Insulin Receptor Substrate-1 Expression Is Regulated by Estrogen in the MCF-7 Human Breast Cancer Cell Line*

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Estrogens can stimulate the proliferation of estrogen-responsive breast cancer cells by increasing their proliferative response to insulin-like growth factors. The mechanism underlying the increased proliferation could involve the induction of components of the insulin-like growth factor signal transduction pathway by estrogen. In this study we have examined the regulation of the expression of insulin receptor substrate-1, a major intracellular substrate of the type I insulin-like growth factor receptor tyrosine kinase. Estradiol increased insulin receptor substrate-1 mRNA and protein levels at concentrations consistent with a mechanism involving the estrogen receptor. Insulin receptor substrate-1 was not induced significantly by the antiestrogens tamoxifen and ICI 182,780, but they inhibited the induction of insulin receptor substrate-1 by estradiol. Analysis of tyrosine-phosphorylated insulin receptor substrate-1 showed that the highest levels were found in cells stimulated by estradiol and insulin-like growth factor-I, whereas low levels were found in the absence of estradiol irrespective of whether type I insulin-like growth factor ligands were present. Insulin receptor substrate-2, -3, and -4 were not induced by estradiol. These results suggest that estrogens and antiestrogens may regulate cell proliferation by controlling insulin receptor substrate-1 expression, thereby amplifying or attenuating signaling through the insulin-like growth factor signal transduction pathway.

Estrogens are important in controlling the proliferation of breast cancer cells, and therapeutic agents that inhibit the synthesis and effects of estrogens are widely used in the treatment of breast cancer. The mechanisms by which estrogens stimulate cell proliferation, however, are not clear. The proliferation of estrogen receptor-positive breast cancer cell lines is stimulated by estrogen in culture, and these cell lines provide useful model systems for determining how estrogens stimulate breast cancer cell proliferation. The proliferation of breast cancer cells in culture is also responsive to insulin-like growth factors, and components of the IGF signal transduction system are expressed by both breast tumors and cultured breast cancer cells (1–5).

We along with others have suggested that estrogens control cell proliferation by modulating the proliferative response of breast cancer cells to IGFs (6–8). Estrogen-responsive breast cancer cells show a much greater proliferative response to high concentrations of insulin (acting through the type I IGF receptor) or physiological concentrations of IGFs in the presence of estrogen, and conversely the proliferative response to estradiol is much greater in the presence of insulin or IGFs.

The proliferative effects of IGFs are mediated by the type I IGF receptor a cell surface receptor comprising two α and two β subunits (9). Binding of the growth factor activates tyrosine kinase activity in the C terminus of the β subunit. The activated tyrosine kinase of the receptor then phosphorylates a number of intracellular signaling molecules, of which insulin receptor substrate 1 (10, 11) is a key signaling molecule involved in mediating the proliferative response. IRS-1 acts as a multisite docking protein that links multiple downstream signaling pathways by binding to the SH2 domains of a variety of signaling molecules, including Syp phosphotyrosine phosphatase (12, 13), phosphatidylinositol 3'-kinase (14), Fyn tyrosine kinase (15), and the adapter proteins Grb-2 (16), Nck (17), and Crk (18).

We have suggested (7, 19, 20) that the ability of estrogens to increase the response of breast cancer cells to IGFs derives from the up-regulation of one or more critical components of the IGF signal transduction system. Components of the IGF signal transduction pathway have been characterized in breast cancer cells in culture and tumors, and the expression of ligands (21, 22), receptors (7, 23–25), and downstream signaling molecules (26, 27) have all been demonstrated.

We originally proposed that the increased response could result from increased expression of the type I IGF receptor (7). However, experiments in which this receptor was constitutively overexpressed in estrogen-responsive breast cancer cells showed that overexpression did not abrogate estrogen responsiveness (20).

In this study, we show that the expression of IRS-1, a major substrate for the type I IGF receptor tyrosine kinase, is regulated by estrogens and antiestrogens, whereas other members of the IRS family, IRS-2, -3, and -4, are not. This regulation could be responsible for the increased proliferative response of breast cancer cells to IGFs in the presence of estrogen.

MATERIALS AND METHODS

Cell Culture—MCF-7 (28) ZR-75-B (29), EFF-3 (30), EFM-19 (31), T47D (32), BT20 (33), and Ha578T (34) human breast cancer cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1 μg/ml insulin. MDA MB-231 cells (35) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum only. Cells were incubated at 37 °C in a humidified atmosphere containing 4% CO₂. In experiments in which the effects of estrogen and/or insulin-like growth factors were measured, cells were first cultured in medium depleted of steroids. This comprised phenol red-free minimal essential medium supplemented with charcoal-treated newborn calf serum and insulin (1 μg/ml). In experiments in which the acute effects of insulin or IGF-I on IRS-1 tyrosine phosphorylation were examined, cells were incubated with the type I IGF receptor ligand in medium comprising phenol red-free min-

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The abbreviations used are: IGF, insulin-like growth factor; IRS-1, insulin receptor substrate-1; kb, kilobase(s).
RNA Preparation—75-cm² flasks of cells were grown to 70% confluence and then withdrawn from steroids and growth factors by culturing for 6 days in withdrawal medium, as described previously (36). Medium was changed daily, and cells were washed twice with phosphate-buffered saline before adding the new medium for the first 3 days. Withdrawn cells were treated for 3 days with steroids and/or growth factors in withdrawal medium as required, with a daily change of medium. Total RNA was prepared from cells by homogenization in lithium chloride/urea and extraction in phenol/chloroform.

Northern Analysis—10 μg of total RNA was electrophoresed on 1.2% agarose/formaldehyde gels. RNA was transferred to Hybond-N nylon membrane by capillary transfer and fixed by baking at 80 °C under vacuum and irradiating with UV light. The 4.1-kb human IRS-1 cDNA insert from plasmid pHIRS-1–5 (obtained from C. R. Kahn (37)) was radiolabeled with [32P]dCTP by random-priming and was hybridized to the immobilized RNA at a concentration of 10³ cpm/ml for 3 days at 42 °C. Membranes were then extensively washed at 65 °C and exposed to preflashed x-ray film for up to 1 week at −70 °C.

Protein Preparation—Cells were grown to 70% confluence in 35-mm wells, withdrawn for 6 days, and treated for 3 days with growth factors and/or steroids as required. Cell monolayers were washed twice with ice-cold phosphate-buffered saline and lysed in 0.5 ml of lysis buffer (50 mM HEPES, pH 7.2, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM Na₂EDTA, 10% glycerol, 1% w/v Triton X-100, 0.4 mM Na₃VO₄, 1 μg/ml aprotinin, 1 μg/ml leupeptin) on ice for 30 min. The lysate from each well was centrifuged at 12,900 × g (12,000 rpm) for 15 min. The protein concentration of the supernatant was measured by BCA assay.

Immunoblotting—Antibodies against IRS-1, -2, -3, and -4 were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). The IRS-1 antibody was raised against the 14 C-terminal amino acids of rat IRS-1, the IRS-2 antibody was raised against amino acids 976–1094 of mouse IRS-2, the IRS-3 antibody was raised against a GST fusion protein containing the N-terminal 120 amino acids of rat IRS-3, and the IRS-4 antibody was raised against amino acids 1240–1257 of human IRS-4. All antibodies were raised in rabbits and were protein A-purified IgG.

RESULTS

Expression of IRS-1 in Breast Cancer Cell Lines—IRS-1 mRNA expression was measured in three estrogen receptor-negative and five estrogen receptor-positive human breast cancer cell lines. The cDNA probe used for the hybridization to total RNA encompassed the entire coding region of human IRS-1 and detected four major mRNAs of 8.5, 6.9, 6.1, and 4.4 kb (Fig. 1). IRS-1 mRNA was detected in all the cell lines apart from BT20, but the levels of expression were extremely variable. For example, in MCF-7 cells, the highest level of expression of all four mRNA, and the level of the 8.5-kb mRNA was more than 10-fold higher than in EFF-3 and MDA-MB 231 cells. Each cell line showed a different pattern of expression of the four transcripts. In MCF-7 and ZR-75 cells, the level of expression of the 8.5-kb transcript was highest, whereas EFM-19 had highest levels of the 6.1-kb transcript. The 4.4-kb mRNA was a minor mRNA in all cell lines in which it was expressed. There was no clear relationship between IRS-1 expression and the estrogen receptor status of the cell lines. However, of the four cell lines that expressed the highest levels of IRS-1 mRNA, three were estrogen receptor-positive.

Regulation of IRS-1 mRNA and Protein by Estrogen in the MCF-7 Breast Cancer Cell Line—To determine whether the expression of IRS-1 is regulated by estrogen in estrogen-responsive breast cancer cells, IRS-1 mRNA and protein were first measured in MCF-7 cells that had been withdrawn from the effects of estrogen in the routine culture medium and then stimulated with estradiol. A large decrease in IRS-1 mRNA expression was observed in MCF-7 cells after 3 days of culture in steroid-free medium (data not shown). To investigate whether this decrease was attributable to the withdrawal of the cells from the estrogen present in the routine culture medium, MCF-7 cells were withdrawn by culturing for 3 days in steroid-free medium and then treated for times varying from 4 h to 6 days with 10⁻¹⁰ m estradiol. RNA was then extracted for the measurement IRS-1 mRNA expression. From the autoradiograph shown in Fig. 2A, it is clear that there is a dramatic induction in the levels of IRS-1 mRNA by estradiol and that the effect is visible after only 4 h treatment. The intensity of hybridization was quantified by scanning densitometry (Fig. 2B), which showed that IRS-1 mRNA levels were increased more than 2-fold after only 4 h of estradiol treatment and continued to increase with time so that after 4 days IRS-1 mRNA levels were more than 20-fold higher in estrogen treated compared with control cells. IRS-1 mRNA levels started to plateau between 4 and 6 days.

To determine whether estradiol induced a similar increase in IRS-1 protein expression, the above experiment was repeated, the cells were lysed, and the IRS-1 protein in the lysates was measured. As shown in Fig. 2C, IRS-1 protein was undetectable in withdrawn cells, was first detected after 16 h of estrogen treatment, and then continued to increase during 4–5 days of estradiol treatment. Compared with the mRNA induction by estradiol, there appeared to be a lag before IRS-1 protein was detected. It was not possible to quantitate the absolute increase in protein levels because of the undetectable amounts of IRS-1 in withdrawn cells, but the increase was more dramatic than the increase in mRNA levels (Fig. 2D). Three forms of IRS-1 were identified. The highest molecular mass form had an apparent molecular mass of approximately 66 kDa. IRS-3 reacted with a protein of correct size (approximately 75 kDa) in MCF-7 cell extracts. 7.5-kb immunoreactive band was observed in MCF-7 cells. The IRS-3 antibody was raised against a GST fusion protein containing the N-terminal 120 amino acids of rat IRS-3. The IRS-2 antibody was raised against amino acids 976–1094 of mouse IRS-2. The IRS-4 antibody was raised against the 14 C-terminal amino acids of rat IRS-4. The IRS-3 antibody was raised against amino acids 1240–1257 of human IRS-4. All antibodies were raised in rabbits and were protein A-purified IgG.

Antibodies against IRS-1, -2, -3, and -4 were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). The IRS-1 antibody was raised against the 14 C-terminal amino acids of rat IRS-1, the IRS-2 antibody was raised against amino acids 976–1094 of mouse IRS-2, the IRS-3 antibody was raised against a GST fusion protein containing the N-terminal 120 amino acids of rat IRS-3, and the IRS-4 antibody was raised against amino acids 1240–1257 of human IRS-4. All antibodies were raised in rabbits and were protein A-purified IgG.

The results were analyzed by Western blotting using the antibodies raised against IRS-1, -2, -3, and -4.

FIG. 1. Expression of IRS-1 mRNA in breast cancer cell lines. Northern transfer of BT20, Hs578T, MDA-MB-231, MCF-7, ZR-75-B, EFF-3, EFM-19, and T47D human breast cancer cell line RNA was hybridized with [32P]labeled IRS-1 cDNA. BT20, Hs578T, and MDA-MB-231 cells are estrogen receptor-negative, whereas the rest are estrogen receptor-positive. The positions of 28 and 18 S ribosomal RNA are shown on the left, and the sizes of the IRS-1 mRNAs are shown on the right.
molecular mass of 185 kDa as described by others. The two lower molecular mass forms that were most prominent in cells cultured for 5–6 days in estradiol are presumed to represent degradation products of the 185-kDa form.

Cells that had been withdrawn from the effects of estradiol in the routine cell culture medium were then stimulated with various concentrations of estradiol for 3 days to determine the concentration required to induce IRS-1 mRNA and protein (Fig. 3). IRS-1 mRNAs were induced coordinately and were induced by the lowest concentration of estradiol tested (Fig. 3).

**Fig. 2.** Time course of the induction of IRS-1 mRNA and protein by estradiol. MCF-7 cells were withdrawn from the steroids present in the routine culture medium for 6 days and then treated for varying lengths of time with 10^{-9} M estradiol. RNA and proteins were extracted, and the relative levels of IRS-1 mRNA and protein were detected by Northern and Western transfer analysis, respectively. A, typical Northern blot showing increased IRS-1 mRNA levels with length of estradiol treatment. The position of 28 S rRNA is shown on the left, and the sizes of the IRS-1 mRNAs are shown on the right. B, graph showing the increase in IRS-1 protein with time of treatment. Each point represents the mean of two experiments.

**Fig. 3.** Dose response effect of estrogen on IRS-1 expression in MCF-7 cells. MCF-7 cells were withdrawn from the steroids present in the routine culture medium for 6 days and then treated with various concentrations of estradiol for 3 days. RNA and proteins were extracted, and the relative levels of IRS-1 mRNA and protein detected by Northern and Western transfer analysis, respectively. A, representative autoradiograph showing the induction of IRS-1 mRNA by different concentrations of estradiol. The positions of 28 and 18 S ribosomal RNA are shown on the left, and the sizes of the IRS-1 mRNAs are shown on the right. B, representative Western transfer showing increased IRS-1 protein by different concentrations of estradiol. The positions of the size markers are shown on the left, and the approximate molecular masses of IRS-1 (185 kDa) and a smaller immunoreactive protein are shown on the right.

The lower molecular mass forms that were most prominent in cells cultured for 5–6 days in estradiol are presumed to represent degradation products of the 185-kDa form.
Maximal induction was observed with concentrations of 10^{-10} M estradiol and higher. The induction of IRS-1 protein showed a similar dose response curve to that of the mRNA with low levels of IRS-1 being detected at 10^{-13} and 10^{-12} M estradiol and maximal induction occurring at 10^{-10} M estradiol and higher (Fig. 3B). These dose response experiments therefore showed that the induction of both IRS-1 mRNA and protein was very sensitive to estradiol consistent with this effect being mediated by the estrogen receptor. The two lower molecular mass forms of IRS-1 were less apparent in this experiment, which is consistent with the protein profile seen in time course experiment after 3 days of estradiol treatment.

**Regulation of IRS-1 mRNA and Protein by Antiestrogens in MCF-7 Cells**—The ability of the antiestrogens to induce the expression of IRS-1 mRNA and to inhibit the induction by estrogen in MCF-7 cells was then examined. Tamoxifen is widely used in the treatment of breast cancer, and although it inhibits the effects of estradiol, it has partial estrogen agonist activity for most estrogen responses measured in breast cancer cells. Cells were withdrawn and then treated for 3 days with various concentrations of tamoxifen alone or in the presence of 2 x 10^{-10} M estradiol, a concentration that induces IRS-1 mRNA to maximal levels. Tamoxifen alone had negligible estrogen agonist activity over a wide range of concentrations (10^{-10}–10^{-5} M) but inhibited the induction of IRS-1 mRNA by 2 x 10^{-10} at concentrations above 10^{-7} M. The concentration of tamoxifen required for half maximal inhibition was approximately 10^{-6} M (Fig. 4).

ICI 182,780 is currently being evaluated for the treatment of breast cancer (38) and has been reported to have little or no estrogen agonist activity. Cells were withdrawn and treated for 3 days with various concentrations of ICI 182,780 tamoxifen alone or in the presence of 2 x 10^{-10} M estradiol. ICI 182,780 alone also had negligible estrogen agonist activity over a wide range of concentrations (10^{-10}–10^{-5} M) but was somewhat more potent than tamoxifen in inhibiting the induction of IRS-1 by estradiol. ICI 182,780 inhibited the induction of IRS-1 mRNA at concentrations above 10^{-9} M, and the concentration required for half-maximal inhibition was approximately 7 x 10^{-8} M. The concentrations of the two antiestrogens required to inhibit the induction of IRS-1 were consistent with the known antiestrogenic potencies of these two estrogen antagonists. Both tamoxifen and ICI 182,780 also inhibited the induction of IRS-1 protein levels by estradiol (data not shown).

**Effects of Insulin and IGF-I on IRS-1 Tyrosine Phosphorylation in Cells Cultured the Presence and Absence of Estradiol**—If the enormously increased amounts of IRS-1 protein present in estradiol-stimulated cells are activated in the presence of the ligands that use this docking protein to transduce their signal, then the high levels of IRS-1 protein may contribute to the increased proliferation induced by ligands in the presence of estradiol.

The amount of tyrosine-phosphorylated IRS-1 was therefore measured in cells grown under the conditions used in cell proliferation assays. Cells were cultured in withdrawal medium for 6 days and then in withdrawal medium alone or in the presence of estradiol or insulin alone and cells grown in the presence of estradiol and insulin together for 3 days. Cell lysate was prepared from the cells, IRS-1 was immunoprecipitated, and its tyrosine phosphorylation was assessed by Western transfer analysis (Fig. 5A). Cells that had been withdrawn from the effects of estrogen in the routine culture medium had no detectable tyrosine-phosphorylated IRS-1 either in the absence or presence of insulin (Fig. 5A). In contrast, large amounts of tyrosine-phosphorylated IRS-1 were detected in cells that had been grown in medium containing estradiol and insulin. Importantly, cells grown in medium containing estradiol but lacking insulin showed similar low levels of tyrosine-phosphorylated IRS-1 to cells that had been grown in medium lacking both estradiol and insulin.

To demonstrate that insulin or IGF-I is directly responsible for the increased tyrosine phosphorylation of IRS-1 in the experiment shown in Fig. 5A, MCF-7 cells were grown in the presence of estradiol and then treated for 5 min with insulin or IGF-I. No tyrosine-phosphorylated IRS-1 was detected in unstimulated cells, but both insulin and IGF-I caused a similar large induction of IRS-1 phosphorylation in these estrogen-stimulated cells. The concentrations of the two ligands used in this experiment are consistent with this effect being mediated by the type I IGF receptor (Fig. 5B).

**Effects of Estrogen on IRS-2, IRS-3, and IRS-4 Expression in MCF-7 Breast Cancer Cells**—IRS-1 belongs to a family of related molecules including IRS-2 (39), IRS-3 (40), and IRS-4 (41) that contain a pleckstrin homology domain and a phosphotyrosine-binding domain toward the N terminus of the protein. The effects of estrogen on IRS-2, -3, and -4 levels in MCF-7 cells were therefore investigated to determine whether the effects of estrogen were specific for IRS-1 or involved other members of this family of proteins. Fig. 6 shows a representative Western
transfer of control and estrogen-stimulated cells reacted with IRS-1, -2, -3, and -4 antibodies together with a histogram showing the results for three separate experiments. The IRS-2 antibody reacted with a protein of approximately 180 kDa, and the IRS-3 antibody reacted with a protein of 66 kDa. These sizes are consistent with those reported in the literature for these proteins. The IRS-4 antibody reacted predominantly with proteins of 130 and 97 kDa. Two minor proteins of 93 and 100 kDa were also identified, and longer exposures of the filter shown in Fig. 6 also revealed a protein of 160 kDa. Immuno-precipitation of IRS-3 and IRS-4 from cell lysates of control or IGF-I-treated MCF-7 cells and immunoblotting with antiphosphotyrosine antibody failed to demonstrate that these proteins were tyrosine-phosphorylated or that tyrosine phosphorylation could be induced by IGF-I. We therefore conclude that although IRS-3 and -4 are expressed in MCF-7 breast cancer cells, they are not tyrosine-phosphorylated by the type I IGF receptor in response to IGF-I. IRS-1 was induced approximately 100-fold in this experiment, whereas IRS-2 and -3 and none of the forms of IRS-4 were induced significantly by estradiol. These experiments show that of the members of the IRS family analyzed, only IRS-1 levels are significantly regulated by estradiol.

**DISCUSSION**

Changes in the structure, function, and expression of intracellular signaling molecules have been implicated in the aberrant control of cell proliferation resulting in malignant transformation. The intracellular signaling pathways involved in the control of breast cancer cell proliferation are not known, although alterations to a number of different signaling molecules have been reported.

Understanding the mechanisms underlying the responsiveness of breast cancer cells to estrogen is clinically important given the incidence of the disease and the widespread use of hormonal manipulation to control the progression of the disease by abrogating the effects of estrogens. A number of laboratories have focussed on the involvement of the IGF signal transduction pathway and have shown: (i) that breast cancer cells are responsive to the proliferative effects of IGFs, (ii) that a number of components of the IGF signal transduction pathway can be regulated by estrogen including IGF-II (21, 22), IGF-binding proteins (42), and the type I IGF receptor (7), and...
(iii) that estrogens control the proliferative response of cells to IGFs (6–8). In this study we report that estrogens regulate the expression of IRS-1, a major substrate of the type I IGF receptor tyrosine kinase, and suggest that this regulation may be responsible for mediating the effects of estrogen on breast cancer cell proliferation.

No previous studies have analyzed IRS-1 mRNA in breast cancer cells. Four major mRNAs were identified of 8.5, 6.9, 6.1, and 4.4 kb in MCF-7 cells. mRNAs of 6 and 6.9 kb have been reported previously in a variety of human tissues (skeletal muscle, heart, and brain) (37, 43), mRNAs of 9 and 6 kb have been found in human pancreas and a variety of cell lines (44), and a single mRNA of 5 kb has been found in normal human liver and hepatocellular carcinoma (45). The reasons for the differences in the sizes of the mRNAs is not known but may result, at least in part, from multiple transcription start points as has been observed for mouse IRS-1 mRNA (46).

The majority of published studies of IRS-1 protein in breast cancer cells present illustrations which showing the 185-kDa form only (27, 47), and it is not possible to evaluate whether other forms were observed. We consistently observed lower molecular mass forms that we presume to be degradation products e.g. Fig. 2. The degradation pathway of IRS-1 is not known, but the identification of well defined intermediates suggests that there may be specific degradation pathways. Because the role of IRS-1 is to act as a docking protein that assembles signaling complexes, IRS-1 degradation may be an important way of regulating IRS-1 signaling. IRS-1 contains PEST sequences and is a substrate for the calcium-dependent protease calpain. Whether, the lower molecular mass forms of IRS-1 seen in this study reflect calpain digestion, however, remains to be established.

IRS-1 levels were highly regulated by estrogen in MCF-7 cells, and, overall, the dose response curve for the induction of IRS-1 by estradiol and the concentrations of the two antiestrogens, tamoxifen andICI 182,780, required to inhibit the effects of estradiol are consistent with this effect being mediated by the estrogen receptor. Although tamoxifen is generally considered to be a partial estrogen agonist, we observed no estrogen agonist activity of tamoxifen on IRS-1 expression. It is recognized, however, that the estrogen agonist activity of tamoxifen varies with the response being measured (48) and that estrogen-responsive genes have been identified that are induced minimally by tamoxifen (49).

These results are, at first sight, in contrast to those of Guvakova and Surmacz (47) who reported that tamoxifen, although reducing the amount of tyrosine-phosphorylated IRS-1, had no effect on IRS-1 protein levels in MCF-7 breast cancer. Our results are, however, consistent with those of Guvakova and Surmacz (47) in that we found no estrogen agonist or antagonist effect of tamoxifen at the concentration used in their study (10 nM). We did find, however, that higher concentrations of tamoxifen inhibited the induction of IRS-1 by estradiol.

The main impetus for studying the effect of estradiol on IRS-1 expression was to explain how estrogens can increase the response of estrogen-responsive breast cancer cell lines to IGFs. Although IGFs alone stimulate the proliferation of breast cancer cells, the stimulation is markedly increased by estrogen in hormone-responsive breast cancer cells (6–8). Binding studies and dose response experiments had suggested that the increased responsiveness was mediated via the type-I IGF receptor, and experiments in breast cancer cells and other cell types had suggested that estrogens can increase the proliferative response to insulin-like growth factors by increasing type I IGF receptor levels (7).

To investigate this hypothesis, Daws et al. (20) used a retroviral expression vector to produce MCF-7 cells that constitutively overexpressed the type-I IGF receptor. The cells overexpressing the type-I IGF receptor showed the same magnitude of response to IGF-I as the parental cells, suggesting that the level of the type I IGF receptor does not control the response of breast cancer cells to IGFs. In addition, estrogen increased the response of both the parental and transfected cells to IGFs, confirming that the increased response of MCF-7 cells to IGFs in the presence of estradiol does not result from the induction of the receptor by estradiol.

The results of the present study provide an alternative explanation for the increased response of breast cancer cells to IGFs in the presence of estrogen. We have shown that IRS-1 is induced by estrogen and suggest that the induction of IRS-1 by estrogen could account for the increased response to IGFs in the presence of this steroid. Although not examining the estrogen regulation of IRS-1 expression by estradiol, the data of Surmacz and Burgaud (50) are consistent with this model. Surmacz and Burgaud derived MCF-7 clones overexpressing IRS-1 and showed a loss of estrogen dependence for growth. They also showed that IRS-1 antisense oligonucleotides completely inhibited the growth of normal MCF-7 cells as well as cells overexpressing IRS-1, again emphasizing the importance of this protein in estrogen-responsive cell proliferation.

Estrogen regulation of other downstream signaling molecules apart from IRS-1 may also be involved in mediating the effects of estrogen on the responsiveness of cells to IGFs. Although we have not analyzed all signaling molecules in this pathway, we investigated the effect of estrogen on three other members of the IRS family. The lack of effect of estrogen on IRS-2, -3, and 4 suggests that estrogen mediates its effects by regulating a limited repertoire of signaling molecules, and the magnitude of the induction of IRS-1 suggests that this molecule may be very important in mediating the effects of estrogen on the responsiveness of these cells to IGFs.

The importance of IRS-1 in mediating the effects of IGFs and estradiol suggests that measurement of IRS-1 expression may be of clinical importance in predicting the proliferative behavior of breast tumors. There has been only one study to date (27) on the prognostic value of IRS-1 expression in breast cancer, and this measured total IRS-1, rather than tyrosine-phosphorylated IRS-1, in a series of 195 node-negative primary cancers. Consistent with the view that IRS-1 expression is regulated by estrogen, IRS-1 expression was positively correlated with expression of estrogen receptor. Importantly, IRS-1 expression was associated with a poor prognosis, and this was most pronounced in small tumors. Although these findings are consistent with the view that IRS-1 is an important molecule in breast cancer cell growth, a stronger association may have been found if phosphorylated IRS-1, i.e. the active form of IRS-1, had been measured, because this may more accurately reflect the activity of the estrogen/IGF pathways.

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