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Insulin-Stimulated Cell Growth in Insulin Receptor Substrate-1–Deficient ZR-75-1 Cells Is Mediated by a Phosphatidylinositol-3-Kinase–Independent Pathway

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Abstract In many human breast cancers and cultured cell lines, insulin receptor expression is elevated, and insulin, via its own insulin receptor, can stimulate cell growth. It has recently been demonstrated that the enzyme phosphatidylinositol-3-kinase (PI3-K) mediates various aspects of insulin receptor signaling including cell growth. In order to understand the mechanisms for insulin-stimulated cell growth in human breast cancer, we measured insulin-stimulable PI3-K activity in a non-transformed breast epithelial cell line, MCF-10A, and in two malignantly transformed cell lines, ZR-75-1 and MDA-MB157. All three cell lines express comparable amounts of insulin receptors whose tyrosine autophosphorylation is increased by insulin, and in these cell lines insulin stimulates growth. In MDA-MB157 and MCF-10A cells, insulin stimulated PI3-K activity three- to fourfold. In ZR-75-1 cells, however, insulin did not stimulate PI3-K activity. In ZR-75-1 cells PI3-K protein was present, and its activity was stimulated by epidermal growth factor, suggesting that there might be a defect in insulin receptor signaling upstream of PI3-K and downstream of the insulin receptor. Next, we studied insulin receptor substrate-1 (IRS-1), a major endogenous substrate for the insulin receptor which, when tyrosine is phosphorylated by the insulin receptor, interacts with and activates PI3-K. In ZR-75-1 cells, there were reduced levels of protein for IRS-1. In these cells, both Shc tyrosine phosphorylation and mitogen-activated protein kinase (MAP-K) activity were increased by the insulin receptor (indicating that the $p21^{ras}$ pathway may account for insulin-stimulated cell growth in ZR-75-1 cells).

The PI3-K inhibitor LY294002 (50 μM) reduced insulin-stimulated growth in MCF-10A and MDA-MB157 cell lines, whereas it did not modify insulin effect on ZR-75-1 cell growth. The MAP-K/Erk (MEK) inhibitor PD98059 (50 μM) consistently reduced insulin-dependent growth in all three cell lines.

Taken together, these data suggest that in breast cancer cells insulin may stimulate cell growth via PI3-K–dependent or–independent pathways. J. Cell. Biochem. 70:268–280, 1998.

Key words: insulin; insulin receptor; breast cancer cells; insulin receptor substrate 1; phosphatidylinositol-3-kinase

Insulin receptors are overexpressed in many human breast cancer specimens [Papa et al., 1990]. Also in many human breast cancer cell lines, functional insulin receptors are overexpressed when compared with nontransformed breast epithelial cells [Milazzo et al., 1992]. In these cultured cells, insulin, via its own insulin receptor, stimulates cell growth [Milazzo et al., 1992]. Moreover, in nonmalignantly transformed breast epithelial cells transfected with and overexpressing insulin receptors, insulin induces a ligand-dependent transformed pheno-

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type [Frittitta et al., 1995; Giorgino et al., 1991]. The insulin receptor is a member of the receptor tyrosine kinase family. Insulin-mediated signals, both metabolic and mitogenic, are mediated by insulin binding to its plasma membrane receptor, followed by insulin receptor tyrosine autophosphorylation [White and Kahn, 1994; Goldfine, 1987]. Subsequently there is rapid phosphorylation of an intermediate downstream docking protein, insulin receptor substrate-1 (IRS-1) [Shoelson et al., 1992; Sun et al., 1991]. IRS-1 is a cytoplasmic protein with m.w. 160–190 kDa and is a major cellular substrate for both the insulin receptor and the insulin-like growth factor-I (IGF-I) receptor [Myers et al., 1993]. IRS-1 contains 20 potential tyrosine phosphorylation consensus sequences that associate with proteins containing SH2 domains [Shoelson et al., 1992; Sun et al., 1991]. Phosphorylated IRS-1 directly interacts with the p85 regulatory subunit of phosphatidylinositol-3-kinase (PI3-K) and subsequently stimulates its p110 catalytic subunit activity [White and Kahn, 1994; Quon et al., 1995; Myers et al., 1992]. PI3-K also directly associates with and is stimulated by many activated growth factor receptors and nonreceptor tyrosine kinases [Kaplan et al., 1987; Courtneidge and Heber, 1987; Varticovski et al., 1989]. PI3-K phosphorylates phosphatidylinositol (PI), PI-4-monophosphate, and PI-4,5-bisphosphate to generate PI-3-monophosphate, PI-3,4-bisphosphate, and PI-3,4,5-trisphosphate, respectively [Whitman et al., 1987, 1988]. Although these D-3–phosphorylated phosphoinositides are not direct substrates for any known phospholipases and their exact physiological roles are unknown [Serunian et al., 1989], increases in PI3-K activity following growth factor stimulation are closely associated with an increase in cell proliferation [Parker and Waterfield, 1992]. Phosphorylated IRS-1 also interacts with an adaptor molecule Grb2, which links IRS-1 to the $p21^{ras}$ signaling system, via Grb2–mSos complex [Skolnik et al., 1993; Baltensperger et al., 1993]. mSos is a mammalian homologue of the *Drosophila* Son-of-sevenless (Sos) protein, a guanine nucleotide exchange factor, and a positive regulator of the $p21^{ras}$ signaling [Egan et al., 1993; Rozakis-Adcock et al., 1993]. Like PI3-K activity, activation of $p21^{ras}$ has also been demonstrated to be important for cell proliferation and differentiation [Szeberenyi et al., 1990; Smith et al., 1986]. Unlike PI3-K activation, however, $p21^{ras}$ activation can also be achieved by an IRS-1–independent pathway via tyrosine phosphorylation of the Shc protein [Skolnik et al., 1993; Yonezawa et al., 1994; Ouwens et al., 1994].

Since insulin and the insulin receptor play a role in breast cancer cell growth, we investigated various components of the insulin receptor signaling system in three insulin-responsive cells. These included a nonmalignantly transformed line (MCF-10A) and two breast cancer cell lines (MDA-MB157 and ZR-75-1). Furthermore, specific PI3-K (LY294002) and MEK (PD98059) inhibitors were used to evaluate the blocking effect of these signaling components, which are involved in insulin-stimulated cell growth. The data indicate that activation of PI3-K may not be a necessary requirement for insulin-stimulated cell growth in certain breast cancer cells.

MATERIALS AND METHODS

Materials

The following materials were purchased from Sigma Chemical Company (St. Louis, MO): bovine serum albumin (BSA), bacitracin, insulin, sodium fluoride, phosphatidylinositol, β-glycerophosphate, HEPES, trypsin, dithiothreitol (DTT), ATP, Triton X-100, sodium orthovanadate, aprotinin, phenylmethylsulfonyl fluoride (PMSF), salmon sperm DNA, epidermal growth factor, porcine insulin, cholera toxin, and hydrocortisone. Cell culture media and antibiotics were obtained from GIBCO (Paisley, Scotland). All sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) reagents and the protein determination kit were obtained from Bio-Rad Laboratories (Richmond, CA). Streptavidin-horseradish peroxidase (HRP) was obtained from Pierce (Rockford, IL); Protein A-Sepharose was from Pharmacia Laboratory Division (Uppsala, Sweden); [γ-^{32}P]ATP (6,000 Ci/mmol) was from Du Pont New England Nuclear (Boston, MA). Silica gel TLC plates were from Alltech Associates Inc. (Deerfield, IL). Nitrocellulose membranes (Hybond-ECL) and the ECL detection system were obtained from Amersham. Anti-Shc antiserum and the anti-phosphotyrosine (α-PY) monoclonal antibody were obtained from Transduction Laboratories (Lexington, UK); rabbit antisera against the 85-kDa and 110-kDa subunits of PI3K, α-PY biotinylated antibody, myelin basic protein, and rabbit α-MAP-K antiserum were obtained from Upstate Biotechnology Inc. (Lake Placid, NY).

The MA-20, a mouse monoclonal antibody to the insulin receptor, was prepared as previously described [Forsayeth et al., 1987; Hawley et al., 1989]. PI3-K inhibitor LY294002 and the MEK inhibitor PD98059 were kindly provided, respectively, by Dr. C.J. Vlahos (Lilly Research Laboratories, Indianapolis, IN) and Dr. A.R. Saltiel (Parke Davis Pharmaceutical Research, Ann Arbor, MI).

Cell Culture

All cell lines were obtained from ATCC. MCF-10A cells were grown in Dulbecco's modified Eagle's medium-F12 (1:1) supplemented with 5% horse serum donor herd, penicillin and streptomycin, hydrocortisone, insulin, epidermal growth factor, and cholera toxin. ZR-75-1 and MDA-MB157 cells were grown in minimum essential medium with 10% fetal bovine serum, nonessential amino acids, glutamine, penicillin, and streptomycin.

Cell Growth and Use of Signaling Inhibitors

Cells plated in 24-well tissue culture plates were grown in regular medium for 24 h and then serum-starved in medium containing 0.1% BSA. After 16 h, insulin (10 or 100 nM, supplemented every 12 h) and either the PI3-K inhibitor LY294002 (50 μM) or the MEK inhibitor PD98059 (50 μM) were added and supplemented every 24 h. Two days (MCF-10A cells) or 4 days (MDA-MB157 and ZR-75-1 cells) later, cells were collected after incubation at 37°C for 5 min in phosphate-buffered saline (PBS) buffer containing 1 mM EDTA and 0.1% BSA. After spinning 2 min 100 *g* at room temperature cells were resuspended in 150 μl of complete medium, and 50 μl was used for cell counting with a Burker chamber. Cells were then pelleted as above and solubilized in 0.03% SDS for DNA measurement [Labarca and Paigen, 1980].

Insulin Receptor Radioimmunoassay and Insulin Receptor Autophosphorylation

The insulin receptor content was measured by a radioimmunoassay as previously described [Pezzino et al., 1989]. The sensitivity of the assay is 0.18 ng; the cross-reactivity with the related IGF-IR is negligible. For studies of insulin receptor autophosphorylation, cells in 100-mm dishes grown at approximately 70% confluency were serum-starved for 16 h and incubated with different concentrations of insulin for 15 min at 37°C. Cells were then washed and solubilized with 50 mM Hepes buffer, pH 7.4, containing 1% Triton X-100, 2 mM sodium orthovanadate, and 1 mM PMSF at 4°C [Labarca and Paigen, 1980; Pezzino et al., 1989]. Cell lysates were cleared by centrifugation (12,000 *g* at 4°C for 15 min) and stored at −80°C until assayed. IR autophosphorylation was detected by a plate-capture enzyme-linked immunosorbent assay (ELISA) [Boge and Roth, 1995]. Ninety-six–well microtiter plates (Nunc Immunoplate I Maxisorp) were coated with 100 μl of a specific monoclonal anti-insulin receptor antibody (2 μg/ml in 50 mM bicarbonate buffer, pH 9.0) for 16 h at 4°C. After blocking of nonspecific binding with 1% BSA (30 min at 56°C), 100 μl of cell lysate (100 μg of protein) was added and incubated at 22°C. Two hours later 100 μl of biotinylated α-PY antibody was added. After 2 h at 22°C, 100 μl of peroxidase-conjugated streptavidin (0.1 μg/ml) was added (30 min at 22°C). Finally, the peroxidase activity was determined colorimetrically by adding 100 μl of OPD substrate (0.67 mg/ml in 0.1 M phosphate-citrate buffer, pH 5.0, and 0.4 μl/ml of 30% H_2O_2). The reaction was stopped by adding 100 μl of 1 M phosphoric acid, and the absorbance was measured at 450 nm.

Western Blot Measurements

Cells in 100-mm dishes grown at approximately 70% confluency were serum-starved for 16 h and incubated with 10 nM insulin (at 37°C) for different times (indicated in the figure legends). At the end of incubation, cells were washed with ice-cold PBS and then solubilized with 20 mM Tris buffer, pH 7.4, containing 100 mM NaCl, 1.3% Nonidet P-40, 10 mM sodium fluoride, 1 mM EDTA, 10 μg/ml leupeptin, 2 mM PMSF, and 2 mM Na-orthovanadate, after which the insoluble material was removed by centrifugation (12,000 *g* at 4°C for 15 min). Samples were either directly immunoprecipitated with specific antibodies or denatured in Laemmli buffer containing 100 mM DTT and then subjected to SDS-PAGE, followed by Western blotting analysis. Membranes were immunoblotted with different antibodies (α-PY, α-IRS-1, α-p85, α-p110, α-Shc), and specific proteins were detected with horseradish peroxidase-conjugated antimouse or antirabbit IgG followed by ECL detection [Sung et al., 1994].

IR and IRS-1 mRNA PCR Measurement

Total RNA was extracted using a commercial kit (RNA Extraction Kit, Pharmacia). cDNA

was synthesized by adding 0.8 to 5 µg of total RNA to 2.5 units/ml murine leukemia virus reverse transcriptase (Perkin Elmer Cetus, Norwalk, CT), 2.5 mM random hexamers, 1 U/ml RNase inhibitor, 1 mM of each dNTP, 5 mM $MgCl_2$, 500 mM KCl, and 100 mM Tris-HCl in a total volume of 20 µl and incubating at 37°C for 1 h. PCR amplification was performed in the same tube by adding 2.5 units Taq polimerase (Perkin Elmer Cetus) and 15 pmol of each primer in 80 µl of a 2 mM $MgCl_2$-50 mM KCl-1 mM Tris-HCl buffer. For IRS-1 mRNA detection the following primer sequences were used: 5′-3′: CT-CTG-CTC-AGC-GTT-GGT-GGT corresponding to the position 989 of the human IRS-1 cDNA [Araki et al., 1993]; 3′-5′:GGT-GCT-TGT-TCT-TGG-AGT-CA. For IR mRNA detection the following sequences were used: 5′-3′: GAG-AAC-AAC-GTC-GTC-CAC-TT; 3′-5′: GAG-ACT-GGC-TGA-CTC-GTT-GA. Conditions for PCR were 95°C × 30 s; 60°C × 30 s; 72°C × 60 s; for 35 cycles. PCR products were analyzed by 2% agarose gel electrophoresis, followed by ethidium bromide staining.

PI3-K In Vitro Assay

Cells were incubated with insulin (5 min, 37°C) and then lysed at 4°C in buffer containing 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% (vol/vol) glycerol, 1% Nonidet P-40, 1 mM DTT, 1 mM PMSF, 0.4 mM sodium orthovanadate. The insoluble material was removed by centrifugation at 13,000 *g* for 15 min. Soluble supernatants were immunoprecipitated with either α-PY or α-85 antibodies and protein A-Sepharose. The immunoprecipitates were washed two times in phosphate-buffered saline containing 1% Nonidet P-40 and 1 mM DTT, two times in 100 mM Tris, pH 7.4, containing 500 mM $LiCl_2$ and 1 mM DTT, and then two times with 10 mM Tris, pH 7.4, containing 100 mM NaCl and 1 mM DTT. PI3-K activity was measured directly in these immunoprecipitates in 50 µl of reaction mixture containing 20 mM Hepes, pH 7.1, 0.4 mM EGTA, 0.4 mM sodium phosphate, 10 mM $MgCl_2$, 0.2 mg/ml PI, and [γ-^{32}P]-ATP (40 µM, 10 µCi). After 5 min at 25°C, the reaction was stopped by the addition of 15 µl of 4N HCl and 130 µl of CHCl3:methanol (1:1). The samples were centrifuged, and the lower organic phase was removed and applied to a silica gel TLC plate which had been coated with 1% potassium oxalate at 100°C for 1 h. Thin-layer chromatography was performed in CHCl3:CH3OH:H2O:NH4OH (60:47:11.3:3.2), dried, and visualized by autoradiography [Sung and Goldfine, 1992].

P70^{S6} Kinase Assay in Cytosolic Extracts

After stimulation for 30 min with 10 nM insulin at 37°C, cells were lysed in buffer containing 80 mM β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 0.1 mM PMSF, 0.2 mM Na-orthovanadate, and 1 µg/ml leupeptin. This material was then sonicated for 15 sec and ultracentrifuged for 30 min at 200,000 *g*. p70^{S6}K activity was measured by incubating 10 µg of cell extracts in 30 µl buffer containing 50 mM MOPS, pH 7.4, 1 mM DTT, 5 µg *Artemia salina* egg 40S ribosome [Zasloff and Ochoo, 1974], and [γ-^{32}P]-ATP (50 µM, 2.5 µCi). After 15 min at 30°C, the reaction was terminated by the addition of 10 µl of 4 × Laemmli buffer. Samples were then boiled for 3 min, and S6 phosphorylation was analyzed by 12% SDS-PAGE followed by autoradiography [Sung et al., 1989].

MAP Kinase Assay

After incubation with 10 nM insulin for 5 min at 37°C, cells were lysed with the same buffer used for PI3 kinase measurement, and the insoluble material was removed by centrifugation at 100,000 *g* at 4°C. α-MAP-K antibody was then added for 16 h at 4°C and collected on Protein A-sepharose beads for 1 h at 22°C. Immunoprecipitates were washed 3 times with lysis buffer and 3 times with 50 mM Tris-HCl (pH 8), 1 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 10% glycerol, 25 mM $MgCl_2$ before being suspended in 30 µl of the same buffer containing 0.5 mg/ml myelin basic protein and [γ-^{32}P]-ATP (20 µM, 1 µCi). After 30 min at 30°C, reactions were stopped by the addition of 10 µl of 0.6% HCl, 1% BSA, 1 mM ATP. Twenty microliters of each sample was then spotted in duplicate on 2 × 2-cm P81 phosphocellulose paper (Whatman Int. Ltd, Maidstone, UK), washed two times in 180 mM phosphoric acid, and the radioactivity on each disc was then measured [Rossomando et al., 1992].

RESULTS

Insulin Receptor Content and Insulin Effect on Cell Growth

Insulin receptor content was examined in one nontransformed human breast cell line (MCF-

TABLE I. Effect of Insulin on Cell Growth*

	−Insulin	+Insulin
MCF-10A	2.8 ± 0.45	5.3 ± 0.08
ZR-75-1	3.85 ± 0.2	6.23 ± 0.32
MDA-MB157	3.72 ± 0.16	7.52 ± 0.23

*Cells in 24 well plates were serum starved for 16 h, treated with and without 10 nM insulin and grown to 70–80% confluency (2 days for MCF-10A; 5 days for ZR-75-1, MDA-MB157). Next, cells were solubilized in 0.03% SDS and DNA contents were measured. Data are presented as μg DNA/well and are mean ± SEM of three separate experiments.

10A) and two human breast cancer cell lines (ZR-75-1 and MDA-MB157) by a radioimmunoassay as previously described (30). MCF-10A, ZR-75-1 and MDA-MB157 cells contained 5.6 ± 1.1, 17.1 ± 1.9 and 36.1 ± 4.4 ng insulin receptor/10^6 cells, respectively (mean ± SEM, n = 6).

To study insulin effect on cell growth, cells were grown with and without 10 nM insulin for 2–5 days, solubilized and their total DNA content was measured. In agreement with the previous findings [Milazzo et al., 1992], insulin stimulated cell growth by 40–50% in all three breast cell lines studied (Table I).

Cell growth in the absence of insulin ranged 1–3% over baseline (data not shown).

Insulin Receptor Tyrosine Autophosphorylation by Insulin

We next measured insulin receptor tyrosine autophosphorylation in response to various concentrations of insulin. Cells were incubated for 15 min with 0.1–100 nM insulin and solubilized. In all three cell lines, insulin increased tyrosine autophosphorylation of the insulin receptor (Fig. 1). In all cells half-maximal effect was observed at 0.2–0.3 nM, and maximal effect was observed at 10 nM.

Lack of an Effect of Insulin on PI3-K Activity and $p70^{s6K}$ Activity in ZR-75-1 Cells

Following 10 nM insulin stimulation of cells for 5 min, we measured PI3-K activity. In both MCF-10A and MDA-MB157 cells, insulin treatment of cells stimulated PI3-K activity three- to fourfold (Fig. 2). In ZR-75-1 cells, however, insulin did not stimulate PI3-K activity. $p70^{S6}$ kinase is a cytosolic enzyme [Chen and Blenis, 1990] slowly activated (maximal activation after 30 min) by insulin downstream to PI3-K [Chung et al., 1994; Cheatham et al., 1994]. To avoid interference by $p90^{S6}K$ activity, we measured enzymatic activity in cytosolic extracts at 30 min (when stimulated $p90^{S6}K$ activity comes down to almost basal levels). In MCF-10A and

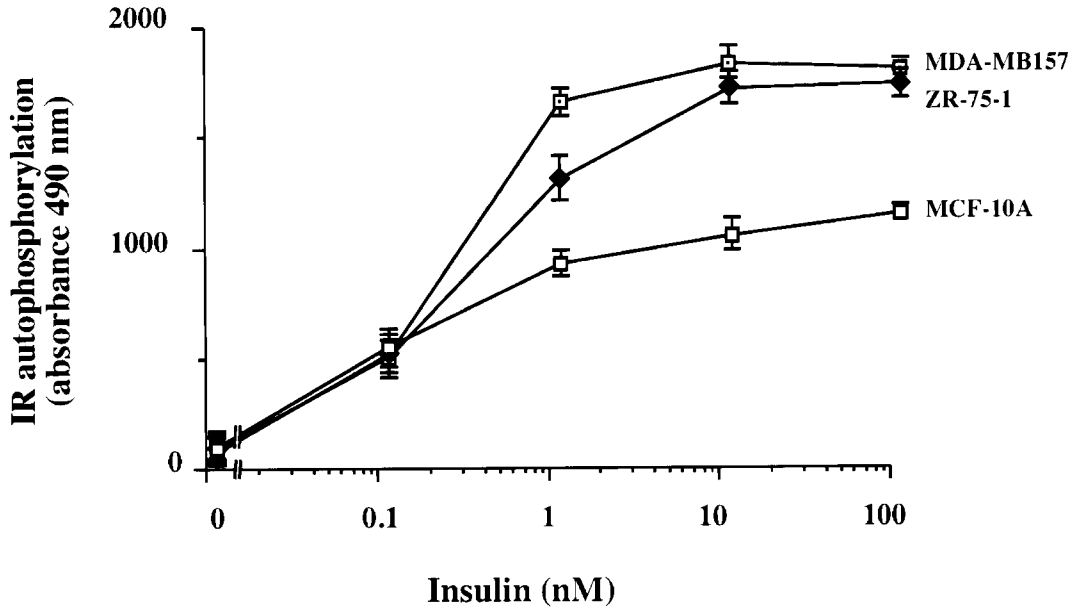

Fig. 1. Insulin stimulation of insulin receptor tyrosine autophosphorylation. Confluent cell monolayers were serum-starved for 16 h and incubated for 15 min with various insulin concentrations at 37°C. Next, cells were solubilized for 1 h at 4°C in 1% Triton X-100 lysis buffer. Tyrosine autophosphorylation of the insulin receptor was measured by a plate immunocapture assay. Each value is the mean ± SEM of three separate experiments.

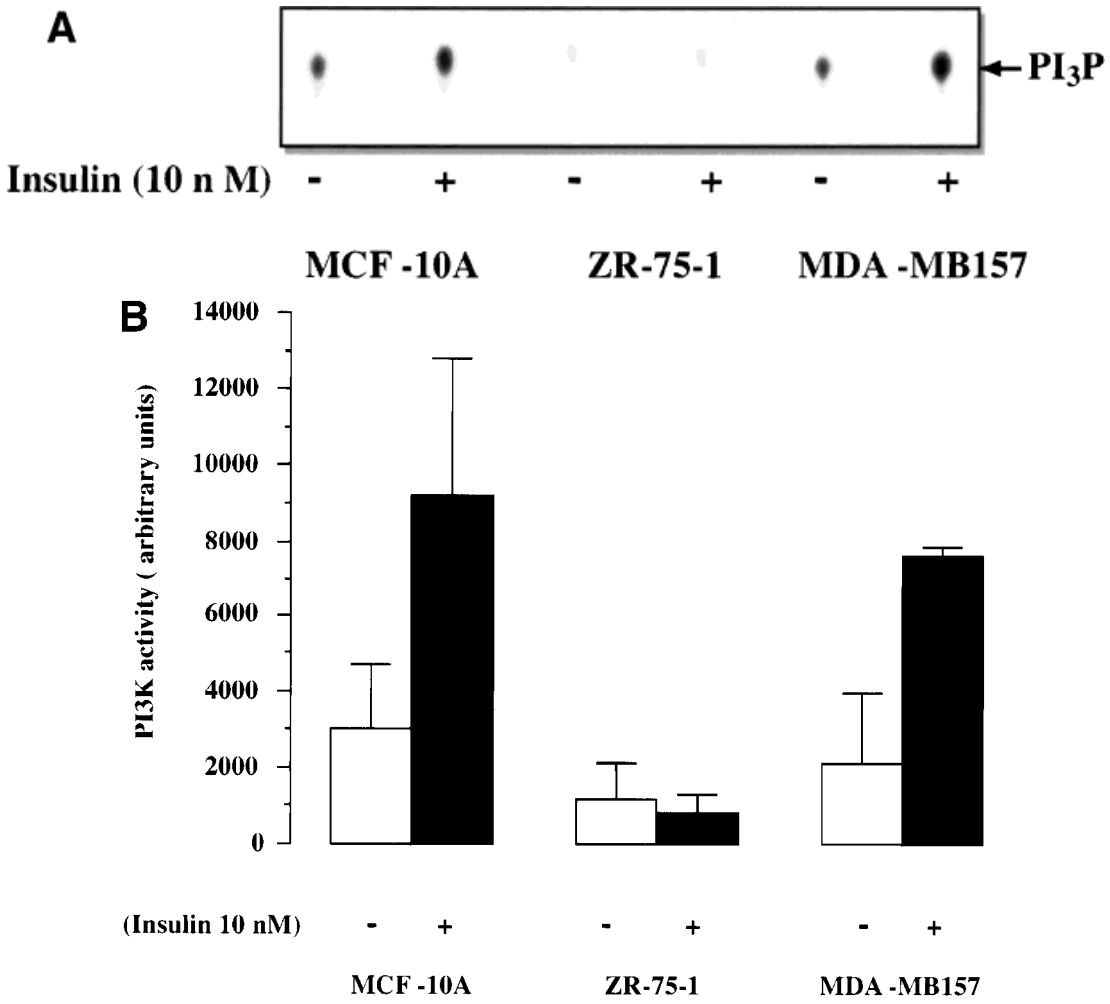

Fig. 2. Stimulation of PI3-K activity by insulin. Cells were incubated for 5 min with or without 10 nM insulin, solubilized, and immunoprecipitated with α-PY antibody. PI3-K activity was then measured directly in these immunoprecipitates in a reaction mixture containing [γ-^{32}P]ATP (40 μM; 10 μCi) and phosphatidylinositol (10 μg). Next, reaction products were analyzed by thin-layer chromatography and subsequent autoradiography. **A:** A representative experiment is shown. **B:** Results of four experiments are shown (mean ± SEM).

MDA-MB157 cells, but not in ZR-75-1 cells, insulin stimulated $p70^{S6}$ kinase activity, further indicating that in ZR-75-1 cells PI3-K was not activated by insulin (Fig. 3).

In order to study whether the lack of insulin stimulable PI3-K activity in ZR-75-1 cells was due to the lack of PI3-K protein per se, we analyzed soluble cell lysates from all three cell lines by western blotting with antibodies to either p110 catalytic subunit or p85 regulatory subunit of PI3-K. Data indicated that ZR-75-1 cells, as well as the other two cell lines, contained both p110 and p85 subunits of PI3-K (Fig. 4).

We next studied whether PI3-K in ZR-75-1 cells can be stimulated by other growth factors. We incubated ZR-75-1 cells for 5 min with either insulin, IGF-I, or EGF and assayed for PI3-K activities (Fig. 5). Both insulin and IGF-I failed to stimulate PI3-K activity in these cells. However EGF, unlike insulin and IGF-I, stimulated PI3-K eight- to tenfold in these cells. These data indicated that in ZR-75-1 cells there may be a signaling defect upstream of PI3-K which is unique for the insulin and IGF-I receptors, but not the EGF receptor. One such problem may reside at the level of IRS-1.

Reduced Levels of IRS-1 Protein in ZR-75-1 Cells

To study whether ZR-75-1 cells contain functional IRS-1, which is necessary for insulin receptor activation of PI3-K, we first measured IRS-1 mRNA. Total RNA was prepared from cells, and cDNA was generated by reverse trans-

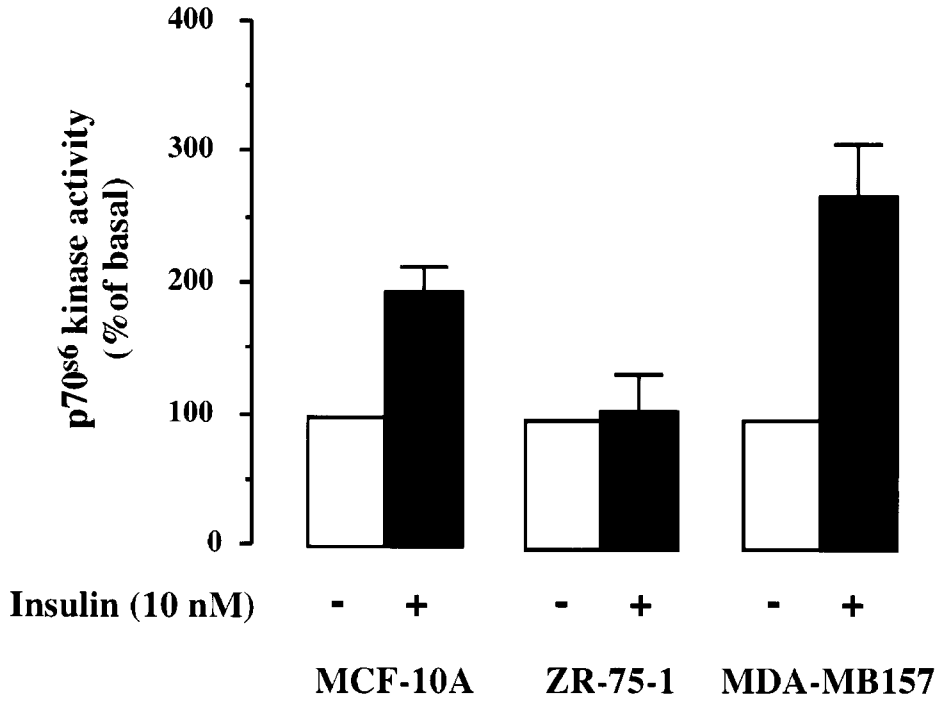

Fig. 3. Stimulation of $p70^{S6K}$ activity by insulin. Cells were incubated for 30 min with or without 10 nM insulin and sonicated. The soluble cellular extracts were then assayed for $p70^{S6K}$ activity in a reaction mixture containing 40S small ribosomal subunits (5 μg) and [γ-^{32}P]ATP (50 μM; 2.5 μCi). S6 phosphorylation was analyzed by SDS-PAGE and subsequent autoradiography. Data are presented as mean ± SEM of three separate experiments.

criptase reaction. With IRS-1–specific primers (20mer), IRS-1–specific cDNA was next amplified by polymerase chain reaction (PCR) and analyzed by 2% agarose gel electrophoresis. In parallel experiments, insulin receptor cDNA was also amplified with insulin receptor–specific primers. Both insulin receptor and IRS-1 mRNAs were present in all three cell lines (data not shown). Next, we measured IRS-1 protein content by immunoprecipitating and blotting with α-IRS-1 antibody. Densitometric analysis indicated that the IRS-1 content ratio was 11.5, 3.7, and 0.8 in MCF-10A, MDA-MB157, and ZR-75-1, respectively. Therefore, IRS-1 protein was normally expressed in MCF-10A and MDA-MB157 cells, but only a negligible amount of IRS-1 was present in ZR-75-1 cells. (Fig. 6, lower panel).

To further address whether in ZR-75-1 cells there was a functional IRS-1 protein which was tyrosine phosphorylated in response to insulin, we treated cells with 10 nM insulin. Cells were solubilized, and the lysates were immunoprecipitated with α-IRS-1 antibody and Western blotted with α-PY antibody. In MCF-10A and MDA-MB157 cells, insulin treatment markedly increased tyrosine phosphorylation of IRS-1 (Fig. 6, upper panel). In contrast, in ZR-75-1 cells, insulin treatment increased IRS-1 phosphorylation to a lesser extent.

Insulin Stimulation of MAP-K Activity and Shc Tyrosine Phosphorylation in ZR-75-1 cells

In addition to PI3-K activity, stimulation of MAP-K, a downstream molecule of $p21^{ras}$ pathway, has also been implicated in cell growth [Thomas, 1992]. Thus, we measured MAP-K activity following insulin treatment of cells. Cells were incubated for 5 min with 10 nM insulin and solubilized. Lysates were next immunoprecipitated with α-MAP-K antibody, and these immunoprecipitates were assayed for their MAP-K enzymatic activity. In all three cell lines, insulin treatment of cells stimulated MAP-K activity up to 60–100% over basal (Fig. 7).

It has been well established that insulin treatment of cells also increases Shc tyrosine phosphorylation, which in turn links the insulin receptor to the $p21^{ras}$ pathway leading to cell growth [Skolnik et al., 1993; Yonezawa et al., 1994; Ouwens et al., 1994]. Thus, we studied tyrosine phosphorylation of p52 Shc following insulin treatment of cells. Cells were incubated

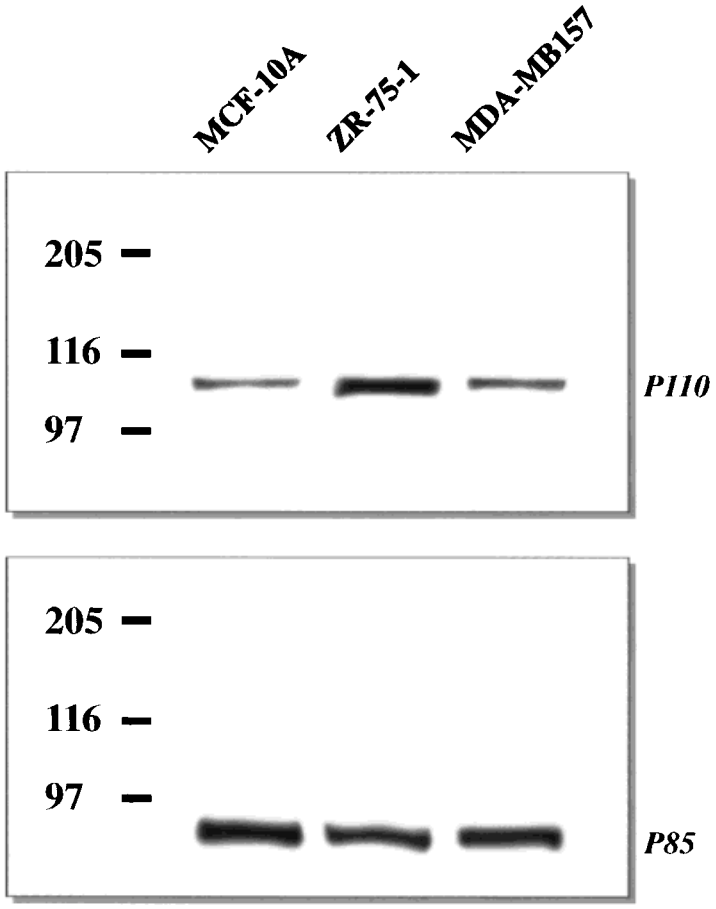

Fig. 4. PI3-K contents by Western blotting analysis. Cells were harvested and solubilized. Soluble cell lysates were then immunoprecipitated with α-p85 or α-p110, and the immunoprecipitates were next analyzed by western blotting with either α-p85 or α-p110 antibodies. A representative experiment is shown.

for 15 min with 10 nM insulin and solubilized. Soluble cell lysates were then immunoprecipitated with α-Shc antibody followed by western blotting analysis with α-PY antibody. In all three cell lines, Shc tyrosine phosphorylation was stimulated following insulin treatment (Fig. 8).

Effect of the PI3-K Inhibitor LY294002 and the MEK Inhibitor PD98059 on Insulin-Stimulated Cell Growth

In order to verify whether PI3-K activity is required for mitogenesis in MCF-10A, MDA-MB157, and ZR-75-1 breast cell lines, we evaluated the effect of the specific PI3-K inhibitor LY294002 on insulin-stimulated (100 nM) cell growth by both DNA synthesis and cell number in all three cell lines. Furthermore, to test the hypothesis that the alternative Shc-p21ras-MAP-K pathway may be responsible for insulin-dependent mitogenesis in ZR-75-1 cells, we also evaluated the effect of the MAP-K/Erk kinase (MEK) inhibitor PD98059 on insulin-stimulated (100 nM) growth in the same cell lines. Both inhibitors were used at 50 μM, a concentration known to be effective for cell growth inhibition [Sanchez-Margalet et al., 1994; Dudley et al., 1992]. Cell viability, tested with trypan blue, ranged from 94% with LY294002 to 98% with PD98059. Exposure to LY294002 slightly decreased insulin-independent (basal) cell growth in ZR-75-1 and MDA-MB157 cells. The MEK inhibitor PD98059 markedly reduced insulin-stimulated growth in terms of DNA content in all three cell lines studied (−76%, −86%, and −81% in ZR-75-1, MDA-MB157, and MCF-10A cells, respectively) (Fig. 9). The PI3-K inhibitor LY294002 markedly suppressed insulin-stimulated cell growth in MCF-10A cells (−82%), blunted it in MDA-MB157 cells (−40%), and had no effect in ZR-75-1 cells (+1%). This latter finding emphasizes that PI3-K activation is not required for insulin-stimulated cell growth in ZR-75-1 cells. The partial suppression of insulin response in MDA-MB157 cells incubated with the PI3-K inhibitor supports the idea that in this cell line additional or alternative pathways are operative for cell growth. The

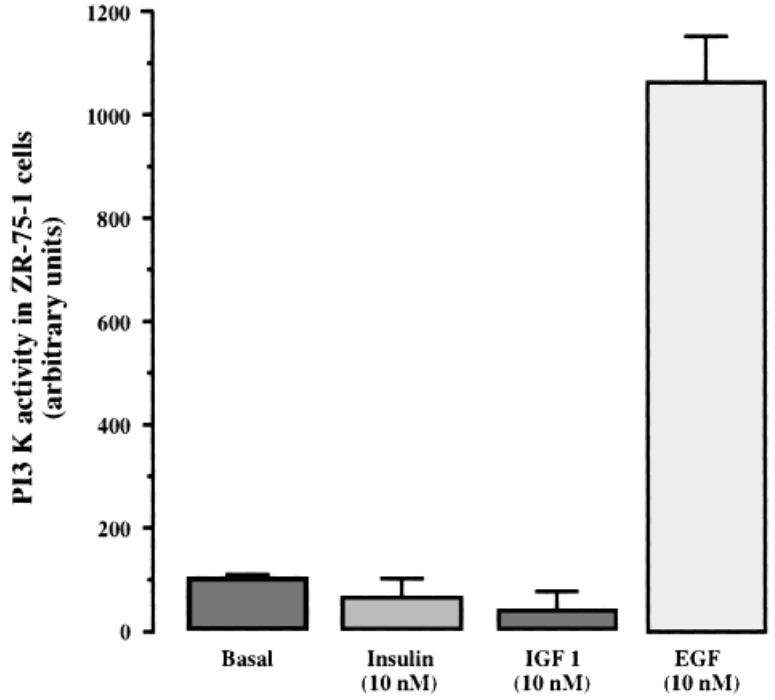

Fig. 5. Stimulation of PI3-K activity by different ligands in ZR-75-1 cells. Cells were incubated for 5 min with 10 nM of either insulin, IGF-I, or EGF. Soluble cell lysates were then immunoprecipitated with α-PY, and the immunoprecipitates were assayed for their PI3-K activities. A representative of three separate experiments is shown.

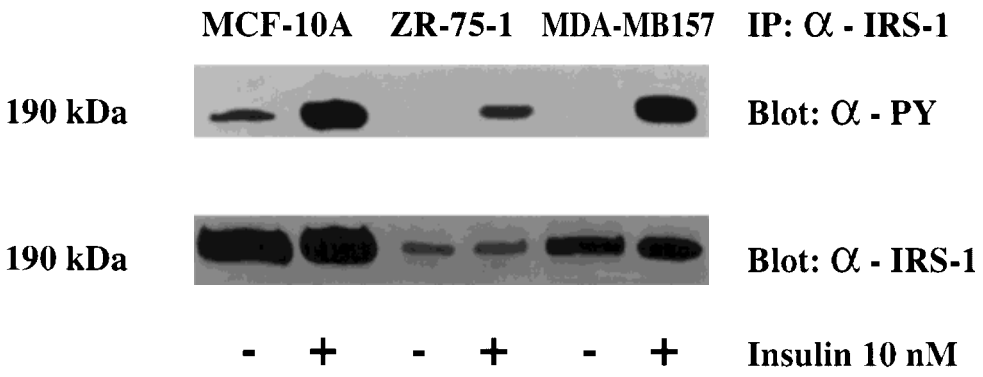

Fig. 6. IRS-1 protein content and phosphorylation. Cells were incubated for 1 min with or without 10 nM insulin and solubilized. Cell lysates were then immunoprecipitated with α-IRS-1 and resolved by SDS-PAGE. After transfer on NC filter, proteins were blotted with α-PY antibody to evaluate ligand-dependent phosphorylation and subsequently reprobed with α-IRS-1 antibody to measure the relative IRS-1 protein content.

observation that in MCF-10A cells both inhibitors markedly suppressed insulin-mediated growth suggests that the two signals are in some way linked [Sjolander et al., 1991] and that PI3-K activation may lead to MAP-K activation through the ras pathway.

Similar results were also observed with both inhibitors in all three cell lines by cell number count (not shown).

DISCUSSION

In the present study we have investigated the relationship between early steps of the insulin receptor signaling system and insulin stimulation of cell growth in three breast epithelial cell lines in culture (MCF-10A, ZR-75-1, and MDA-MB157). In all three cell lines, insulin stimulated insulin receptor tyrosine autophosphorylation and cell growth. In addition, in all of them insulin activated the $p21^{ras}$ pathway as evidenced by MAP-K activation. Although it is well accepted that IRS-1 plays a major role in insulin-stimulated cell growth [Waters et al., 1993], more controversial is the role of PI3-K. A more direct link between PI3-K and growth factor–mediated mitogenesis comes from studies on the role of the platelet-derived growth factor (PDGF) receptor and HGF in different

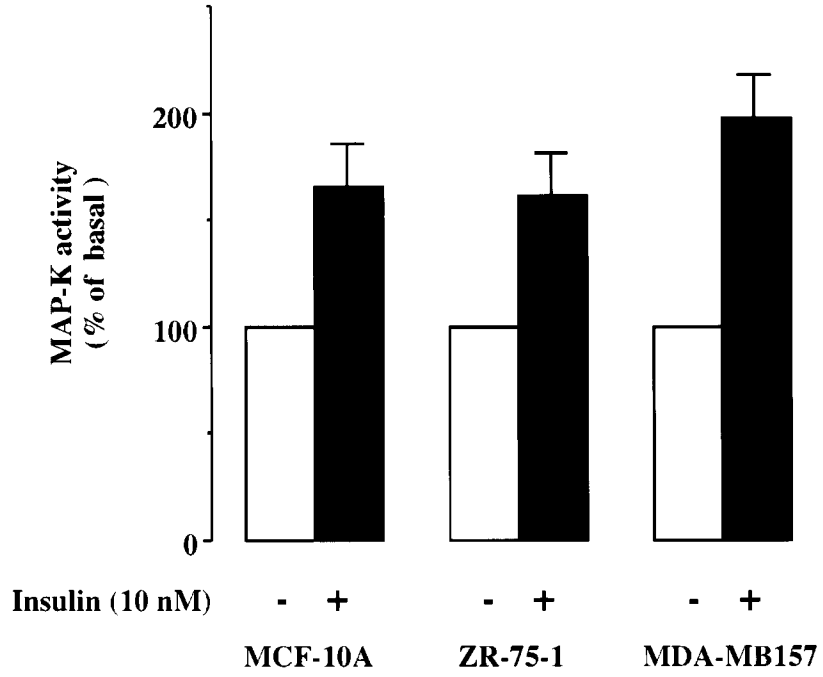

Fig. 7. Stimulation of MAP-K activity by insulin. Cells were incubated for 5 min with or without 10 nM insulin and solubilized. Soluble cell lysates were then immunoprecipitated with α-MAP-K, and the immunoprecipitates were assayed for their MAP-K activities in reaction mixtures containing myelin basic protein (15 μg) and [γ-^{32}P]ATP (20 μM; 1 μCi). Reaction products were next spotted on P81 phosphocelluose paper, and radioactivity was measured. Data indicate the mean ± SEM of three separate experiments.

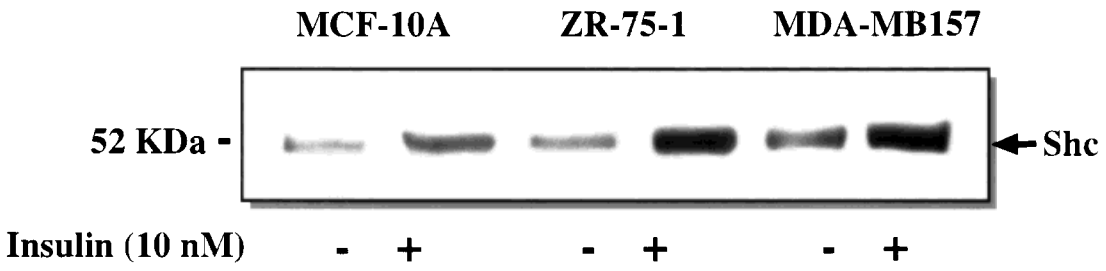

Fig. 8. Tyrosine phosphorylation of Shc by insulin. Cells were incubated for 15 min with or without 10 nM insulin and solubilized. Soluble cell lysates were then immunoprecipitated with α-Shc antibody, and the immunoprecipitates were analyzed by western blotting with α-PY antibody. A representative of three experiments is shown.

cellular models [Valius and Kazlaus, 1993; Rahimi et al., 1996].

In our studies insulin was able to activate the PI3-K pathway in MCF-10A and MDA-MB157 cells, whereas in ZR-75-1 breast cancer cells no PI3-K activation was observed after insulin stimulation, suggesting that in this cell line insulin-stimulated growth occurs independently of PI3-K.

In ZR-75-1 cells, the PI3-K protein content was similar to that of the other cell lines, and PI3-K was readily activated by EGF stimulation, indicating that the protein was functionally active. It is known that the insulin receptor and the related IGF-I receptor activate PI3-K indirectly, via the docking protein IRS-1 [White and Kahn, 1994; Quon et al., 1995; Myers et al., 1992], whereas EGF and its receptor stimulate PI3-K activity by physical association with HerbB3, which mimics the effect of IRS-1 by recruiting the cytosolic PI3-K and making it available as a substrate for the EGF-R kinase [Soler et al., 1994; Soltoff et al., 1994]. It should be noted that in ZR-75-1 cells only a little amount of IRS-1 protein was present. This observation may suggest that the amount of IRS-1 in ZR-75-1 cells is not sufficient to activate PI3-K or that in these cells some other regulatory impairment occurs at this level. In agreement with this possibility, in ZR-75-1 cells both insulin and IGF-I (which require functional IRS-1 for PI3-K activation) but not EGF (which does not require functional IRS-1 for PI3-K activation) failed to stimulate PI3-K activity and also p70^{S6}K, which is activated downstream of PI3-K.

In ZR-75-1 cells, however, insulin induced Shc tyrosine phosphorylation and MAP-K acti-

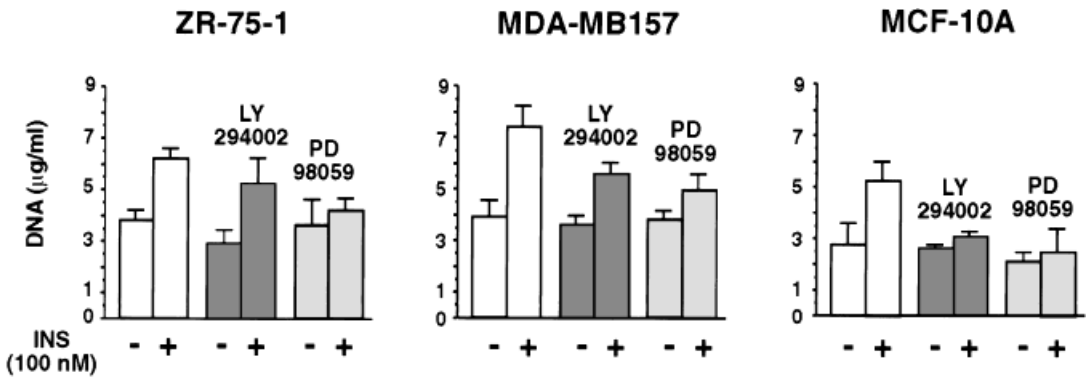

Fig. 9. Effect of inhibitors LY294002 and PD98059 on cell growth. Cells were grown in regular medium for 24 h and serum starved in 0.1% BSA containing medium for 16 h. Insulin (100 nM, supplemented every 12 h) and/or PI3-K inhibitor LY294002 (50 μM) or MEK inhibitor PD98059 (50 μM) were added and supplemented every 24 h. Two days (MCF-10A cells) or 4 days (MDA-MB157 and ZR-75-1) later cells were solubilized in 0.03% sodium dodecyl sulfate (SDS) for DNA measurement. Values represent the mean ± SD of two or three experiments in triplicate.

vation. These experiments suggest, therefore, that in these cells insulin stimulated cell growth most likely via the Shc-mediated $p21^{ras}$/MAP-K pathway rather than via an IRS-1/PI3-K–dependent pathway. It has previously been reported that the insulin receptor can activate p21ras/MAP-K pathway via Shc in an IRS-1–independent manner [Skolnik et al., 1993; Yonezawa et al., 1994; Ouwens et al., 1994]. This hypothesis is supported by studies with the PI3-K inhibitor LY294002 and the MAP-K/Erk (MEK) inhibitor PD98059. In fact, LY294002, a specific blocker of PI3-K activity, did not affect insulin-stimulated growth in ZR-75-1 cells, confirming that PI3-K does not play a major role in this insulin effect. By contrast, LY294002 markedly or partially reduced insulin-stimulated growth in MCF-10A and MDA-MB157 cell lines, respectively, suggesting a relevant role of PI3-K in insulin-mediated mitogenesis in these two cell lines. It should be noted, however, that in MDA-MB157 cells, there was only a partial decrease (≈40%) in insulin-dependent cell growth in the presence of LY294002 (i.e., no PI3-K activity), suggesting a presence of a PI3-K–independent pathway.

The observation that the MEK inhibitor PD98059 consistently reduced insulin-dependent growth in all cell lines studied suggests that the block of the ras-MAP-K mitogenic pathway is crucial for breast cancer cell proliferation.

Taken together, the present data suggest that a strategy to inhibit the action of hormones and growth factors on breast cancer cell growth should take into account diverse signaling pathways utilized by different malignant cells to activate the mitogenic process.

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