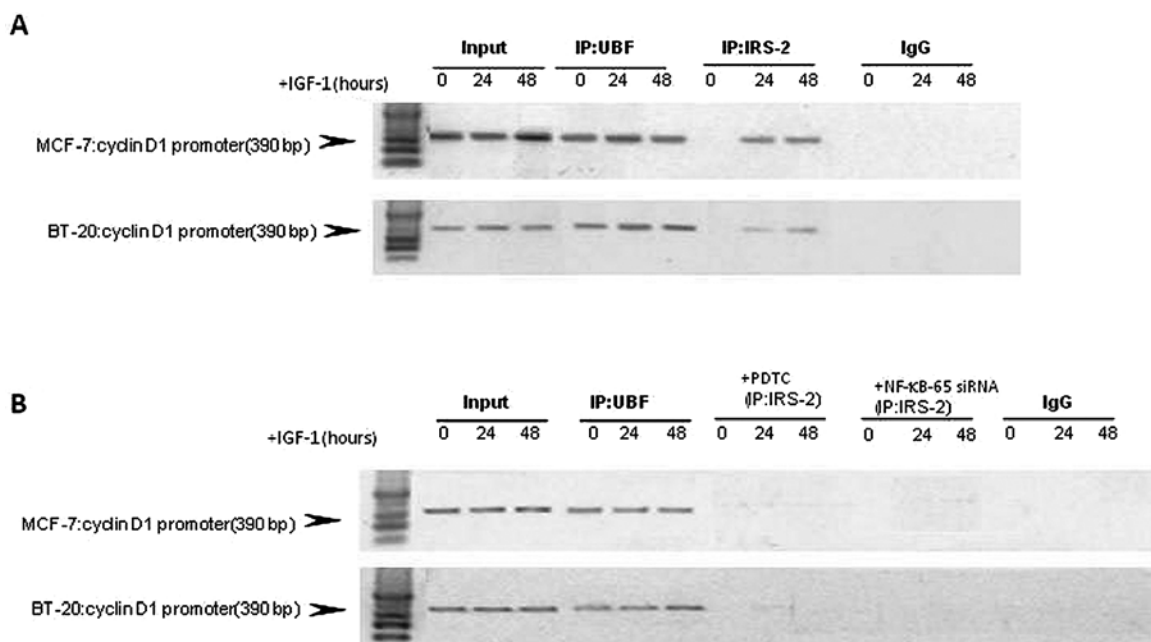

Figure 6. Recruitment of IRS-2 to the rDNA and cyclin D1 promoter. The main panel shows the results of experiments in which the sonicated chromatin from MCF-7 and BT-20 cells was immunoprecipitated with antibodies to IRS-2 and UBF (positive control). The interval for IGF-1 stimulation is 24 h. (B) The effects of selective inhibition of NF-κB-65 on nuclear IRS-2 association with cyclin D1 promoters. NF-κB-65 activity was silenced by pyrrolidine dithiocarbamate (PDTC, a recognized and specific NF-κB inhibitor) or NF-κB-65 siRNA.

cell lines that have enhanced metastatic potential (11,37). The fact that IRS proteins have non-redundant functions is clear, but how much of these functions are compensatory has yet to be determined. Thus far, most of the studies on breast cancer have focused upon IRS-1, the biological pattern of IRS-2 is limited (3).

In our studies, depletion of endogenous IRS-2 by anti-sense strategies impaired cell proliferation after serum withdrawal, blunted PI3K/Akt activation in IGF-1induced response in MCF-7 and BT-20 breast cancer cells. These results indicate that IRS-2 is capable of activating a unique signaling pathway that is essential for tumor progression and that this pathway is not activated sufficiently, or at all, through IRS-1. Moreover, the absence of IRS-2 abolished the effects of IGF-1 on NF-κB activation, supporting the hypothesis that IRS-2 effects were associated with, at least in part, NF-κB via the PI3K/Akt pathway.

The serine/threonine kinase Akt, is activated by lipid products of phosphatidylinositol 3-kinase (PI3K). Akt phosphorylates numerous protein targets that control cell survival, proliferation and motility. Previous studies suggest that Akt regulates transcriptional activity of the nuclear factor-κB (NF-κB) by inducing phosphorylation and subsequent degradation of inhibitor of κB (38). Nuclear factor-κB, is a family of transcription factors that regulates diverse cellular activities and the common form of NF-κB is p65/RelAp50 (39). The constitutive activation of NF-κB is identified in breast cancer and the constitutively active NF-κB is responsible for overexpression of pro-metastatic and anti-apoptotic genes in breast cancer cells. Previous studies suggest that Akt regulates transcriptional activity of NF-κB by inducing phosphorylation and subsequent degradation of inhibitor of κB (23,40).

Our results also indicated that IGF-1 promotes nuclear colocalization of insulin receptor substrates 2 and NF-κB in MCF-7 and BT-20 breast cancer cells. Canonical IRS signaling is defined by the binding of IRS-1 or IRS-2, via the conserved PH and PTB domains, to ligand-phosphorylated IGF-1R. Upon receptor activation, IRSs are rapidly phos-phorylated, resulting in activation of many downstream pathways such as the phosphatidylinositol 3'-kinase (PI3K) (41). IRS proteins may also have non-canonical functions within the nucleus. Nuclear localization of IRS-2 has recently been described in mouse embryo fibroblasts stimulated with IGF-1 (1). The mechanism by which IRS-2 enters cell nucleus is still not clear. Although IRS-2 contains putative NLS, it is thought that IRS-2 is chaperoned to the nucleus by other proteins. The transporting molecules involved in IGF-1R-dependent IRS-2 nuclear translocation are yet unknown.

Our experiments suggest that a fraction of IRS-2 might be present in the nucleus even without IGF-1 treatment according to rough estimations based on subcellular fractions procedures (Fig. 2B), because a detectable amount of IGF-1 can be produced in MCF-7 and BT-20 cells in serum-free condition 2), it is understandable that nuclear accumulation of IRS-2 might occur in both cell lines. In our experimental system, the prerequisite for nuclear translocation of IRS-2 in response to IGF-1 is most likely the formation of the NF-κB/IRS-2 complex in the cytoplasm. NF-κB association

with cytoplasmic signaling molecules is not unusual. Recently, NF-κB has been shown to bind the PI3K/Akt complex (42) and our data suggest that the interaction between IRS-2 and NF-κB requires the PI3-K binding sites of IRS-2, and it is possible that NF-κB not only directly associate with IRS-2, but also dependent on IRS-2 tyrosine phosphorylation for mitogenic and transforming activity.

The mechanism by which IRS-2 induces tumor cell motility and invasiveness remains still elusive. In our present studies, nuclear IRS-2 can interact with cyclin D1 promoters. A hypothesis could be proposed on the basis that the distinct intracellular compartmentalization of IRS-2 might play a role in the metastasis-promoting functions in breast cancer (3-6). IRS-2 contains potential binding motifs for many intracellular signaling molecules and many of these effectors function in signaling pathways that could regulate metastasis.

Accumulating literature indicates important roles for cyclin D1 in diverse physiological and pathological processes. Cyclin D1 regulates normal cell cycle progression and diffe-rentiation, and its overexpression is associated with a variety of neoplasias, including breast cancer (43). In addition to its key role in cell cycle regulation and differentiation, overexpression of cyclin D1 is associated with a tumor invasiveness and metastasis. Herein, cyclin D1-deficient mouse embryo fibroblasts (MEFs) exhibited increased adhesion and decreased motility compared with wild-type MEFs. In addition, cyclin D1 promotes cellular motility through inhibiting ROCK signaling and repressing the metastasis suppressor TSP-1 (44,45).

With respect to the molecular mechanisms linking IRS-2 and NF-κB, recent evidence indicated that the activation of cyclin D1 is involved in the PI3K/Akt/NF-κB pathway in breast cancer cells (46-49). Our experiments confirmed the effects of IRS-2 on IGF-1 induced responses in cyclin D1 promoter activities and PI3K binding tyrosine residues of IRS-2 are important for its activities. It is noteworthy that IRS-2 is detectable in cyclin D1 promoters and neutralization of NF-κB abolished IRS-2 recruitment further supporting a functional interaction between IRS-2 and NF-κB. IRS-2 modification of cyclin D1 promoter activities is probably restricted to certain transcriptional complexes though PI3K/ Akt and NF-κB pathway, as we did not observe significant effects of MAPK inhibition on IRS-2 occupancy to cyclin D1 promoters.

In addition to significant homology between IRS proteins, the other domain discrepancy might be relevant to the distinct biological actions of IRS-2 versus IRS-1. We have previously shown that unlike IRS-1, both wild-type IRS-2 and a truncated IRS-2 lacking the PH and PTB domains inhibit the differentiation and sustain the IGF-1-dependent cell proliferation (49). Studies in breast cancer cell lines and in mice have shown divergent roles for IRS-1 and IRS-2 in proliferation, migration, invasion, and metastasis. Despite all of this evidence, studies in human breast cancer are inconsistent and require more investigation. Further studies are required to determine a careful dissection of the relative roles of IRS-1 versus IRS-2 and the upstream activators/downstream effectors for IRS-2 during breast cancer transformation.

In conclusion, our study suggests that IRS-2 plays a significant role by activating, at least in part, NF-κB via PI3K/Akt pathway in IGF-1 induced-response in breast cancer cells and the crosstalk between nuclear IRS-2 and NF-κB might be responsible for transcriptional progression of the breast cancer cells.

Acknowledgements

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