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Inducible Expression of Dominant Negative Insulin-like Growth Factor I Receptor in MCF-7 Breast Cancer Cells

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The insulin-like growth factor I receptor (IGF-IR) is expressed in many cell types and is critical for normal growth and development. In the healthy mammary gland, the role of IGF-IR is not fully elucidated. However, IGF-IR, which is primarily expressed in the mammary epithelial cells, is known to play an obligatory role in cellular transformation, facilitating the progression to breast cancer. We have utilized the tetracycline regulatory (tet-on) system to generate an in vitro model system to allow us to further investigate IGF-I/IGF-IR function in mammary epithelial cells. A plasmid construct containing a mutant IGF-I receptor (IGF-IR-DN) fused to the tetracycline operator (tetOPh$_{CMV}$-IGF-IR-DN) was stably transfected into MCF-7 human breast cancer cells. The conditional regulation of the IGF-IR-DN gene expression was studied in four independent clonal lines. The translated IGF-IR-DN protein was detected only in the stably transfected doxycycline-induced cells, and its expression was up-regulated (three- to sixfold) following induction. IGF-I stimulated cell proliferation diminished (twofold) in doxycycline-induced cells compared to uninduced cells, demonstrating that the transgene construct was functional and ruling out any pleiotropic effect that may be attributed to doxycycline. Interestingly, autophosphorylation of the IGF-IR and phosphorylation of the downstream substrate, insulin receptor substrate-1 (IRS-1), was not inhibited in doxycycline/IGF-I treated cells, suggesting the possibility that activation of downstream substrates other than the IRS-1 may be critical for optimal cell proliferation. This novel in vitro model should allow us to more directly examine the role of IGF-I/IGF-IR signaling and function in mammary epithelial cells.

Key Words: IGF-IR; dominant negative; tetracycline-inducible system; mammary epithelial cells.

Introduction

The insulin-like growth factors (IGF-I and IGF-II) are growth modulators for many different cell types *(1–3)*. Both IGF ligands have been shown to play an essential role in promoting growth *in utero* and *ex utero (4–6)*. The most convincing evidence that IGF is important for development comes from gene knockout studies. Mice lacking IGF-I or IGF-II are born with reduced birth weight, while IGF-IR gene knockout mice were born with reduced weight and died soon after birth *(4)*. The IGF-I receptor (IGF-IR), which mediates the mitogenic action of IGF-I, is involved in cell cycle regulation *(7)*, apoptosis *(8)*, and cell transformation *(9)*.

The role of the IGF-I/IGF-IR in growth and morphogenesis in the mammary gland is not fully understood. Current knowledge about the importance of IGF-I in the breast has been derived primarily from breast cancer studies. The IGF-IR has been shown to be overexpressed *(10–13)* and amplified *(14)* in many malignant breast cancers. Several studies have indicated that IGF-IR has an obligatory role in cellular transformation *(3,15,16)*. Such studies, in addition to the observation that the inhibition of IGF-I receptor function can impede the growth of cancer cells *(17–19)*, has led to the proposal that IGF-IR may be a target for cancer therapy *(20)*.

In healthy mammary glands, IGF-I and its receptor have been localized to stromal and epithelial cells, respectively *(21)*, and IGF-I has been shown to elicit mitogenic responses in normal mammary epithelial cells *(9,22,23)*. The overexpression of the IGF-I gene in the mammary glands of transgenic mice was shown to delay gland involution *(7,24,25)*, suggesting that the IGF-I/IGF-IR may be specifically important at that phase of development. In addition, by measuring terminal end bud formation and branching of ducts in IGF-I null female mice, Ruan and Kleinberg *(26)* have demonstrated that mammary development was not possible unless IGF-I was present. Moreover, our laboratory has recently shown that the level of IGF-IR gene expression is highest during early pregnancy and late involution (d 10) and lowest during pregnancy and lactation *(27)* demonstrating that a developmental pattern of IGF-IR gene expression does exist in the mouse mammary gland. Such data suggest that IGF-I/IGF-IR may be important at specific stages of mammary gland development.

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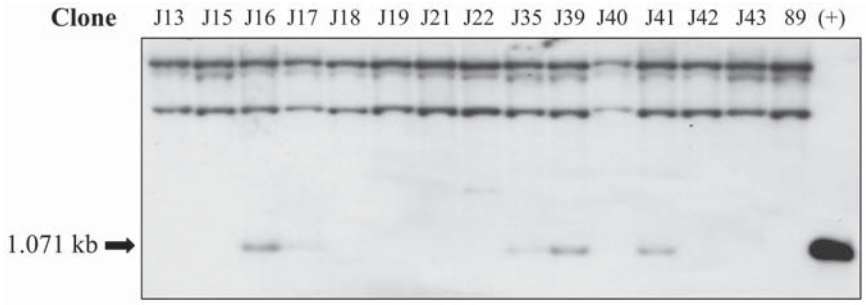

Fig. 1. Integration of the IGF-IR-DN gene in stably transfected clonal cell lines. Integration of the IGF-IR-DN transgene into the genome of the cells stably transfected with the tetO-IGF-IR-DN plasmid was examined by Southern blot analysis. Genomic DNA (10 μg) from 38 clonal cell lines was digested with *Eco*RV and electrophoresed on a 0.8% gel. The gel was transferred onto a nitrocellulose membrane and hybridized with a ^{32}P-labeled probe specific for a 1071 bp fragment unique to the IGF-IR-DN cDNA. A total of 10 positive clonal cell lines were identified, of which five are shown. The presence of the 1.071 kb band was used as a marker for transgene integration. Clone 89 (an rtTA-expressing parental clonal cell line) was used as a negative control. IGF-IR-DN plasmid DNA was used as the positive control.

The signaling pathway of the IGF-I/IGF-IR is partially elucidated *(28)*. The IGF-I receptor is a tyrosine kinase protein composed of two heterodimers, each containing an extracellular α-subunit and a transmembrane β-subunit. Upon activation by the ligand, the IGF-I receptor autophosphorylates and subsequently activates downstream substrates including the major substrate, insulin receptor substrate-1 (IRS-1), phosphotidylinositol (PI) 3-kinase, mitogen-activated protein kinases (MAP kinases), the src homology domain containing adapter protein (Shc), the growth factor receptor bound 2 (Grb2), and the CrkII and Crk-like (CrkL) proteins *(28–30)*. Although many of the downstream signaling substrates have been identified and characterized, signaling pathways that are related to particular functions of the IGF-I receptor, such as cell growth, transformation, or apoptosis are still unclear.

To further understand IGF-IR action in mammary epithelial cells, we have utilized the tetracycline regulatory system to allow us to conditionally regulate the expression of IGF-IR in vitro. The MCF-7 human breast cancer cell line is a useful cell line to examine inhibition of IGFI/IGF-IR function because it is a mammary epithelial cell line that overexpresses IGF-IR *(31,32)*, and the enhancement of growth and inhibition of apoptosis by IGF-I/IGF-IR is well documented in MCF-7 cells *(13,33)*. Here we report the characterization of a stably transfected mammary epithelial cell line in which IGF-IR gene expression is regulated by the tetracycline operator sequence. Using this model, we demonstrate that expression of both the mutant IGF-I receptor transgene and protein is inducible and cell proliferation is partially inhibited despite normal levels of phosphorylation of IGF-IR and the IRS-1.

Results

Identification of IGF-IR-DN Stably Transfected Cells

MCF-7 cells were cotransfected with the tetOPh$_{CMV}$-IGF-IR-DN and pUDHD172-1neo plasmids. Resistant clonal cell lines were identified by screening with neomycin and zeocin selection agents. To confirm the integration of the IGF-IR-DN transgene into the genome, DNA was extracted from the clonal cell lines and analyzed by Southern blotting, using a ^{32}P-labeled IGF-IR-DN probe. A total of 10 positive clonal cell lines were isolated (Fig. 1). The presence of a 1.071 kb band confirmed that the transgene was stably integrated. Five out of the 10 lines (J10, J11, J35, J39, and J41) were selected for further characterization.

Analysis of IGF-IR-DN Gene Expression in the Stably Transfected Mammary Epithelial Cells

The maximum level of transgene expression was observed following administration of 1 μg/mL doxycycline for 24 h (Fig. 2). IGF-IR-DN gene expression was induced by doxycycline in four out of five clonal lines (clones J10, J11, J35, and J41), previously identified to be positive for transgene integration (Fig. 3). The highest level (4.3-fold) of induction was observed in clone J35. IGF-IR-DN gene expression was not induced by doxycycline in one clonal line, clone J39 (Fig. 3). This cell line was subsequently used as a negative control for IGF-IR-DN gene expression.

IGF-IR-DN Protein Expression in the Stably Transfected Cells

IGF-IR-DN protein expression was detected by immunoblotting with the antibodies specific to the α- (N-20) and β-subunit (H-60) of the IGF-I receptor. The 135 kDa α-subunit was identified in both the doxycycline-induced and uninduced cells (Fig. 4). An increase (high, low, and moderate) in the IGF-IR protein (135 kDa) expression was observed in the clonal cell lines treated with doxycycline. Clone J11, in which high levels of IGF-IR-DN gene expression were previously demonstrated (Fig. 3), also displayed high levels of protein expression (threefold; Fig. 4). In addition, a sixfold increase in protein expression was observed in clone J35 (data not shown).

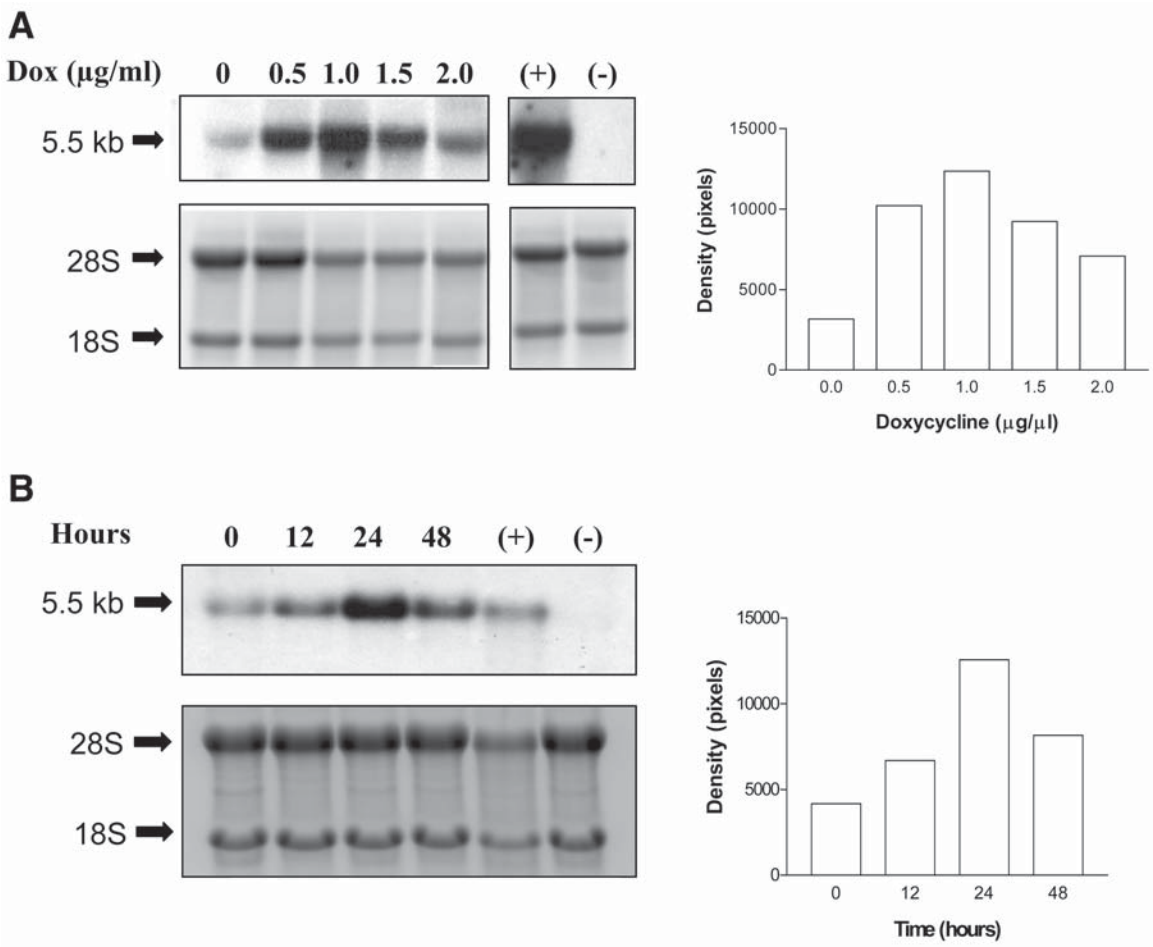

Fig. 2. Determination of optimal time and doxycycline concentration required for maximal induction of the IGF-IR-DN transgene expression. (**A**) Stably transfected cells were grown for 24 h in the presence of doxycycline (0, 0.5, 1, 1.5, 2.0 µg/mL). (**B**) Cells were grown in the presence of 1.0 µg/mL doxycycline for 0, 12, 24 and 48 h. Optimal expression was observed after 24 h in cells treated with 1.0 µg/mL doxycycline (dox). Ethidium bromide staining was used to reflect RNA loading. The 18S and 28S ribosomal RNA bands are indicated. Clone J1, previously identified as expressing IGF-IR-DN, was used as a positive (+) control. Clone 89 (an rtTA-expressing parental clonal cell line) was used as a negative (–) control. Densitometric values from a representative experiment are shown.

Not unexpectedly, the 90 kDa endogenous IGF-IR β-subunit, detected with the antibody against the β-subunit of the IGF-I receptor (H-60), was identified in both induced and uninduced cells (Fig. 4). The mutant receptor (50 kDa), also detected with the same H-60 antibody, was present only in the stably transfected cells treated with doxycycline (Fig. 4A). The mutant receptor was not detected in uninduced cells or in the control clonal line J39 (Fig. 4B).

Cell Proliferation Assays

Growth of the clonal cell lines was evaluated under different conditions (see Materials and Methods). Data are presented as means ± standard deviation (SD). No change (Fig. 5) in growth was observed at d –1 and 0 of treatment (prior to addition of IGF-I). No difference in growth rate was observed between the two control cell lines, clone 89 (data not shown) and J39 (Fig. 5), treated with or without doxycycline following IGF-I stimulation (d 2 and 4) ruling out any pleiotropic effects of doxycycline (1 µg/mL) on cell growth. In contrast, cell growth was significantly reduced in doxycycline-treated cells of clones J11 (twofold) and J35 (1.3-fold) compared to untreated cells following IGF-I stimulation (d 2, 4, and 6). In comparison to control cells, cell growth was not observed in either doxycycline-induced or uninduced cells (clones J11, J35 and J39) in the absence of IGF-I.

Detection of Phosphorylated Proteins

To determine whether the IGF-IR-DN protein was functional, its ability to inhibit the autophosphorylation of the IGF-I receptor and phosphorylation of the downstream substrate, insulin receptor substrate 1 (IRS-1), was examined. In a separate experiment we determined that the optimal protein phosphorylation was observed following administration of 5 n*M* IGF-I for 10 min (data not shown). IGF-I induced autophosphorylation of the IGF-IR β-subunit (90

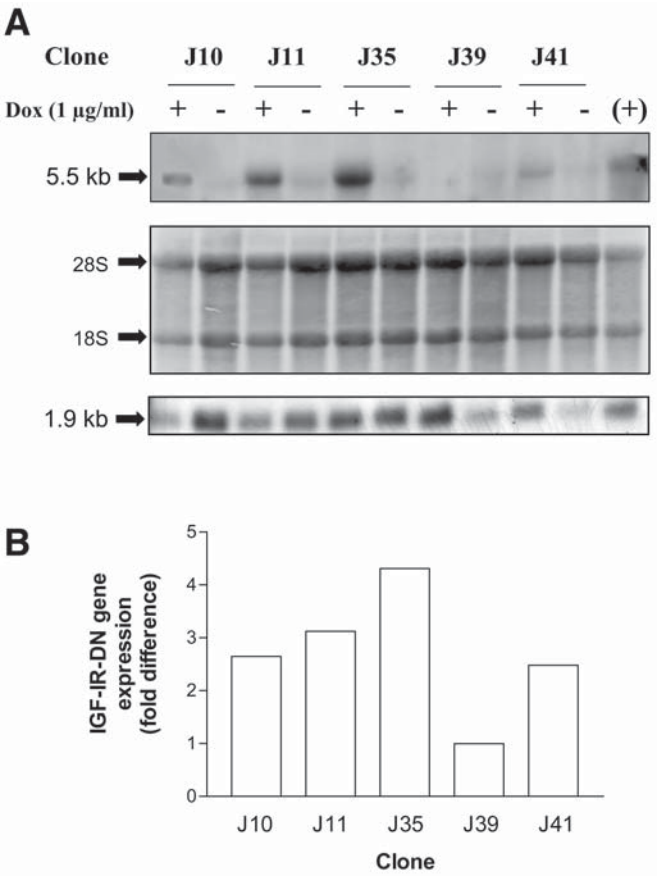

Fig. 3. Northern blot analysis of IGF-IR-DN gene expression in clonal cell lines. (**A**) Total RNA (30 μg) extracted from doxycycline-induced (+) and uninduced (–) cells was electrophoresed in a 0.8% denaturing agarose-formaldehyde gel, transferred, and hybridized with a ^{32}P-labeled IGF-IR-DN cDNA probe. The 5.5 kb IGF-IR-DN transcript was observed only in doxycycline (dox)-induced cells. Clone J39, positive for IGF-IR-DN gene integration but negative for gene expression, was used as a negative control. Clone J1, an IGF-IR-DN gene expressing clone, was used as a positive (+) control. IGF-IR-DN gene expression was highest (4.3-fold) in clone J35. The fold increase in gene expression was obtained by comparing the expression levels with that of the basal gene expression level in clone J39. The 18S and 28S ribosomal RNA bands are indicated. The membranes were probed with GAPDH cDNA probe to indicate sample loading. Densitometric values from a representative assay are shown in Panel **B**.

kDa) and phosphorylation of the IRS-1 protein (185 kDa) using the mouse monoclonal antiphosphotyrosine antibody (PY-20; *34*) was observed in all clonal lines (Fig. 6A) including the negative controls (clones 89 and J39; data not shown). However, following normalization with actin (the loading control; Fig. 6C), we determined that the doxycycline induction of the transgene expression did not alter the phosphorylation status of the IGF-IR (90 kDa) and the IRS-1 (185 kDa) in these lines (Fig. 6A). These results were repeated in two other independent experiments using clones J11 and J35 (data not shown). To verify that the identity of the 185 kDa band (Fig. 6A) was that of the IRS-1 phosphoprotein, the blots previously used for the phosphorylation studies were immunoblotted with an antibody against the phosphorylated IRS-1 (Ab-1). The antibody recognized a 185 kDa band, which corresponded to the size of the phosphorylated protein in the lanes containing protein isolated from IGF-I-treated MCF-7 cells (data not shown). Following this step, the same blots were then stripped and immunoblotted with an antibody against the IRS-1 protein to detect the presence of the IRS-1 in both the IGF-I-treated and -untreated clonal lines. The IRS-1 protein (185 kDa) was expressed under all conditions examined (Fig. 6B). Again, upon normalizing the IRS-1 protein levels with the actin control (Fig. 6C), we found no difference in IRS-1 protein levels between the experimental groups.

Morphological Studies

No change in cell morphology was observed following doxycycline treatment (data not shown).

Discussion

In this study we have used the tetracycline regulatory system to conditionally regulate IGF-IR gene expression in MCF-7 human breast cancer cells, in vitro. Using a mutant IGF-I receptor (IGF-IR-DN) that functions as a dominant negative receptor *(15,35)*, we were able to inhibit IGF-I-induced cell growth. Interestingly, cells continued to grow but at a much slower rate when doxycycline was added, suggesting that the mutant receptor partially but not completely inhibited cell proliferation.

We further investigated the extent to which the IGF-I/IGF-IR signaling pathway was inhibited by the expression of the mutant receptor by examining both the autophosphorylation of the IGF-IR and the phosphorylation of the downstream substrate, insulin receptor substrate-1 (IRS-1). Neither was the autophosphorylation of the IGF-IR nor the IGF-I-induced phosphorylation of the IRS-1 significantly inhibited following doxycycline administration. Because the IGF-IR-DN mutant receptor has previously been shown to block IRS-1 phosphorylation and inhibit endogenous IGF-IR function in rat pituitary cells *(35)* and in rat fibroblasts *(15)*, it appears that the cell growth signal transduction pathway of the IGF-I in these MCF-7 cells is unlike that observed in the fibroblasts and in the pituitary cells.

Based on these observations, it appears that the phosphorylation of IRS-1 may not be critical for optimal cell proliferation. However, it is possible that the method used in our study is not sensitive enough to detect small changes in phosphorylation in the presence of doxycycline. By Western blot analysis we may have failed to detect a small change in IGF-IR autophosphorylation and phosphorylation of the IRS-1. This could account for the observed growth inhibition of cells treated with doxycycline.

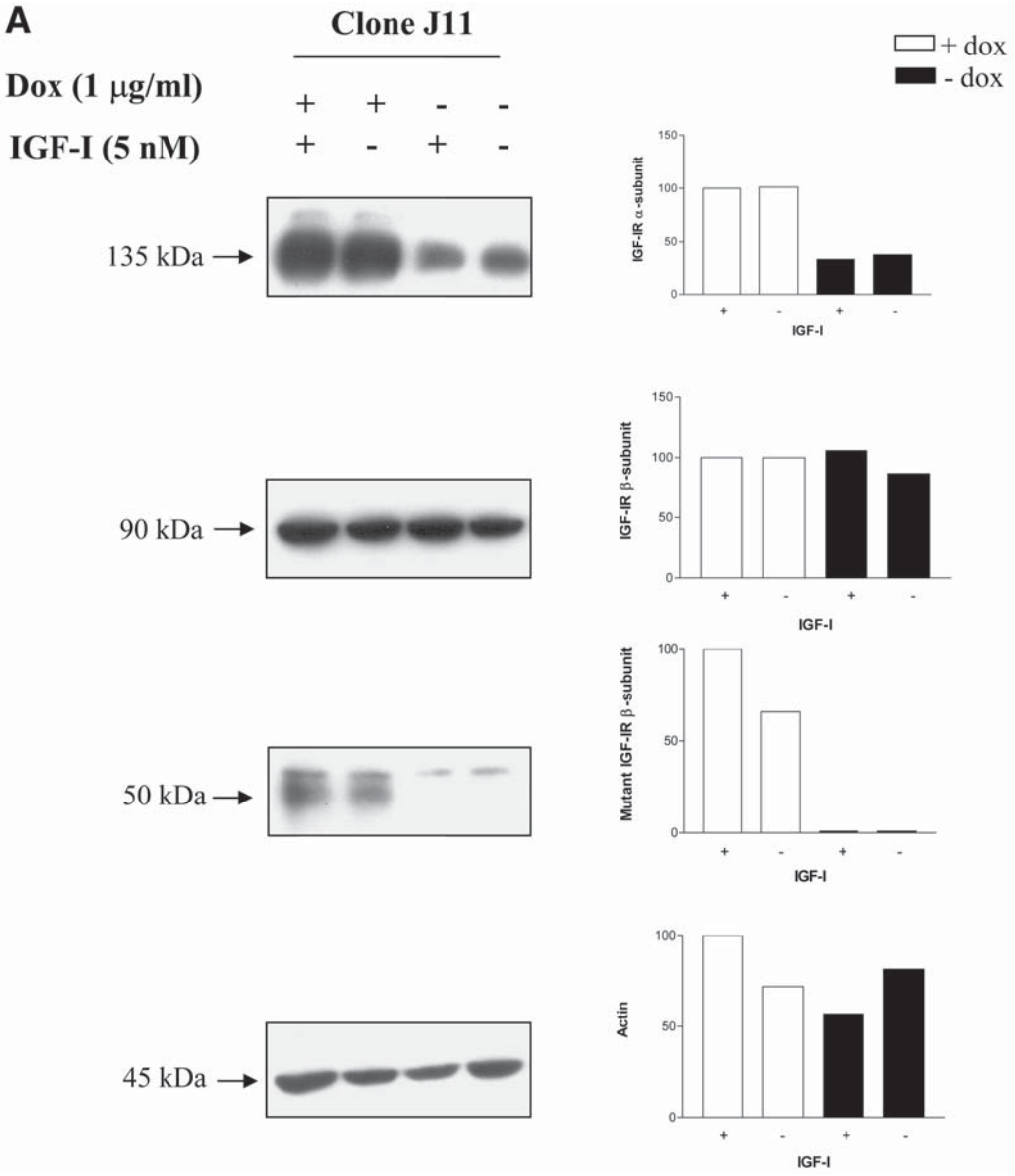

Fig. 4. Expression of the mutant IGF-I receptor protein in the IGF-I stimulated cells. (**A**) The IGF-IR protein (135 kDa) was overexpressed in clones J11 (threefold) and J35 (sixfold; data not shown) cells treated with doxycycline (dox) compared to uninduced cells. The endogenous IGFIR (90 kDa) was identified in both doxycycline-induced and uninduced cells, while the mutant receptor (50 kDa) was detected only in the cells treated with doxycycline. Actin (45 kDa) expression was used to indicate sample loading. Densitometric values representing the levels of the endogenous and mutant IGF-I receptors in doxycycline induced and uninduced cells from representative assays are shown.

Alternatively, other IGF-IR downstream substrates may also be involved. Cellular proliferation in MCF-7 cells has also been reported to be mediated via the MAP kinase pathway *(11)* in which the src-homology domain containing adapter protein (Shc) acts as a docking protein and not the IRS-1 *(28)*. Whether the MAP kinase is involved here is not known.

It has also been suggested that the adapter proteins CrkII and CrkL, which mediate protein–protein interactions, play distinct roles in IGF-I receptor signal transduction *(36,37)*. There is some evidence that Crk proteins are preferentially or specifically activated by the IGFIR *(28)*. Thus, Crk proteins may directly associate with phosphotyrosine residues on IGF-IR and, consequently, do not require IRS-1 as a docking protein *(37)*. In addition, Crk proteins have been shown to activate the Ras pathway which plays an important role in IGF-IR induced cell proliferation *(28,38,39)*. CrkII phosphorylation has been observed in IGF-I stimulated human embryonic kidney and NIH 3T3 fibroblast cells *(37)*, and the overexpression of CrkII has been shown to increase the mitogenic response to IGF-I in mouse fibroblasts *(40)*. The role of the Crk proteins in IGF-I receptor signal transduction in MCF-7 cells has not been elucidated, however, it is

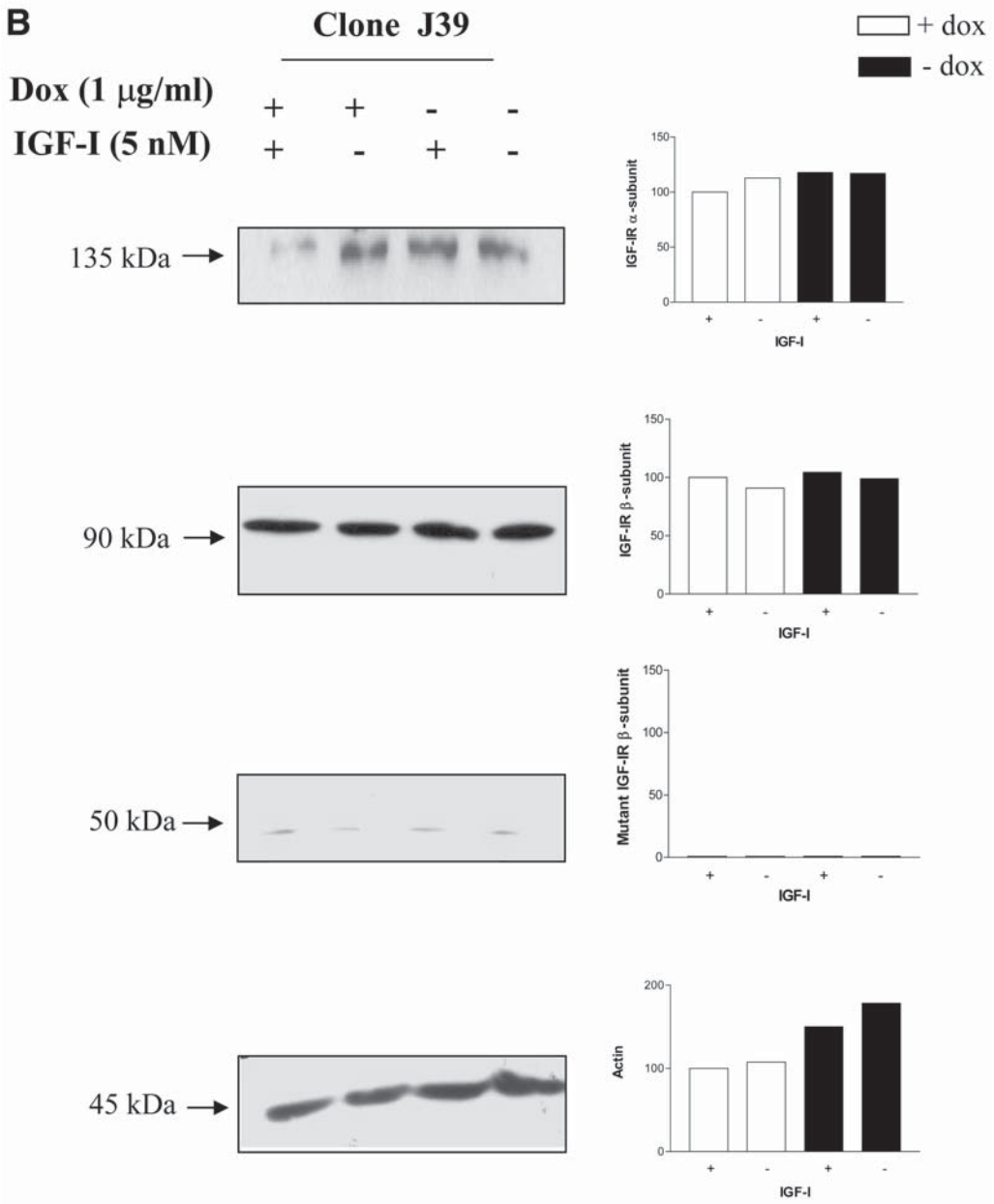

Fig. 4. (**B**) Clonal cell line J39 (that was negative for IGF-IR-DN gene expression) was used as a negative control.

plausible that Crk proteins may also play an important role in MCF-7 cell growth.

In summary, we have developed a novel in vitro model system in which expression of the dominant negative IGF-I receptor is conditionally regulated. We have demonstrated that in the presence of IGF-IR, the overexpression of the dominant negative mutant IGF-IR can inhibit cell proliferation. We speculate that the phosphorylation of IRS-1 may not be critical for cell growth in MCF-7 human breast cancer mammary epithelial cells. This in vitro model system will allow us to examine the IGF-I/IGF-IR signaling pathway in mammary epithelial cells and to further understand the role of IGF-I/IGF-IR function in normal mammary gland development, and ultimately breast cancer.

Materials and Methods

The pUHD172-1neo and pUHD10-3 Plasmid Constructs

The regulator plasmid, pUHD172-1neo, contains the reverse tetracycline transactivator (rtTA) downstream of the cytomegalovirus (P_{CMV}) promoter and a neomycin-resistance cassette. The response plasmid, pUHD10-3, contains seven

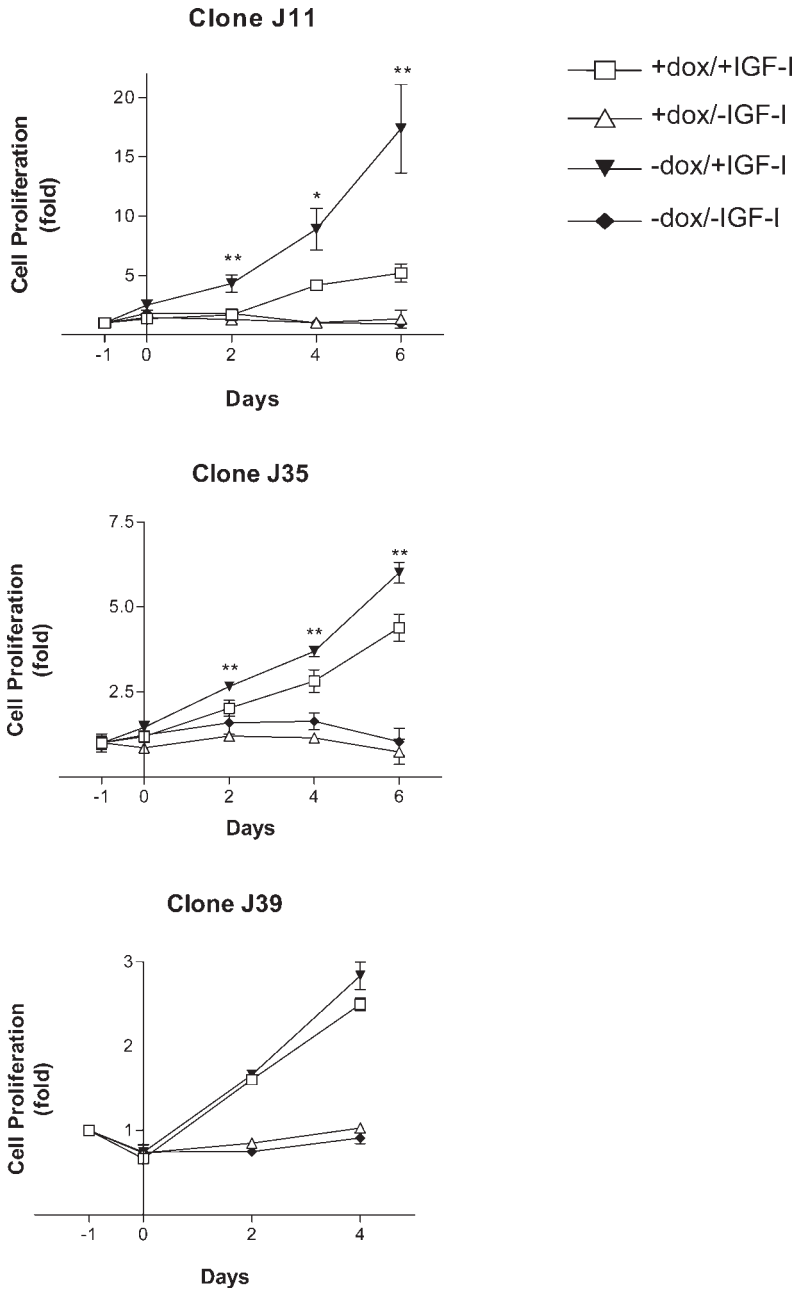

Fig. 5. Expression of the IGF-IR-DN inhibits cells growth in the stably transfected MCF-7 cells. Cells were grown and treated as described in Materials and Methods. IGF-I stimulated growth was significantly increased in clonal lines J11 and J35 in the absence of doxycycline (▼), whereas cell growth was lower in doxycycline-induced cells (□). No stimulated cell growth was observed in either doxycycline-induced (△) or uninduced cells in the absence of IGF-I (◆). Doxycycline treatment did not inhibit IGF-I-induced cell growth in clone J39, which was used as a negative control. Data collected from two experiments (six measurements per experiment for each time point) were combined and are represented as means ± SD ($n = 12$). Significant differences between the +dox/+IGF-I treatment group and the other treatment groups are illustrated. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (by Tukey test).

copies of tetracycline operator sequences ($tetO_7$) upstream of a minimal human cytomegalovirus promoter (Ph_{CMV}) and was modified to contain a zeocin-resistance cassette. The truncated human IGF-I receptor (IGF-IR-DN) cDNA cloned into the pBSK+ expression vector was provided by Dr. Shlomo Melmed *(35)*. The IGFIR-DN cDNA was subcloned into the multiple cloning site (EcoRI/BamHI) downstream of the PhCMV promoter in the pUHD10-3zeo *(41)*.

Generation of Stable Cell Lines

MCF-7 cells were grown and maintained as a monolayer culture in complete media (CM) containing Dulbecco's mod-

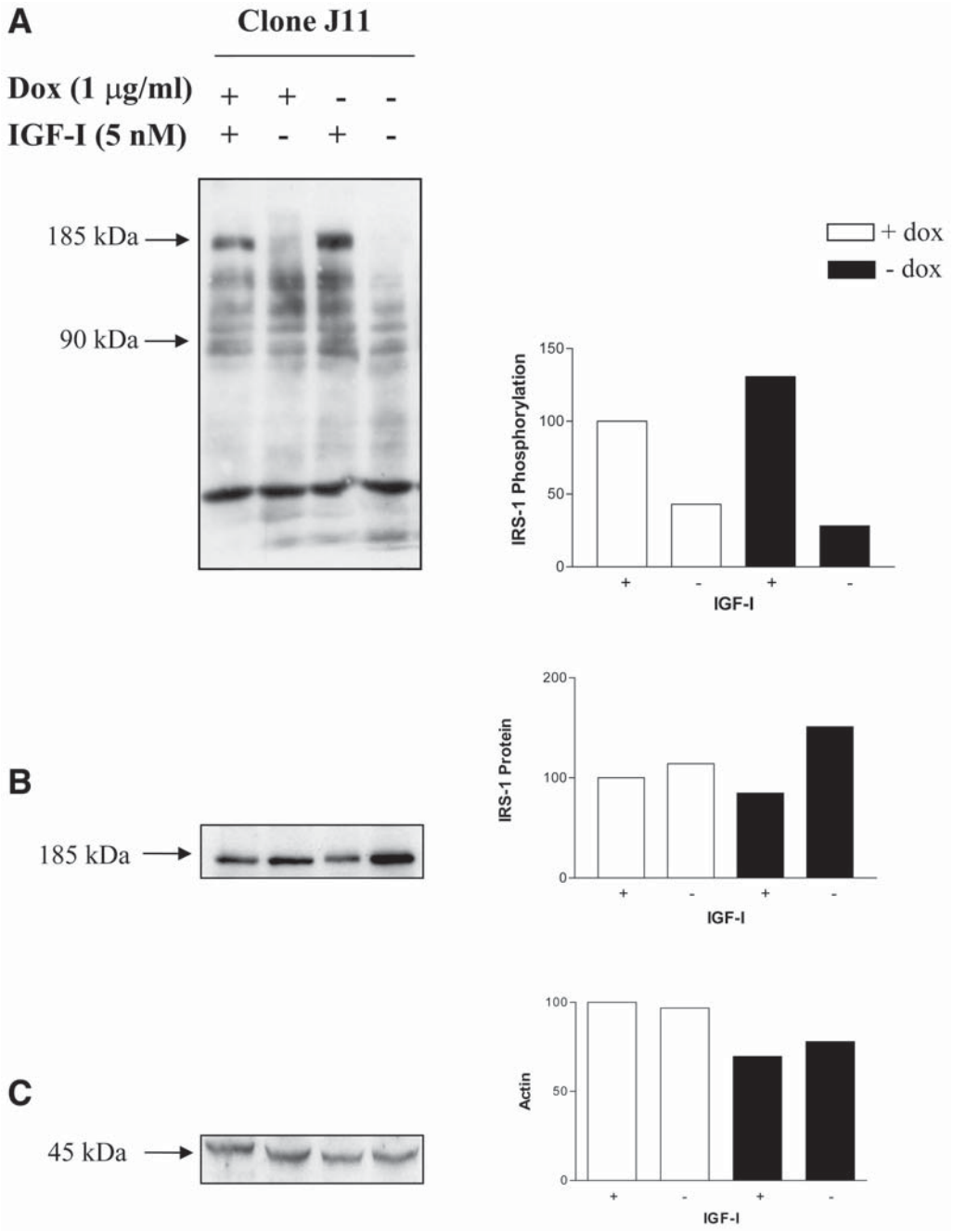

Fig. 6. The truncated IGF-I receptor does not inhibit autophosphorylation of the IGF-I receptor and phosphorylation of the downstream substrate, insulin receptor substrate 1 (IRS-1). Tyrosine phosphorylation studies were carried out as described in Materials and Methods. Cell lysates were collected and subjected to SDS-PAGE (6.5% gel). Western blot analysis was carried out using an anti-phosphotyrosine antibody (PY-20). In clonal cell lines J11 (Panel **A**) and J35 (data not shown) autophosphorylation of the IGF-IR and the phosphorylation of the IRS-1 were not inhibited by the IGF-IR-DN. Autophosphorylation of the IGF-IR β-subunit (90 kDa) and phosphorylation of the IRS-1 (185 kDa) were detected both in IGF-I stimulated cells grown in the presence and absence of doxycycline. (**B**) The IRS-1 protein (185 kDa) expression was observed in all samples. (**C**) The membranes were immunoblotted with an actin antibody (I-19; 45 kDa) to indicate the loading of samples. Densitometric values from a representative assay from three independent experiments are shown.

ified Eagle's medium (DMEM; Sigma Chemical Company) supplemented with 5% (v/v) dialyzed fetal bovine serum (Invitrogen Corporation), 10 units/mL penicillin (Invitrogen Corporation), 10 units/mL streptomycin, 0.3% (w/v) glucose, and 2 m*M* L-glutamine (Invitrogen Corporation). The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in 75 cm^2 polystyrene culture flasks (Corning Inc., Corning, NY, USA).

MCF-7 cells previously transfected with the pUHD172-1neo (clonal cell line 89) *(42)* were stably cotransfected with

the tetOPh$_{CMV}$-IGF-IR-DN plasmid linearized by *Sal*I using the calcium phosphate method (Stratagene, La Jolla, CA, USA). Zeocin (Invitrogen Corporation) at 250 µg/mL was used for the selection of resistant transfectants. Resistant clones were passaged and grown in 800 µg/mL Geneticin (G-418 sulfate; Invitrogen Corporation) and 250 µg/mL Zeocin to select for double transfectants.

Genomic DNA was extracted from the stably transfected MCF-7 cells and analyzed by Southern blot for IGF-IR-DN gene integration. *Eco*RV (100 U/µL; NEB) digested DNA samples were electrophoresed in a 0.8% agarose gel at 25 V overnight in a running buffer of 1X TBE. The gel was then agitated in a denaturing solution (1.5 *M* NaCl, 0.5 *M* NaOH) for 1 h at room temperature. The solution was poured off and replaced by neutralization solution (1 *M* NH_4OAc, 0.02 *M* NaOH) and agitated for 1 h under the same conditions. The DNA was transferred onto a nitrocellulose membrane (0.45 µm pore size; Micron Separations, Westborough, MA, USA), which was then baked for 2 h at 80°C. The blot was prehybridized at 42°C for 2 h in 50% (v/v) formamide, 1X SSPE buffer (0.15 *M* NaCl, 0.01 *M* NaH_2PO_4, 1 m*M* EDTA, pH 7.7), 1X Denhardt's solution (0.02% [w/v] each of Ficoll 400, polyvinylpyrrolidone, and BSA), 0.1% sodium dodecyl sulfate (SDS), and 250 µg/mL denatured salmon sperm DNA. For hybridization, a fresh 10 mL of the same solution was used with the addition of 30 ng of a random primed ^{32}P-labeled IGF-IR-DN cDNA probe and hybridized overnight at 42°C. Nitrocellulose membranes were washed once with 6.6X SCP/1% sarkosyl at room temperature for 15 min on a shaker and followed by washing in 1X SCP/1% sarkosyl at 65°C for an additional 15 min. All membranes were exposed to X-ray film at –70°C with an intensifying screen.

Doxycycline-Inducible IGF-IR-DN Gene Expression

Time course and dosage analysis experiments were performed to determine the appropriate parameters for doxycycline required to induce maximal transgene expression. For these studies, the clonal cell line J35, positive for the IGF-IR-DN by Southern blot analysis, was used. Cells were grown to 70–80% confluency in 140 mm culture dishes in CM containing doxycycline at concentrations of 0, 0.5, 1.0, 1.5, 2.0 µg/mL. The cells were incubated at 37°C with 5% CO_2 for 24 h. The media were aspirated and the cells were washed twice with 1X PBS and collected for RNA analysis. In a separate experiment, Clone J35 was grown and treated with CM containing 1.0 µg/mL doxycycline for 0, 12, 24, and 48 h to determine the optimal time required to induce gene expression.

Upon determining the optimal dose of doxycycline and time, cells were grown to 70–80% confluency in 140 mm culture dishes in CM. The media were aspirated and replaced with CM containing doxycycline (1 µg/mL) and incubated at 37°C, 5% CO_2 for 24 h. The cells were washed twice with 1X PBS after aspiration of the medium and collected for RNA analysis.

Northern Blot Analysis

Total RNA was extracted from the clonal cell lines using TRIzol Reagent (Invitrogen Corporation) according to the manufacturer's instructions. Doxycycline-induced IGF-IR-DN gene expression in the stably transfected cell lines was analyzed by Northern blot analysis. Total RNA (30 µg) samples isolated from the MCF-7 cells were electrophoresed in a 0.8% agarose gel containing formaldehyde. The agarose gel was transferred overnight to nitrocellulose membrane and baked at 80°C for 2 h in a dry oven. The membrane was prehybridized in 10 mL prehybridization solution [50% (v/v) formamide, 5X SSPE (1X SSPE = 0.15M NaCl, 0.01 NaH_2PO_4, 1 m*M* EDTA, ph 7.7), 5X Denhardt's Solution, 0.1% sodium dodecyl sulfate (SDS), and 250 µg/mL salmon sperm DNA] for 2 h at 42°C. The prehybridization solution was poured off and replaced by 10 mL of hybridization solution containing the same components in addition to 25 ng of a random primed ^{32}P-labeled IGF-IR-DN cDNA probe. The blots were hybridized at 42°C overnight. Following hybridization, the membranes were washed once with 2X SSC/0.1% SDS at room temperature for 5 min and then washed in 0.1X SSC/0.1% SDS at 65°C for 5 min. All membranes were exposed to X-ray film at –70°C with an intensifying screen. Bands on the exposed film were analyzed using an image analyzer (MCID-M4 Imaging Research, Inc.) and quantified by densitometry (Quantity One Version 4.2 software; Bio-Rad Laboratories).

Western Blot Analysis

Cells were washed twice in ice cold 1X PBS and lysed with modified radioimmunoprecipitation (RIPA) buffer [50 m*M* Tris-HCl pH 7.4, 1% NP-40, 150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* phenylmethyl sulfonyl fluoride (PMSF; Sigma Chemical Company), protease inhibitor cocktail, 1 m*M* sodium orthovanadate (Na_3VO_4; Sigma Chemical Company)]. The lysates were gently rocked for 15 min at 4°C, centrifuged at 27,000*g* for 15 min at 4°C and the supernatants collected and frozen at –20°C until further use.

Total protein concentrations were determined by the Bradford's method using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Broad range precision prestained standards (Bio-Rad Laboratories, Hercules, CA) were used as molecular weight markers. Cell lysates collected using the modified RIPA buffer were boiled in the presence of 4% SDS and 0.4 *M* DTT and separated on a 6.5% SDS-PAGE gel (Bio-Rad Protean II, Hercules, CA) and transferred onto 0.45 µm nitrocellulose membranes (Osmonics, Westborough, MA).

IGF-IR-DN protein expression was detected by immunoblotting the nitrocellulose blots with the rabbit polyclonal antibody (1:200 dilution; N-20) raised against the IGF-IR α-subunit (Santa Cruz Biotechnology, Inc.). The N-20 anti-

body was used to detect the ratio of endogenous and mutant receptor in the doxycycline-induced cells. The blots were also immunoblotted with a rabbit polyclonal antibody raised against the IGF-IR β-subunit (1:200 dilution; H-60; Santa Cruz Biotechnology, Inc.) to discriminate between the truncated β-subunit of the IGF-IR-DN from the wild-type or endogenous IGF-IR. The H-60 antibody recognizes the amino acids 741–800 of the β-subunit of the IGF-IR, above the truncation site (amino acid 950). An antibody raised against actin (1:500 dilution; I-19; Santa Cruz Biotechnology, Inc.) was used to reflect protein loading. All blots were blocked for 2 h in 1X Tris-buffered saline and 0.05% Tween-20 (TBST), pH 7.4, containing 5% skim milk powder followed by incubation with the primary antibody at 4°C overnight. The blots were washed in 1X TBST and incubated with goat anti-rabbit horseradish peroxidase conjugated IgG (1:2500 dilution; Bio-Rad Laboratories) for 1 h. Following a second washing in 1X TBST, color development was performed using the Super Signal substrate kit (Pierce).

Cell Proliferation Studies

A total of 3×10^3 cells were plated in 96-well microtiter plates and allowed to attach overnight. The media were then removed and replaced with supplemented serum-free media (SFM) and left for 24 h. The following day the media was aspirated and replaced with either (a) SFM + doxycycline (1 μg/mL) or (b) SFM – doxycycline for 24 h. For IGF-I stimulation, 5 n*M* recombinant human IGF-I was added to the medium at various time points as indicated. Fresh media were added every other day.

Cellular proliferation was assessed by the crystal violet staining assay *(43)*. Cell density at d –1, 0, 2, 4 and 6 was determined using an enzyme-linked immunosorbent assay (ELISA) SpectraMAX plus Microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) and the SOFT max PRO version 3.0 (Molecular Devices Corporation). Data were collected from two independent experiments (with six measurements per experiment for each condition: $n = 12$). The ANOVA and Tukey tests were used to assess the statistical significance of the data using the GraphPad Prism version 3.02 (GraphPad Software, Inc.). Cell growth in each of the experimental groups was compared. A probability of $p < 0.05$ was considered to be statistically significant.

Protein Phosphorylation Studies

Initial studies were carried out to determine the optimal dose of IGF-I required for receptor phosphorylation. Cells were grown in phenol-red free CM to 70% confluency in 100 mm culture dishes. The media were replaced with phenol-red free, SFM, supplemented with 200 μg/mL bovine serum albumin (BSA) and 10 μg/mL iron-poor human apotransferrin (Sigma Chemical Company) for 24 h. The cells were stimulated with recombinant human IGF-I (Sigma Chemical Company) at concentrations of 0, 2.5, 5.0, 10 n*M* for 10 min at 37°C, after which time the media were removed and the cells were washed in 1X PBS and lysed with modified RIPA buffer.

For the protein phosphorylation studies, the cells were incubated with the supplemented serum-free media containing doxycycline (1 μg/mL) and recombinant human IGF-I. Four experimental groups were established: Group 1, SFM + doxycycline + IGF-I; Group 2, SFM + doxycycline – IGF-I; Group 3, SFM – doxycycline + IGF-I; Group 4, SFM – doxycycline - IGF-I.

To detect tyrosine phosphorylated proteins, the nitrocellulose blots were blocked for 2 h at room temperature in 1X Tris-buffered saline and 0.1% Tween 20 (TBST; pH 7.4) containing 3% BSA (Fisher Scientific). The mouse monoclonal antiphosphotyrosine antibody (1:500 dilution; PY-20; BD Biosciences, Mississauga, ON, Canada) that recognizes all phosphorylated tyrosine residues and the rabbit phospho-insulin receptor substrate-1 antibody (1:200 dilution; Ab-1; Oncogene Research Products, Boston, MA, USA) were used. The blots were incubated overnight with the primary antibody in blocking buffer containing 1% BSA at 4°C. The blots were washed in 1X TBST and incubated with goat anti-mouse horseradish peroxidase conjugated IgG (1:2500 dilution; Bio-Rad Laboratories) for 1 h. Following a second washing in 1X TBST, color development was performed using the Super Signal substrate kit (Pierce).

IRS-1 expression was detected using a 1:200 dilution of the rabbit polyclonal antibody raised against the insulin receptor substrate-1 (IRS-1; Upstate Biotechnology, Lake Placid, NY, USA). The primary antibody was diluted in a blocking buffer of 1X TBST containing 5% skim milk powder.

Morphological Studies

Cells were examined under the light microscope (Nikon Inverted Microscope Model MS; Nippon Kogaku, K.K., Tokyo, Japan). Cells were photographed using a Nikon FE camera and Nikon inverted microscope DiaPhot-Tmd (Nippon Kogaku K.K.) before and after doxycycline treatment and following IGF-I stimulation.

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