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Clinical and experimental evidence suggest that the insulin-like growth factor receptor (IGF-IR) is involved in breast cancer etiology. For instance, IGF-IR is overexpressed in breast tumors (relative to normal breast epithelium) and high levels of IGF-I (an IGF-IR ligand) correlate with breast cancer risk in premenopausal women. In vitro, IGF-IR regulates the growth and survival of breast cancer cells and plays a role in the development of estrogen-independence. Antiestrogens inhibit IGF-I-dependent growth by interfering with IGF-IR signaling. The mechanisms of this interference are not clear. Here we discuss the evidence that a pure antiestrogen ICI 182,780 specifically blocks breast cancer growth through downregulation of the expression of IRS-1, a major IGF signaling intermediate.

Although the role of IGF-IR is breast cancer cell proliferation is clear, its function in growth-unrelated processes, such as cell adhesion, migration and metastasis is less well understood. Here, we report that an IGF-IR signaling substrate SHC, through its dynamic interactions with $\alpha 5\beta 1$ integrin (a fibronectin receptor) participates in the regulation of breast cancer cell adhesion and motility.

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INTRODUCTION

Clinical and experimental evidence strongly suggest a role of IGF-IR in breast cancer etiology and progression (1, 2). In particular, IGF-IR is up to 14-fold overexpressed in breast cancer compared with its levels in normal epithelial cells or in benign breast tumors (3-5). The IGF-IR ligands, IGF-I and IGF-II (IGFs) are potent mitogens for breast cancer cells in culture and have been detected in the epithelial and/or stromal component of breast tumors (6). In addition, a recent large prospective study found a strong correlation between circulating levels of IGF-I and breast cancer risk in premenopausal women (7). Moreover, new clinical data correlated high levels of IGF-IR or its major substrate IRS-1 with cancer recurrence at the primary site (4, 8). Besides promoting cell growth, IGF-IR, especially the IRS-1 pathway, protects breast cancer cells from apoptosis induced by different anti-tumor drugs or by low concentrations of mitogens (growth factors and steroid hormones) (9, 10).

One important consequence of the amplification of IGF-IR signaling is development of hormone-independence. Often, the growth of breast cancer cells is under synergistic control of polypeptide growth factors such as IGFs and steroid hormones such as estrogen (1, 2). Overexpression of growth factor receptors or signaling molecules may result in loss of steroid requirements and estrogen-independence. Indeed, we have shown that overexpression of IGF-IR or IRS-1 abrogates estrogen requirements in MCF-7 cells (11, 12). The cells, however, are still expressing estrogen receptors and are sensitive to antiestrogens. Our results with Tamoxifen, a non-steroidal antiestrogen, demonstrated that the drug inhibits the growth of MCF-7 cells and MCF-7 cells with amplified IGF-IR or IRS-1 through the interference with IRS-1 signaling (13). Here, we present data on the effects of a novel pure antiestrogen ICI 182,780 on IGF-IR signaling in breast cancer cells.

Although ample evidence suggests that abnormal activation of IGF-IR may contribute to the autonomous growth and increased survival of breast tumor cells, the role of this receptor in breast cancer metastasis is not clear. Relevant clinical data are scarce and inconsistent. For instance, IGF-IR expression was described as either positive or non-significant marker of overall survival. Some small clinical studies demonstrated a correlation between IGF-IR expression in node-positive tumors and worse prognosis (1). Other studies linked IGF-IR expression with better prognosis as IGF-IR was

predominantly expressed in a subset of breast tumors with good prognostic characteristics (estrogen and progesterone receptor-positive, node-negative, low mitotic index, diploid) (1, 5). In the experimental setting, IGF-I stimulates cell motility in MCF-7 and MDA-MB-231 cells on collagen, which may suggest a role of the IGF-IR in cell spread (14).

Recent experimental evidence indicates that IGF-IR signaling substrates SHC (SH2 homology/collagen homology proteins p47, p52, and p66) are involved in the regulation of cell adhesion and motility of many different cell types. Regarding adhesion, SHC can couple with a class of extracellular matrix (ECM) receptors, specifically with integrins $\alpha1\beta1$, $\alpha5\beta1$, $\alpha v\beta3$, and $\alpha6\beta4$ (but not with $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha6\beta1$) (15). Association of SHC with integrins is induced by the ligation of an integrin to an ECM substrate and leads to tyrosine phosphorylation of SHC, followed by SHC/GRB2-SOS binding and activation of Ras/MAP pathway. This MAP activation contributes to intracellular pathways regulating growth survival (15).

SHC also appears to be involved in cell motility and chemoattraction. For instance, overexpressed SHC mediated scattering through the NGF in MDCK cells, and downregulation of SHC reduced EGF-dependent motility in MCF-7 breast cancer cells (10, 16). The impact of integrin/SHC/MAP signaling in the regulation of cell motility is not known.

SHC is overexpressed in many tumors (17), but its status in breast cancer is not known. Here, we present our data on the role of amplified SHC in cell growth, adhesion and motility.

<u>The long-term goal of this project</u> has been:
1) To understand the role of IGF-IR and its various signaling pathways in the development and neoplastic progression of breast tumors, and:
2) To investigate the mechanisms of IGF-IR/estrogen receptor cross-talk in breast cancer cells.

<u>The following aims have been planned for Year 2:</u>

(a) Studies on antiestrogen interference with the IGF-IR signaling: the effects of pure antiestrogen ICI 182,780 on tyrosine

phosphorylation, protein and RNA expression of the IGF-IR and its signaling molecules.

(b) Development and characterization of MCF-7 cell lines overexpressing SHC. Studies on antiestrogen effects on SHC pathway in MCF-7/SHC cells.

(c) Development and characterization of MCF-7 cell lines expressing mutants of the IGF-IR.

TECHNICAL REPORT

The experiments proceeded according to the modified Statement of Work.

(a) Studies on antiestrogen interference with the IGF-IR signaling: the effects of pure antiestrogen ICI 182,780 on tyrosine phosphorylation, protein and RNA expression of IGF-IR and its signaling molecules.

A pure antiestrogen ICI 182,780 inhibits insulin-like growth factor (IGF)-dependent proliferation in hormone-responsive breast cancer cells (18). However, the interactions of ICI 182,780 with IGF-IR intracellular signaling pathways have not been characterized. Here, we studied the effects of ICI 182,780 on IGF-I-dependent growth in MCF-7 cells and different MCF-7-derived cell lines with amplified IGF-IR signaling, specifically in: 1) MCF-7/IGF-IR cells with a 10-fold IGF-IR overexpression over the level in MCF-7 cells; 2) MCF-7/SHC cells with a 5-fold SHC overexpression; and 3) in several MCF-7/IRS-1 cells exhibiting a 3-9-fold IRS-1 amplification. The cells were treated with 0.1-300 nM ICI 182,780 in phenol red-free serum-free medium (PHF-SFM) in the presence or absence of 20 ng/ml IGF-I.

ICI 182,780 blocked the basal and IGF-I-induced growth in all studied cells in a dose-dependent manner, however, the clones with the greatest IRS-1 (but not IGF-IR or SHC) overexpression were clearly least sensitive to the drug. Pursuing ICI 182,780 interaction with IRS-1, we found that the antiestrogen reduced IRS-1 expression and tyrosine phosphorylation in several cell lines in the presence or absence of IGF-I. Moreover, in IRS-1-overexpressing cells, ICI 182,780 decreased IRS-1/p85 and IRS-1/GRB2 binding.

The effects of ICI 182,780 on IGF-IR protein expression were not significant, however the drug suppressed IGF-I-induced (but not basal) IGF-IR tyrosine phosphorylation. The expression and tyrosine phosphorylation of SHC as well as SHC/GRB binding were not influenced by ICI 182, 780.

The results suggest that downregulation of IRS-1 is one of the mechanisms by which ICI 182,780 inhibits the growth of estrogen receptor-positive breast cancer cells. IRS-1 overexpression in breast tumors may therefore contribute to antiestrogen resistance [results are submitted for publication, see Appendix for the manuscript].

(b) Development and characterization of MCF-7 cell lines overexpressing SHC.

SHC is overexpressed in different tumor cells, including different breast cancer cell lines. The biological consequences of SHC amplification in breast cancer cells have been unknown. Here, we addressed this question by developing and studying MCF-7-derived cell lines with ectopic SHC expression (MCF-7/SHC cells). The 3-8-fold overexpression of SHC produced a moderately increased responsiveness to IGF-I and EGF (20-70%) but did not significantly modulate MAP kinase activity in response to growth factors or the rate of proliferation in serum-containing medium. Similarly, high levels of SHC did not improve the ability of cells to grow under anchorage-independent conditions. SHC, however, was found involved in breast cancer cell adhesion and motility.

In MCF-7/SHC cells, the amount of SHC associated with $\alpha 5\beta 1$ integrin, a fibronectin (FN) receptor, was ~ 6-fold greater that in the parental cells or in four control cell lines expressing normal SHC levels. When plated on FN, MCF-7/SHC cells attached faster than control cell lines (1 h vs. 2-3 h). This fast attachment was accompanied by earlier decline of adhesion-induced MAP kinase activity. The attachment of cells to FN was associated with decreased binding of p47 SHC to $\alpha 5\beta 1$ integrin. Conversely, addition of EGF caused partial detachment of cells from FN and stimulated cell motility, which was associated with increased binding of p47 to $\alpha 5\beta 1$ integrin. The motility of MCF-7/SHC cells tested in FN-coated inserts was inhibited compared with that of the control cell lines. However, in the presence of EGF or IGF, the motility MCF-7/SHC cells was greatly (up to 300 %) increased,

whereas it was only moderately altered in cells with normal SHC levels (from -20 to + 40%).

The results suggest that SHC is an important signaling element participating in the regulation of breast cancer cell adhesion and motility. The activity of SHC can be modulated by both integrin and by growth factor-dependent pathways. [The manuscript containing these results is in preparation, see Appendix].

Studies on antiestrogen effects on SHC pathway in MCF-7/SHC cells.

We studied the effects of ICI 182,780 on IGF-IR signaling in MCF-7/SHC cells. The drug did not interfere with SHC expression, tyrosine phosphorylation or GRB2 binding in the presence or absence of IGF-I [Data reported, for the manuscript, see Appendix]. Because of the involvement of SHC in cell motility, we investigated whether ICI 182,780 or Tamoxifen (Tam) affected the motogenic abilities of MCF-7/SHC cells on FN or collagen (COL). Our previous studies indicated that Tam induced tyrosine phosphorylation of SHC in growth arrested cells and according to the results of others, Tam stimulated motility in breast cancer cells. However, in our hands, neither Tam nor ICI 182,780 affected the motility of MCF-7 or MCF-7/SHC cells.

(c) Development and characterization of MCF-7 cell lines expressing mutants of the IGF-IR.

The involvement of different signaling pathways in controlling the phenotype of breast cancer cells can be studied in cells expressing dominant negative mutants of IGF-IR. Mutant receptors, through dimerization with normal receptors, block certain receptor domains, which interferes with the activation of different signaling pathways. To investigate the role of the C-terminal domain of IGF-IR in cell growth, transformation, and cell-cell adhesion, we overexpressed IGF-IRs with a 108 aa C-terminal deletion (IGF-IR/TC) in MCF-7 cells. The MCF-7/IGF-IR/TC clones expressed approximately equal amounts of the wild-type (endogenous) and the mutant receptors. The monolayer growth of MCF-7/IGF-IR/TC cells was comparable to that of MCF-7 cells expressing corresponding levels of wild-type IGF-IRs, however, transforming abilities (anchorage-independent growth) of MCF-7/IGF-IR/TC cells were reduced by at least ~40%. This indicates that the C-terminal region of IGF-IR transmits signals necessary to support anchorage-independent growth but is dispensable for monolayer growth. The aggregation of MCF-7/IGF-

IR/TC cells in 3-dimensional culture was normal, suggesting that signals necessary for transformation do not overlap with that important for cell-cell adhesion. [Published in Breast Cancer Res. Treatm. (1)].

Cell lines expressing other mutant IGF-IRs are being developed. We are particularly interested in identifying signaling pathways of IGF-IR that regulate cell-cell and cell-substrate adhesion.

CONCLUSIONS

Clinical data suggest that overexpression of IGF-IR and/or IGF is an important factor in breast cancer etiology. However, the mechanisms by which IGF-IR contributes to the development of breast tumors and to breast cancer metastasis are still not clear. This project has been designed to address these questions using a cellular model. In particular, we have been investigating if amplified IGF signaling affects proliferation, survival, hormone-dependence, tumorigenicity, cell-cell interactions in three-dimensional culture, motility and invasiveness, the features that are important markers of breast tumor progression.

Previously, we documented the impact of IGF-IR overexpression on breast cancer growth, transformation, and cell-cell interactions. The focus of the present report is on the involvement of two major IGF-IR signaling pathways, the IRS-1- and SHC-dependent pathways, in the neoplastic progression of breast cancer cells.

The IRS-1 pathway is transmitting signals supporting the growth and survival of breast cancer cells. This pathway is also a target for antiestrogens blocking IGF-dependent proliferation. Here we report that downregulation of IRS-1 represents one of the mechanisms by which a pure antiestrogen ICI 182,780 inhibits the growth of breast cancer cells. Our data suggest that overexpression of IRS-1 in breast tumors may contribute to the development of antiestrogen resistance. The SHC pathway is not affected by ICI 182,780, which points to a specificity in the interactions between antiestrogens and IGF-IR signaling pathways.

Our pursuit of the role of SHC pathway in breast tumor cells revealed a new information regarding growth-unrelated functions of this

substrate. Specifically, our data suggest that SHC is involved in the regulation of cell adhesion and motility in breast cancer cells.

PUBLICATIONS and COMMUNICATIONS RESULTING FROM THIS WORK

Publications

Surmacz, E., Guvakova, M., Nolan, M., Nicosia, R., and Sciacca, L. Type I insulin-like growth factor receptor function in breast cancer. Breast Cancer Res. Treatm. 47: 255-267, 1998.

Guvakova, M.A. and Surmacz, E. Tamoxifen interferes with the insulin-like growth factor I receptor (IGF-IR) signaling pathway in breast cancer cells. Cancer Res. 57: 2606-2610, 1997.

Nolan, M. K., Jankowska, L., Prisco, M., Xu, S., Guvakova, M. A., Surmacz, E. Differential roles of IRS-1 and SHC signaling pathways in breast cancer cells. Int. J. Cancer 72: 828-834, 1997.

Ando, S., Panno, M. L., Salerno, M., Sisci, D., Mauro, L., Lanzino, M., Surmacz, E. Role of IRS-1 signaling in insulin-induced modulation of estrogen receptors in breast cancer cells. In revision.

Salerno, M., Sisci, D., Guvakova, M., Ando, S., Surmacz, E. Insulin receptor substrate 1 (IRS-1) is a target of a pure antiestrogen ICI 182,780 in breast cancer cells. Submitted.

Meeting Communications and Invited Talks:

Surmacz, E. IGF-IR and breast cancer. Department of Medical Genetics, University of Ferrara, Italy, May 13, 1998 (invited speaker)

Surmacz, E. IGF-IR signaling in breast cancer. Oncology Clinic, University of Modena, Italy, May 12, 1998 (invited speaker)

Surmacz, E. IGF-IR signaling in breast cancer. Department of Pathology, Allegheny University, Philadelphia, PA, December 16, 1997 (invited speaker)

Guvakova, M., Surmacz, E. IGF-IR modulates breast cancer cell motility through the regulation of p125 FAK/p130 CAS. 37th American Society for Cell Biology Annual Meeting, Washington D.C., December 13-17, 1997

Ando, S., Panno, M. L., Salerno, M., Sisci, D., Surmacz, E. Role of IRS-1 signaling in the modulation of estrogen receptor function by insulin. Annual Breast Cancer Meeting, San Antonio, TX, December 3-6, 1977

Surmacz, E., Sisci, D., Salerno, M., Guvakova, M., Ando, S. Insulin receptor substrate 1 (IRS-1) is a target for a pure antiestrogen ICI 182,780. Annual Breast Cancer Meeting, San Antonio, TX, December 3-6, 1997

Surmacz, E. Overexpressed IGF-I Receptors promote cell-cell adhesion and enhance survival of breast cancer cells, Era of Hope, Breast Cancer Research Program Meeting, Washington, D.C. October 31-November 3, 1997

Guvakova, M., Surmacz, E. Molecular mechanisms of tamoxifen effect on IGF-IR signaling in breast cancer cells. The 2nd World Congress on Advances in Oncology, Vouliagmenti, Athens, Greece, October 16-18, 1997

REFERENCES

1. Surmacz, E., Guvakova, M., Nolan, M., Nicosia, R., Sciacca, L. type I insulin-like growth factor receptor function in breast cancer. Breast Cancer Res. Treat. 47: 255-267, 1998

2. Lee, A. V., Yee, D. Insulin-like growth factors and breast cancer. Biomed. Pharmacother. 49: 415-421, 1995.

3. Resnik, J. L., Reichart, D. B., Huey, K., Webster, N. J. G., Seely, B. L. Elevated insulin-like growth factor I receptor autophosphorylation and kinase activity in human breast cancer. Cancer Res. 58: 1159-1164, 1998.

4. Turner, B.C., Haffty, B.G., Narayanann, L., Yuan, J., Havre, P.A., Gumbs, A., Kaplan, L., Burgaud, J-L., Carter, D., Baserga, R., Glazer, P.M. IGF-I receptor and cyclin D1 expression influence cellular

radiosensitivity and local breast cancer recurrence after lumpectomy and radiation. Cancer Res.57: 3079-3083, 1997.

5. Pezzino, V. , Papa, V., Milazzo, G., Gliozzo, B., Russo, P., Scalia, P. L. Insulin-like growth factor-I (IGF-I) receptors in breast cancer. Ann NY Acad Sci 784: 189-201, 1996.

6. Ellis, M.J.C., Singer, C., Hornby, A., Rasmussen, A., Cullen, KJ. Insulin-like growth factor mediated stromal-epithelial interactions in human breast cancer. Breast Cancer Res. and Treat., 31: 249-261.1994.

7. Hankinson, S. E., Willet, W. C., Colditz, G. A., Hunter, D. J., Michaud, D. S., Deroo, B., Rosner, B., Speitzer, F. E., Pollak, M. Circulating concentrations of insulin-like growth factor and risk of breast cancer. Lancet 35: 1393-1396, 1998.

8. Rocha, R. L., Hilsenbeck, S. G., Jackson, J. G., Van Der Berg, C. L., Weng, C-W., Lee, A. V. Yee, D. Insulin-like growth factor binding protein 3 and insulin receptor substrate 1 in breast cancer: correlation with clinical parameters and disease-free survival. Clin. Cancer Res. 3: 103-109, 1997.

9. Dunn, S.E., Hardman, R.A., Kari, F.W., Barrett, J.C. Insulin-like growth factor 1 (IGF-1) alters drug sensitivity of HBL100 human breast cancer cells by inhibition of apoptosis induced by diverse anticancer drugs. Cancer Res., 57: 2687-2693, 1997.

10. Nolan, M., Jankowska, L., Prisco, M., Xu, S., Guvakova, M., Surmacz, E. Differential roles of IRS-1 and SHC signaling pathways in breast cancer cells. Int. J. Cancer,72: 828-834, 1997.

11. Guvakova, M. A., Surmacz, E. Overexpressed IGF-I receptors reduce estrogen growth requirements, enhance survival and promote cell-cell adhesion in human breast cancer cells. Exp. Cell Res. 231: 149-162, 1997.

12. Surmacz, E., Burgaud, J-L. Overexpression of IRS-1 in the human breast cancer cell line MCF-7 induces loss of estrogen requirements for growth and transformation. Clin. Cancer Res. 1: 1429-1436, 1995.

13. Guvakova, M. A., Surmacz, E. Tamoxifen interferes with the insulin-like growth factor I receptor (IGF-IR) signaling pathway in breast cancer cells. Cancer Res. 57: 2606-2610, 1997.

14. Doer, M., Jones, J. The roles of integrins and extracellular matrix proteins in the IGF-IR-stimulated chemotaxis of human breast cancer cells. J. Biol. Chem. 271: 2443-2447, 1996.

15. Wary, K. K., Mainiero, F., Isakoff, S., Marcantonio, E. E., Giancotti, F. G. The adaptor protein SHC couples a class of integrins to the control of cell cycle progression. Cell 87: 733-743, 1996.

16. Sachs, M., Weidner, K. M., Brinkmann, V., Walther, I., Obremeier, A., Ullrich, A., Birchmeier, W. Motogenic and morphogenic activity of epithelial receptor tyrosine kinases. J. Cell Biol. 133: 1095-1107, 1996.

17. Pelicci, G., Lanfrancone, L., Salcini, A. E., Romano, A., Mele, S., Borrello, M. G., Segatto, O., Di Fiore, P. P., Pelicci, P. G. Constitutive phosphorylation of SHC proteins in human tumors. Oncogene 11: 899-907, 1995.

18. Coradini, D., Biffi, A., Cappelletti, V., and Di Fronzo, G. Activity of Tamoxifen and new antiestrogens on estrogen receptor positive and negative breast cancer cells. Anticancer Res. 14: 1059-1064, 1994.

APPENDIX

MANUSCRIPTS:

1. Salerno, M., Sisci, D., Guvakova, M., Ando, S., Surmacz, E. Insulin receptor substrate 1 (IRS-1) is a target of a pure antiestrogen ICI 182,780 in breast cancer cells. Submitted.

2. Ando, S., Panno, M. L., Salerno, M., Sisci, D., Mauro, L., Lanzino, M., Surmacz, E. Role of IRS-1 signaling in insulin-induced modulation of estrogen receptors in breast cancer cells. In revision.

3. Nolan, M. K., Jankowska, L., Prisco, M., Xu, S., Guvakova, M. A., Surmacz, E. Differential roles of IRS-1 and SHC signaling pathways in breast cancer cells. Int. J. Cancer 72: 828-834, 1997.

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INSULIN RECEPTOR SUBSTRATE 1 (IRS-1) IS A TARGET FOR A PURE ANTIESTROGEN ICI 182,780 IN BREAST CANCER CELLS

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ABSTRACT

The pure antiestrogen ICI 182,780 inhibits insulin-like growth factor (IGF)-dependent proliferation in hormone-responsive breast cancer cells. However, the interactions of ICI 182,780 with IGF-I receptor (IGF-IR) intracellular signaling have not been characterized. Here, we studied the effects of ICI 182,780 on IGF-IR signal transduction in MCF-7 breast cancer cells and in MCF-7-derived clones overexpressing either the IGF-IR, or its two major substrates: IRS-1 (insulin receptor substrate 1) or SHC (src/collagen homology proteins).

ICI 182,780 blocked the basal and IGF-I-induced growth in all studied cells in a dose-dependent manner, however, the clones with the greatest IRS-1 overexpression were clearly least sensitive to the drug. Pursuing ICI 182,780 interaction with IRS-1, we found that the antiestrogen reduced IRS-1 expression and tyrosine phosphorylation in several cell lines in the presence or absence of IGF-I. Moreover, in IRS-1-overexpressing cells, ICI 182,780 decreased IRS-1/p85 and IRS-1/GRB2 binding.

The effects of ICI 182,780 on IGF-IR protein expression were not significant, however the drug suppressed IGF-I-induced (but not basal) IGF-IR tyrosine phosphorylation. The expression and tyrosine phosphorylation of SHC as well as SHC/GRB binding were not influenced by ICI 182, 780.

In summary, downregulation of IRS-1 may represent one of the mechanisms by which ICI 182,780 inhibits the growth of breast cancer cells. Thus, overexpression of IRS-1 in breast tumors could contribute to the development of antiestrogen resistance.

INTRODUCTION

ICI 182,780, an alpha-alkylsulphinylamide, is a new generation pure antiestrogen that has shown a great promise as a second line endocrine therapy agent in patients with advanced breast cancer resistant to the non-steroidal antiestrogen Tamoxifen (Tam) (1-5). In several in vitro and in vivo studies, the anti-tumor effects of ICI 182,780 were greater than that of Tam (1-6). Moreover, unlike Tam, ICI 182,780 lacks agonist (estrogenic) activity and its administration does not appear to be associated with deleterious site effects such as induction of endometrial cancer or retinopathy (4). ICI 182,780 antagonizes multiple cellular effects of estrogens by impairing the dimerization of the estrogen receptor (ER) and by reducing ER half-life (3, 4). ICI 182,780 also interferes with growth factor-induced growth, but it is not clear if this activity is mediated exclusively through the ER, or some ER-independent mechanism is implicated (6). Despite their great antitumor effects, pure antiestrogens do not circumvent the development of antiestrogen-resistance, as most breast tumor cells initially sensitive to ICI 182,780 eventually become unresponsive to the drug (3, 4, 7, 8). The mechanism of this resistance is not clear, but it has been suggested that both mutations of the ER as well as alterations in growth factor-dependent mitogenic pathways may be involved (3, 8-10).

The IGF system (IGFs, the IGF-IR, and IGF binding proteins (IGFBP)) plays a critical role in the pathobiology of hormone-responsive breast cancer (11-13). In the experimental setting, the IGF-IR has been shown to stimulate growth and transformation, improve survival as well as regulate cell-cell and cell-substrate interactions in breast cancer cells (11, 12, 14-19). Moreover, overexpression of different elements of the IGF system, such as IGF-II, the IGF-IR or IRS-1, provides breast cancer cells with growth advantage and reduces or abrogates estrogen growth requirements (15, 20, 21). On the other hand, downregulation of IGF-IR expression, inhibition of IGF-IR signaling, or reduced bioavailability of the IGFs have been demonstrated to block proliferation and survival as

well as interfere with motility or intercellular adhesion in breast cancer cells (11, 12, 14-19).

Clinical studies confirm the role of the IGF-I system in breast cancer development. First, the IGF-IR has been found up to 14-fold overexpressed in breast cancer compared with its levels in normal mammary epithelium (22-24). Moreover, cellular levels of the IGF-IR or its substrate IRS-1 correlate with cancer recurrence at the primary site (25, 26).The ligands of the IGF-IR, IGF-I and IGF-II, are often present in the epithelial and/or stromal component of breast tumors indicating that an autocrine or a paracrine IGF-IR loop may be operative and involved in the neoplastic process (11, 27). In addition, endocrine IGFs probably also contribute to breast tumorigenesis since the levels of circulating IGF-I correlate with breast cancer risk in premenopausal women (28).

ICI 182,780 interferes with the IGF-I system in breast cancer cells. The antiestrogen has been shown to attenuate IGF-I stimulated growth (6), modulate expression of IGFBPs (18, 19) and downregulate IGF binding sites (3). The interactions of ICI 182,780 with the IGF-IR signaling pathways, however, have not been characterized.

Our previous work demonstrated that in breast cancer cells, Tam interferes with the IGF-IR signaling acting upon IGF-IR substrates IRS-1 and SHC (29). Normally, activation of the IGF-IR results in the recruitment and tyrosine phosphorylation of IRS-1 and SHC, followed by their association with several downstream effector proteins and induction of various signaling pathways (12, 13, 30). For instance, association of either IRS-1 or SHC with GRB-2/SOS complexes activates Ras/MAP pathway, whereas binding of IRS-with p85 stimulates PI-3 kinase (13, 30). Tam treatment blocks IGF-dependent growth, which coincides with decreased tyrosine phosphorylation of IRS-1 and the IGF-IR and with hyperphosphorylation of SHC (29). Here, we demonstrate the interactions of ICI 182,780 with IGF-IR signaling and discuss the relevant similarities and differences in the modes of action of the two antiestrogens.

MATERIALS AND METHODS

Cell Lines and Cell Culture Conditions. In this study we used MCF-7 cells and several MCF-7-derived clones overexpressing either the IGF-IR (MCF-7/IGF-IR cells), IRS-1 (MCF-7/IRS-1 cells) or SHC (MCF-7/SHC cells). MCF-7/IGF-IR, clone 17 and MCF-7/IRS-1 clones 9, 3 and 18 were developed by stable transfection of MCF-7 cells with expression vectors encoding either the IGF-IR or IRS-1 and were characterized previously (15, 21). MCF-7/SHC cells are MCF-7-derived cells transfected with the plasmid pcDNA3/SHC; compared with MCF-7 cells, the level of $p55^{SHC}$ and $p47^{SHC}$ overexpression in MCF-7/SHC cells is approximately 5-fold (manuscript submitted)[3]. The above MCF-7-derived clones express ERs and respond to E2, similar to MCF-7 cells (15, 21). The levels of IRS-1 in MCF-7/IGF-IR and MCF-7/SHC cells are similar to those in MCF-7 cells (Fig. 2B and unpublished results).

MCF-7 cells were grown in DMEM:F12 (1:1) containing 5% calf serum (CS). MCF-7-derived clones were maintained in DMEM:F12 plus 5% CS plus 200 ug/ml G418 (15, 21). In the experiments requiring E2-free conditions, the cells were cultured in phenol red-free DMEM containing 0.5 mg/ml BSA, 1 uM FeSO4 and 2 mM L-glutamine (PRF-SFM) (15, 17).

Cell Growth Assay. Cells were plated at a concentration $2x10^5$ in 6-well plates in a growth medium; the following day (day 0), the cells were shifted to PRF-SFM containing different doses of ICI 182,780 (1-300 nM) with or without 50 ng/ml IGF-I and incubated for 4 days. The increase in cell number from day 0 to day 4 in PRF-SFM was designated as 100 % growth increase.

Immunoprecipitation and Western Blotting. The expression and tyrosine phosphorylation of IGF-I signaling proteins were measured by immunoprecipitation (IP) and Western blotting (WB), as described before (15, 21). Protein lysates (500 ug) were immunoprecipitated with the following antibodies (Abs): for the IGF-IR: anti-IGF-IR monoclonal Ab (mAb) alpha-IR3 (Oncogene Science); for IRS-1: anti-C-terminal IRS-1

polyclonal Ab (pAb) (UBI); for SHC: anti-SHC pAb (Transduction Laboratories). Tyrosine phosphorylation was probed by WB with an anti-phosphotyrosine mAb PY20 (Transduction Laboratories). The levels of IRS-1, IGF-IR, SHC expression were determined by stripping the phosphotyrosine blots and reprobing them with the following Abs: for IRS-1: anti-IRS-1 pAb (UBI); for IGF-IR: anti-IGF-IR mAb (Santa Cruz); for SHC: anti-SHC mAb (Transduction Laboratories). The association of GRB2 or p85 with IRS-1 or SHC was visualized in IRS-1 or SHC blots using an anti-GRB2 mAb (Transduction Laboratories) or an anti-p85 mAb (UBI), respectively. The intensity of bands was measured by laser densitometry scanning.

Northern Blotting. The levels of IRS-1 mRNA were detected by Northern blotting in 20 ug of total RNA using a 631 bp probe derived from a mouse IRS-1 cDNA (nt 1351-2002). This fragment (99.8% homology with the human IRS-1 sequence) hybridizes with both human and mouse IRS-1 mRNA (31, 32).

Statistical Analysis. The results in cell growth experiments were analyzed by ANOVA or Student t-test, where appropriate.

RESULTS

ICI 182,780 inhibits the growth of MCF-7 cells with amplified IGF-IR signaling. Sensitivity to ICI 182,780 is determined by the cellular levels of IRS-1. All cell lines used in this study secrete autocrine IGF-I-like mitogens and are able to proliferate in PRF-SFM (15, 17, 21). The basal (autocrine) growth of the cells was enhanced in the presence of IGF-I (Fig. 1A and B). Short (1-2 days) treatments with ICI 182,780 were not sufficient to inhibit cell growth (data not shown), but a 4-day culture in the presence of the antiestrogen produced evident cytostatic effects (Fig. 1A and B). In general, the response to ICI 182,780 was dose-dependent (Fig. 1A and B), however, compared with the other cell lines, the cells highly overexpressing IRS-1(MCF-7/IRS-1 clones 3 and 18) were more resistant to the drug (Fig. 1B and C). Specifically, 1 nM ICI

182,780 inhibited the basal growth by 80, 55, and 50% in MCF-7, MCF-7/IGF-IR, and MCF-7/SHC cells, respectively, but the same dose produced only a 20-30% growth inhibition in MCF-7/IRS-1, clones 3 and 18 (Fig. 1A and B). Higher concentrations of ICI 182,780 (10 and 100 nM) effectively suppressed the autocrine growth, or even induced cell death in all cell lines, except MCF-7/IRS-1, clone 18, where the maximal reduction (32%) of the basal growth occurred with a 100 nM dose (Fig. 1B).

In the presence of IGF-I, the effects of ICI 182,780 were attenuated. 1 nM ICI 182,780 was never cytostatic (data not shown), while 10 nM and 100 nM doses inhibited (by 30-50 % and 47-78%, respectively) IGF-I-dependent proliferation of cells with low IRS-1 levels (Fig. 1A and B). The same doses, however, were less efficient in MCF-7/IRS-1, clones 3 and 18, where growth reduction was 20-25% for 10 nM and 41-47% for 100 nM. Similarly, 300 nM ICI 182,780 produced a prominent cytostatic effect in all cell lines with low IRS-1 expression, but was less active in the clones highly overexpressing IRS-1 (70-93% versus 45-60% growth inhibition) (Fig. 1A, B and C).

The above results suggested that IRS-1 may be an important target for ICI 182,780 action. Consequently, in the next set of experiments we studied the effects of ICI 182,780 on the expression and function of IRS-1.

ICI 182,780 reduces IRS-1 levels and impairs IRS-1 signaling in MCF-7/IRS-1, MCF-7 and MCF-7/IGF-IR cells. In MCF-7/IRS-1 cells grown under basal conditions, IRS-1 was tyrosine phosphorylated for up to 4 days (Fig. 2A). IGF-I induced a rapid and marked (5-fold) increase of IRS-1 phosphorylation that persisted for up to 1 day and declined thereafter reaching close to the basal phosphorylation status at day 4. A short (≤1 day) treatment with ICI 182,780 had no consequences on IRS-1 expression or tyrosine phosphorylation. (Fig. 2A, panels a and b). However, p85/IRS-1 association was ~30% reduced under the basal conditions at day 1 of the treatment (Fig. 2A panel c).

The evident effect of ICI 182,780 action on IRS-1 expression and signaling occurred at day 4-day, and was especially pronounced in the absence of IGF-I. Specifically, without IGF-I, the drug suppressed IRS-1 protein expression by 60%, which was paralleled by a 60% reduction of IRS-1 tyrosine phosphorylation, and coincided with an almost complete (~95%) inhibition of p85/IRS-1 and GRB2/IRS-1 binding. The addition of IGF attenuated ICI 182,780 action, however, the effects of the treatment were still well detectable: IRS-1 levels were downregulated by 30%, IRS-1 tyrosine phosphorylation by 20%, p85/IRS-1 binding by 30%. Under IGF-I conditions, GRB2/IRS-1 binding was not appreciably affected (Fig. 2A, panels a-d).

Importantly, analogous action of ICI 182,780 on IRS-1 expression and tyrosine phosphorylation was seen in other studied cell lines (Fig. 2B). In both MCF-7/IGF-IR and MCF-7 cells containing only endogenous IRS-1, ICI 182,780 inhibited the IRS-1 expression under basal conditions by ~60%, which was paralleled by the reduced IRS-1 tyrosine phosphorylation (by ~90-95%). In the presence of IGF-I, the antiestrogen suppressed the IRS-1 levels by ~50% and IRS-1 tyrosine phosphorylation by ~40%.

ICI 182,780 attenuates IRS-1 mRNA expression. ICI 182,780 reduced the levels of ~5 kb IRS-1 mRNA (31) in MCF-7 and MCF-7/IGF-IR cells in the absence or presence of IGF-I, by 50 and 70%, respectively (Fig. 3). Moreover, the 5 kb message transcribed from the CMV-IRS-1 plasmid was downregulated (by ~70%) in MCF-7/IRS-1 cells treated with both IGF-I and ICI 182,780 (data not shown).

ICI 182,780 inhibits IGF-I-induced but not basal tyrosine phosphorylation of the IGF-IR. In MCF-7/IGF-IR cells, IGF-I moderately increased the expression of the IGF-IR. This effect was slightly (by 20%) blocked in the presence of ICI 182,780. Under the same conditions, the drug significantly (by 80%) reduced tyrosine phosphorylation of the IGF-IR (Fig. 4). ICI 182,780 had no effect on the basal expression of the IGF-IR, however, it produced a 30% increase in the basal tyrosine phosphorylation of the IGF-IR (Fig. 4). The latter peculiar effect of the antiestrogen occurred in several

repeat experiments. Short treatments with ICI 182,780 (≤1 day) were not associated with any significant changes in IGF-IR expression (data not shown).

Long-term ICI 182,780 treatment does not affect SHC signaling. In the presence of IGF-I, SHC tyrosine phosphorylation was moderately induced, with the maximum seen at 1 h upon stimulation. On the other hand, GRB2/SHC binding peaked at 15 min after IGF-I addition and declined thereafter with the minimal binding found at day 4 (Fig. 5). ICI 182,780 treatment, in the presence or absence of IGF-I, failed to induce significant changes in the levels or tyrosine phosphorylation of SHC proteins, except a transient stimulation of the basal SHC tyrosine phosphorylation at 15 min (Fig. 5). Importantly, at all time points, SHC/GRB2 association was not influenced by the drug.

Interestingly, at day 4, SHC tyrosine phosphorylation and SHC/GRB2 binding were suppressed in the presence of IGF-I (Fig. 5). This characteristic regulation of SHC by IGF-I, documented by us previously in MCF-7 cells and MCF-7-derived clones, was not affected by ICI 182,780 (29).

Similar lack of ICI 182,780 effects on SHC expression and signaling was noted in MCF-7 and MCF-7/IGF-IR cells (data not shown).

DISCUSSION

Pure antiestrogens have been shown to interfere with one of the most important systems regulating the biology of hormone-dependent breast cancer cells, namely the IGF-I system (1, 3, 6, 18, 19, 33-35). The compounds inhibit IGF-induced proliferation, which is associated with such phenomena as downregulation of IGF binding sites and reduction of IGF availability (3, 18, 19). Similar action has been ascribed to non-steroidal antiestrogens such as Tam or 4-OH-Tam (4).

The effects of pure antiestrogens on the IGF signal transduction have been unknown. Here, we studied if and how ICI 182,780 modulates the IGF-IR intracellular pathways in breast cancer cells. We focused on the relationship between drug efficiency

and signaling capacities of the IGF-IR or IRS-1 since these molecules appear to control proliferation and survival in breast cancer cells (11, 12, 21, 25, 26, 36).

Previously we found that cytostatic action of Tam involves its interference with IGF signaling pathways. In particular, Tam suppressed tyrosine phosphorylation of IRS-1 and caused hyperphosphorylation of SHC (29). The most important conclusion of the present work is that inhibition of IRS-1 expression is an important element of ICI 182,780 mode of action. The first observation was that amplification of IGF signaling did not abrogate sensitivity to ICI 182,780. Next, ICI 182,780 appeared to affect a specific IGF signaling pathway, as the efficiency of the drug was dictated by the cellular levels of IRS-1, but not that of SHC or the IGF-IR. For instance, MCF-7/IGF-IR clone 17 was very sensitive to ICI 182,780 despite a 12-fold IGF-IR overexpression, whereas MCF-7/IRS-1, clones 3 and 18 (7 and 9-fold IRS-1 overexpression, respectively) were quite resistant to the drug (15, and Fig. 1). Moreover, ICI 182,780 reduced IRS-1 levels and tyrosine phosphorylation in several cell lines in the presence or absence of IGF-I, while its action on the IGF-IR was limited to the inhibition of IGF-I-induced tyrosine phosphorylation, and its effects on SHC were none.

The reduction of IRS-1 expression by ICI 182,780 occurred in all studied cell lines, however it was clearly more pronounced in the cells expressing low levels of the substrate (Fig. 2). This suggests that downregulation of IRS-1 by ICI 182,780 is a saturable process and overexpression of IRS-1 may provide resistance to the drug. Indeed, although we did not notice a strict correlation between IRS-1 levels or IRS-1 tyrosine phosphorylation and ICI 182,780-dependent growth inhibition, IRS-1 overexpressing cells tended to be more resistant to the cytostatic action of the antiestrogen (Fig. 1). Interestingly, overexpression of IRS-1 clearly had a greater impact on the response to high doses of ICI 182,780 (≥100 nM) than on the effects of low drug concentrations. This could suggest that ICI 182,780 action is multiphased, with the initial inhibition being IRS-1-independent (but perhaps, ER-

dependent) and the strong growth reduction associated with the blockade of IRS-1 function (Fig. 1 and 2).

ICI 182,780 affected IRS-1 expression not only on the level of protein but also on the level of mRNA. In our experiments, the antiestrogen reduced the expression of IRS-1 mRNA in the presence or absence of IGF-I. However, the mechanism by which ICI 182,780 interferes with IRS-1 mRNA expression was not studied here and it remains speculative. Regarding transcriptional regulation, no estrogen responsive elements have been mapped in the IRS-1 promoter, but it can not be ruled out that ICI 182,780 acts indirectly through some other regulatory sequences in the 5' untranslated region of IRS-1 gene, such as AP1, AP2, Sp1, C/EBP, E box (37, 38). A postranscriptional component may be argued by the fact that the inhibition of IRS-1 mRNA by ICI 182,780 was evident in IGF-I-treated MCF-7/IRS-1 cells, in which the majority of IRS-1 message originated from the expression plasmid devoid of any IRS-1 promoter sequences (CMV-driven IRS-1 cDNA)(39) (data not shown). In addition, the finding that ICI 182,780 similarly inhibited IRS-1 mRNA levels under the basal and IGF-I conditions, but IRS-1 protein was significantly more reduced in the absence of IGF-I (Fig. 3 vs. Fig. 2 A) could suggest that the drug acts upon some IGF-I-dependent mechanism controlling mRNA stability, translation, or posttranslational events. In fact, in other experimental systems, IGF-I or insulin regulated various messages, including IRS-1 mRNA, on the postranscriptional level (38, 40, 41).

In its action on IRS-1, ICI 182,780 appeared more potent than Tam which decreased tyrosine phosphorylation of IRS-1 but did not cause any detectable changes in IRS-1 expression. Our results with Tam suggested that this antiestrogen may influence the activity of tyrosine phosphatases (29). An interaction with the phosphatase system has also been suggested for pure antiestrogens (42). In the present work, ICI 182,780 effects on phosphatases acting on IRS-1 were impossible to assess, since the drug affected also IRS-1 expression (Fig. 3). However, some interference of ICI 182,780 with the

phosphorylation/dephosphorylation events could be indicated for instance by our experiments with the IGF-IR, where the compound induced IGF-IR phosphorylation under the basal conditions and IGF-IR dephosphorylation in the presence of IGF-I, without evident modifications of the receptor expression (Fig. 4).

Other important observations stemming form this work concern similarities and differences between the effects of ICI 182,780 and Tam on the IGF-IR and SHC. While Tam did not modulate the expression of IGF-IR protein (29), ICI 182,780 moderately decreased IGF-IR levels in the presence of IGF-I. The action of ICI 182,780 and Tam on IGF-IR tyrosine phosphorylation was similar, namely, both compounds inhibited IGF-I-induced but not basal tyrosine phosphorylation of the IGF-IR. The effects of ICI 182,780 and Tam on SHC were different. With Tam, we observed elevated tyrosine phosphorylation of SHC proteins and increased SHC/GRB2 binding in growth arrested cells, while ICI 182,780 did not affect SHC phosphorylation or expression (29). Thus, induction of a non-mitogenic SHC signaling is a peculiarity of Tam but not ICI 182,780 mechanism of action.

In summary, cytostatic effects of ICI 182,780, similar to Tam, are associated with the inhibition of IGF-IR signaling. The mitogenic/survival IRS-1 pathway is a target for both antiestrogens. Both drugs reduce the levels of tyrosine phosphorylated IRS-1, but only ICI 182,780 clearly inhibits expression of the substrate. High cellular levels of IRS-1 hinder the response to higher doses of ICI 182,780, thus overexpression of IRS-1 in breast tumors may represent an important mechanism of antiestrogen resistance.

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REFERENCES

1. Wakeling, A. E., Dukes, M., and Bowler, J. (1991)*Cancer Res.* **51**, 3867-3873.

2. Robertson, J. F. R., Howell, A., DeFriend, D. J., Blamey, R. W., and Walton, P. (1997) *Breast* **6**, 186-189.

3. de Cupis, A., and Favoni, R. E. (1997) *Trends in Pharmcol. Sci.* **18**, 245-251.

4. Chander, S. K., Sahota, S. S., Evans, T. R. J., and Luqmani, Y. A. (1993) *Crit. Rev. Oncol. Hematol.* **15**, 243-269.

5. Osborne, C. K., Coronado-Heinsohn, E. B., Hilsenbeck, S. G., McCue, B. L., Wakeling, A. E., McClelland, R. A., Manning, D. L., and Nickolson, R. I. (1995) *J. Natl. Cancer Inst.* **87**, 746-750.

6. de Cupis, A., Noonan, D., Pirani, P., Ferrera, A., Clerico, L, and Favoni, R. E. (1995) *Br. J. Pharm.* **116**, 2391-2400.

7. Nicholson, R. I., Gee, J. M., Manning, D. L., Wakeling, A. E., Montano, M. M., and Katzenellenbogen, B. S. (1995) *Ann. NY Acad. Sci.* **761**, 148-163.

8. Pavlik, E. J., Nelson, K., Srinivvasan, S., Depriest, P. D., and Kenady, D. E (1996) *in* Estrogens, Progestins, and their Antagonists (Pavlik, E. J., Ed.) pp. 116-159, Vol. 1, Birkhauser, Boston.

9. Larsen, S. S., Madsen, M. W., Jensen, B. L., and Lykkesfeld, A. E. (1997) *Int. J. Cancer* **72,** 1129-1136.

10. Wiseman, L. R., Johnson, M. D., Wakeling, A. E., Lykkesfeldt, A. E., May, F. E., and Westley, B. R. (1993) *Eur. J. Cancer* **29A**, 2256-2264.

11. Lee, A. V., and Yee, D. (1995)*Biomed. Pharmecother.* **49**, 415-421.

12. Surmacz, E., Guvakova, M. A., Nolan, M. K., Nicosia, R., and Sciacca, L. (1998) *Breast Cancer Res. Treat.* **47**, 255-267.

13. Dickson, R. B., and Lippman, M. E. *Endocrin. Rev.* (1995) **16**, 559-589.

14. Doerr, M., and Jones, J. (1996) *J. Biol. Chem.* **271**, 2443-2447.

15. Guvakova, M. A., and Surmacz, E. (1997) *Exp. Cell. Res.* **231**, 149-162.

16. Bracke, M. E., Vyncke, B. M., Bruyneel, E. A., Vermeulen, S. J., De Bruyne, G. K., Van Larebeke, N. A., Vleminck, K., Van Roy, F. M., and Mareel, M. M. (1993) *Br. J. Cancer* **68**, 282-289.

17. Nolan, M. K., Jankowska, L., Prisco, M., Xu, S., Guvakova, M., and Surmacz, E. (1997) *Int. J. Cancer* **72**, 828-833.

18. Hung, H., and Pollak, M. (1995) *Prog. Growth Factor Res.* **6**, 495-501.

19. Huynh, H., Yang, X. F., and Pollak, M. (1996) Cell Growth Different. **7**, 1501-1506.

20. Cullen, K.J., Lippman, M.E., Chow, D., Hill, S., Rosen, N., and Zwiebel, J.A. (1992) *Mol. Endocrinol.* **6,** 91-100.

21. Surmacz, E., and Burgaud, J-L. (1995) *Clin. Cancer Res.* **1**, 1429-1436.

22. Peyrat, J.P., and Bonneterre, J. (1992) *Breast Cancer Res. Treat.* 22, 59-68.

23. Papa, V., Gliozzo, B., Clark, G. M., McGuire, W. L., Moore, D., Fujita-Yamaguchi, Y., Vigneri, R., Goldfine, and I. D., Pezzino, V. (1993) *Cancer Res. 53*, 3735-3740.

24. Resnik, J. L., Reichart, D. B., Huey, K., Webster, N. J. G., Seely, B. L. (1998) *Cancer Res.* **58**, 1159-1164.

25. Rocha, R. L., Hilsenbeck, S. G., Jackson, J. G., and Yee, D. (1997) *Clin. Cancer Res.* **3,** 103-109.

26. Turner, B. C., Haffty, B. G., Nayarannan, L., Yuan, J., Havre, P. A., Gumbs, A., Kaplan, L., Burgaud, J-L., Carter, D., Baserga, R., and Glazer, P. (1997) *Cancer Res.* **57**, 3079-3083.

27. Ellis, M. J. C., Singer, C., Hornby, A., Rasmussen, A., and Cullen, K. J. (1994) *Breast Cancer Res. Treat.* **31**, 249-261.

28. Hankinson, S., Willet, W. C., Colditz, G., Hunter, D. J., Michaud, D. S., Deroo, B., Rosner, B., Speizer, F., and Pollak, M. (1998) *Lancet* **351**, 1393-1396.

29. Guvakova, M. A, and Surmacz, E. (1997) *Cancer Res.* **57**, 2606-2610.

30. Sepp-Lorenzino, L. (1998) *Breast Cancer Res. Treat.* **47,** 235-253.

31. Nishiyama, M., and Wands, J. R. (1992) *Bioch. Biophys. Res. Comm.* **183,** 280-285.

32. Keller, S. R., Aebersold, R., Garner, C. W., and Lienhard, G. E. (1993) *Bioch. Biophys. Acta* **1172**, 323-326.

33. Nicholson, R. I., Gee, J. M., Bryant, S., Francis, A. B., McClelland, R. A., Knowlden, J., Wakeling, A. E., and Osborne, C. K. (1996) *Ann. NY Acad. Sci.* **784,** 325-335.

34. Wakeling, A. E., Newboult, E., and Peters, S. W. (1989*) J. Mol. Endocrin.* **2**, 225-234.

35. Coradini, D., Biffi, A., Cappelletti, V., and Di Fronzo, G. (1994) *Anticancer Res.* **14**, 1059-1064.

36. Dunn, S. E., Hardman, R. A., Kari, F. W., and Barrett, J. C. (1997) *Cancer Res.* **57**, 2687-2693.

37. Matsuda, K., Araki, E., Yoshimura, R., Tsuruzoe, K., Furukawa, N., Kaneko, K., Motoshima, H., Yoshizato, K., Kishikawa, K., and Shichiri, M. (1997) *Diabetes* **46**, 354-362.

38. Araki, E., Haag, B. L., Matsuda, K., Shichiri, M., and Kahn, C. R. (1995) *Mol. Endocrin.* **9**, 1367-1379.

39. D'Ambrosio, C., Keller, S. R., Morrione, A., Lienhard, G. E., Baserga, R., and Surmacz, E. (1995) *Cell Growth Different.* **6**, 557-562.

40. Miura, M., Li, S-W., Dumenil, G., and Baserga, R. (1994) *Cancer Res.* **54**, 2472-2477.

41. Lee, A. V., Weng, C-N., Jackson, J. G., and Yee, D. (1997) *J. Endocrin.* **152**, 39-47.

42. Freiss, G., and Vignon, F. (1994) *Mol. Endocrinol.* **8**, 1389-1396.

FOOTNOTES

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[2]The abbreviations used are: CS, calf serum; ER, estrogen receptor; E2, 17-beta estradiol; GRB2, growth factor receptor-bound protein 2; IGF-I, insulin-like growth factor I; IGF-IR, IGF-I receptor; IGFBP, IGF binding protein; IP, immunoprecipitation; IRS-1, insulin receptor substrate 1; MCF-7/IGF-IR, MCF-7 cells overexpressing IGF-IRs; MCF-7/IRS-1, MCF-7 cells overexpressing IRS-1; MCF-7/SHC, MCF-7 cells overexpressing SHC; PI-3 kinase, phosphatidilinositol 3 kinase; PRF-SFM, phenol red-free serum-free medium; SHC, src/collagen homology proteins; Tam, Tamoxifen; 4-OH-Tam, 4-hydroxytamoxifen; WB, Western immunoblotting.

[3]Sisci, D., Mauro, L., Salerno, M., Kim, J., Tam, T., Guvakova, M., Ando, S., and Surmacz, E. "Role of SHC in adhesion and motility in breast cancer cells", submitted.

FIGURE LEGENDS

Fig. 1. ICI 182,780 inhibits the growth of MCF-7 cells overexpressing different elements of IGF-IR signaling. IRS-1 levels determine ICI 182,780 sensitivity. A) ICI 182,780-induced growth inhibition in the parental MCF-7 cells ($8x10^4$ IGF-IRs/cell), MCF-7/IGF-IR, clone 17 ($1x10^6$ IGF-IRs/cell), MCF-7/SHC (5-fold SHC overexpression over the level in MCF-7 cells). **B)** Growth reduction in MCF-7/IRS-1 clone 9 (3-fold IRS-1 overexpression over the levels in MCF-7 cells), clone 3 (7-fold overexpression), and clone 18 (9-fold overexpression). The cells were treated with different doses of ICI 182,780 in the presence or absence of 50 ng/ml IGF-I, as described under Materials and Methods. The increase in cell number between day 0 and day 4 is taken as 100%. The results are means from at least 4 experiments. Bar, SE. **C)** Levels of IRS-1 protein in different MCF-7/IRS-1 cell lines. IRS-1 levels were determined by immunoprecipitation and Western blotting as described under Materials and Methods. Representative results from 3 experiments are shown.

Fig. 2. ICI 182,780 inhibits IRS-1-mediated signaling. A) Effects of ICI 182,780 in MCF-7/IRS-1, clone 3. IRS-1 tyrosine phosphorylation (IRS-1 PY) (panel a), protein levels (IRS-1)(panel b) as well as IRS-1-associated p85 of PI-3 kinase (panel c) and GRB2 (panel d) were determined in cells treated for 15 min, 1h, 1 day or 4 days with 100 nM ICI 182,780 in the presence or absence of 50 ng/ml IGF-I. In 1h treatment, the lane IGF-(-) ICI (-) is underloaded. Representative results from 5 experiments are shown. **B)** Effects of ICI 182,780 on IRS-1 in MCF-7/IGF-IR and MCF-7 cells. IRS-1 tyrosine phosphorylation (IRS-1 PY) and protein levels (IRS-1) were examined in cells treated with 100 nM ICI 182,780 for 4 days. Representative blots of 5 experiments are shown.

Fig. 3. ICI 182,780 attenuates the expression of IRS-1 mRNA. IRS-1 mRNA levels in MCF-7 and MCF-7/IGF-IR cells. The expression of IRS-1 mRNA was determined in cells treated with 100 nM ICI 182,780 for 4 days in the presence or absence of IGF-I. Panel a, IRS-1 mRNA ~5 kb; panel b, control RNA loading: 28S and 18 S RNA in the same blot.

Fig. 4. Effects of ICI 182,780 on the IGF-IR. IGF-IR tyrosine phosphorylation (IGF-IR PY) and protein levels (IGF-IR) in MCF-7/IGF-IR, clone 17 treated for 4 days with 100 nM ICI 182,780 in the presence or absence of 50 ng/ml IGF-I. Representative results of 3 different experiments are shown.

Fig. 5. Effects of ICI 182,780 on SHC signaling. SHC tyrosine phosphorylation (SHC PY), protein levels (SHC), and SHC-associated GRB2 were studied in MCF-7/SHC cells treated for 4 days with 100 nM ICI 182,780 in the presence or absence of 50 ng/ml IGF-I. Representative results of 5 experiments are shown.

FIGURE 1

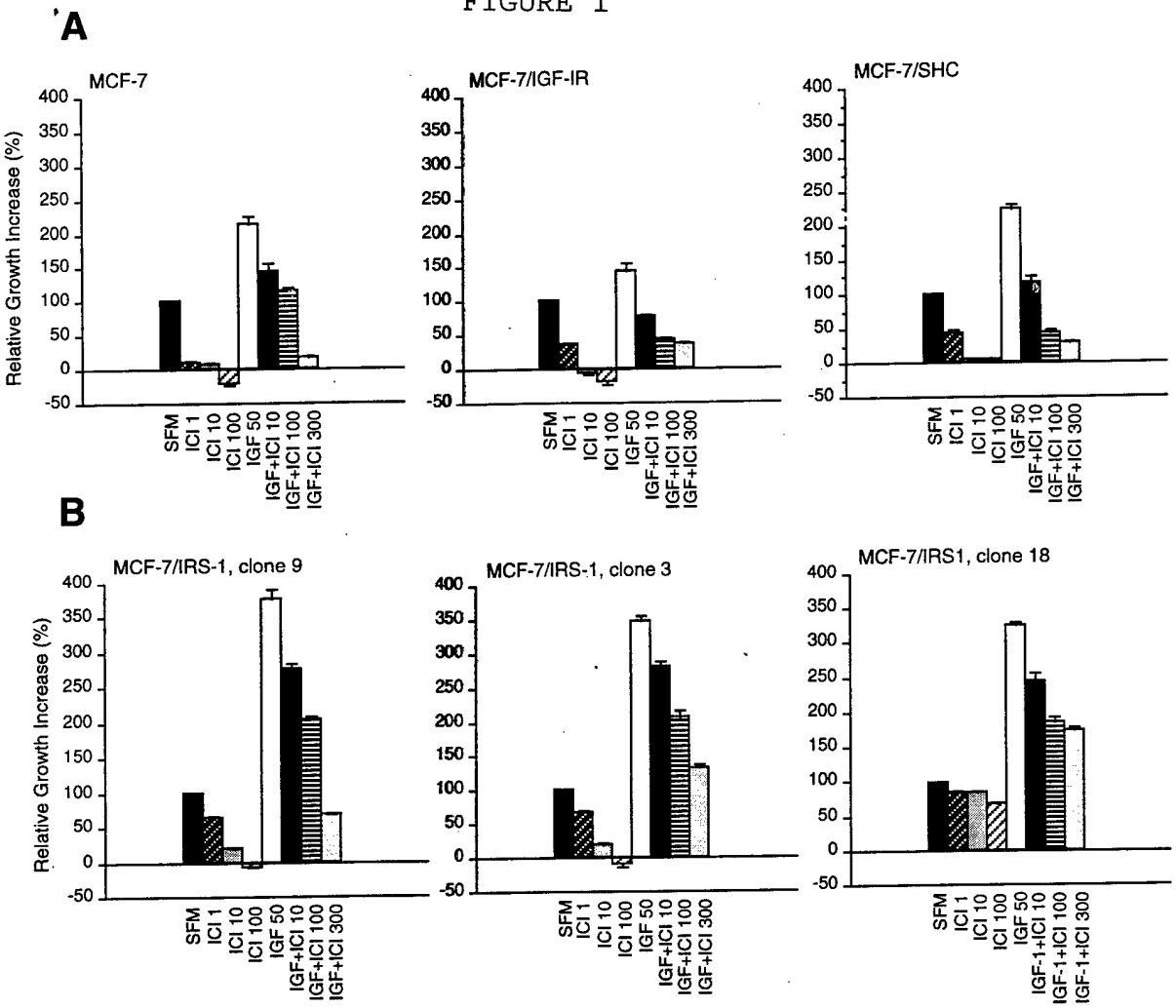

A
MCF-7
MCF-7/IGF-IR
MCF-7/SHC
Relative Growth Increase (%)
400
350
300
250
200
150
100
50
0
-50
SFM
ICI 1
ICI 10
ICI 100
IGF 50
IGF+ICI 10
IGF+ICI 100
IGF+ICI 300
B
MCF-7/IRS-1, clone 9
MCF-7/IRS-1, clone 3
MCF-7/IRS1, clone 18
Relative Growth Increase (%)
SFM
ICI 1
ICI 10
ICI 100
IGF 50
IGF+ICI 10
IGF+ICI 100
IGF+ICI 300
IGF-1+ICI 10
IGF-1+ICI 100
IGF-1+ICI 300

FIGURE 2A

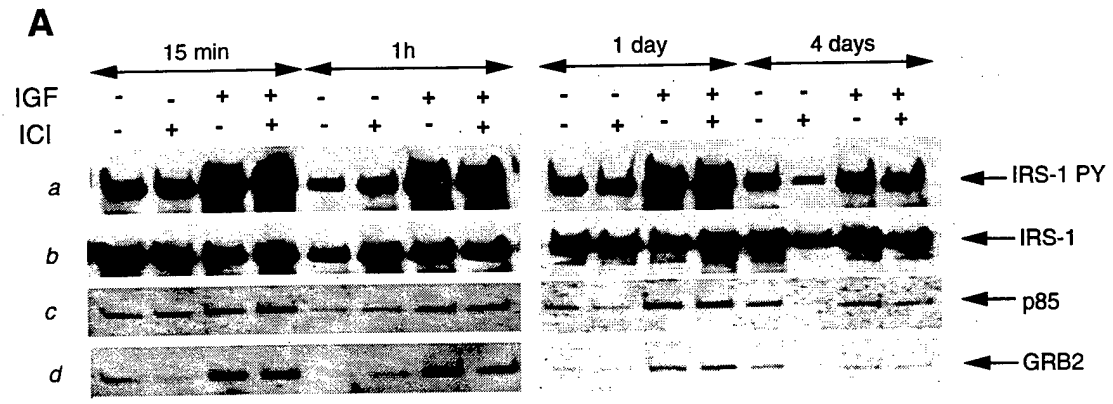


FIGURE 2B

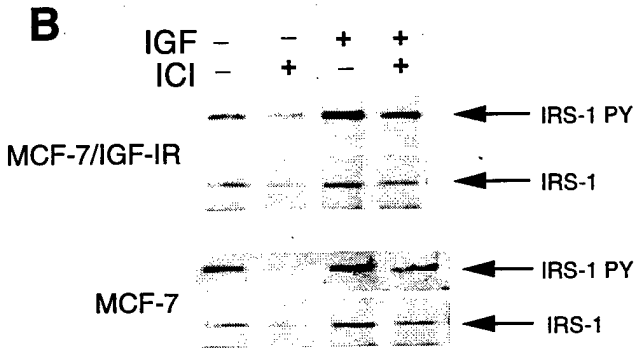


FIGURE 3

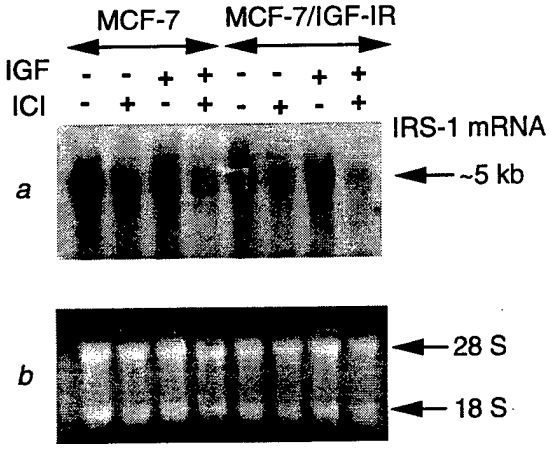


FIGURE 4

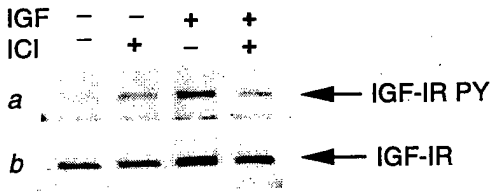
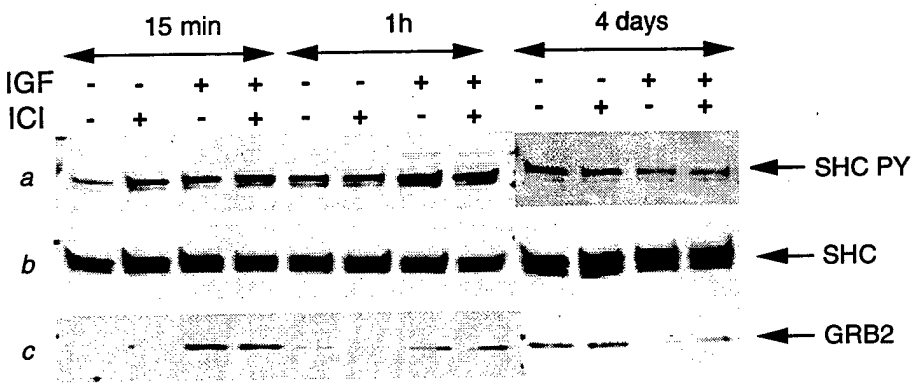


FIGURE 5



ROLE OF IRS-1 SIGNALING IN INSULIN-INDUCED MODULATION OF ESTROGEN RECEPTORS IN BREAST CANCER CELLS

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ABSTRACT

Cross-talk between steroid hormones and polypeptide growth factors regulates the growth of hormone-responsive breast cancer cells. For example, in MCF-7 human breast cancer cell line, insulin up-regulates estrogen receptor (ER) content and binding capacity. Since the insulin receptor (IR) substrate 1 (IRS-1) is one of the core signaling elements transmitting mitogenic and metabolic effects of insulin, we investigated whether IRS-1 is also required for the insulin-induced function of the ER. The effects of insulin on the ER were compared in MCF-7 cells and MCF-7-derived cell lines with decreased levels (by ~80%) of IRS-1 due to the expression of IRS-1 antisense RNA. The severe IRS-1 deficiency in MCF-7 cells was associated with: 1) reduced mitogenic response to 20 ng/ml insulin and 10% calf serum (CS), but not to 1 nM estradiol (E2); 2) loss of insulin-E2 synergism; 3) up-regulation of ER protein expression and binding capacity; and 4) loss of insulin-induced regulation of ER tyrosine phosphorylation. In conclusion, the data confirm the existence of the IR-ER cross talk and suggest that IRS-1-dependent signaling may contribute to the negative regulation of the ER expression and function in MCF-7 cells.

INTRODUCTION

The growth of hormone-responsive breast cancer cells in vitro and in vivo is controlled by steroids and polypeptide growth factors (1,2). Accumulating evidence indicates that this growth control involves complex interactions, or cross-talk, between the two mitogenic systems. For instance, E2 stimulates the expression of several growth factors [such as insulin-like growth factors (IGFs), transforming growth factor alpha and beta, and amphiregulin], alters the levels or activity of different growth factor receptors [such as IGF-I and IGF-II receptors, and the epidermal growth factor (EGF) receptor], as well as modulates the expression of different IGF binding proteins (1-4). E2 is also able to modulate intracellular growth factor signaling pathways. For example, one of the acute effects of E2 binding to the ER in MCF-7 cells is stimulation of c-src tyrosine kinase and activation of c-src-dependent

signaling substrates, including SHC (src-homology/collagen protein), which in consequence induces classic growth factor-responsive Ras/MAP (mitogen-activated kinase) cascade of kinases (5,6). In addition, antiestrogens can block mitogenic action of growth factors on breast cancer cells through an ER-dependent or -independent mechanism (1-4,7).

The other significant element of the cross-talk is modulation of ER expression and function by polypeptide growth factors (8-10). For example, different peptide mitogens can stimulate ER transcriptional activity even in the absence of E2 (11-17), probably through the phosphorylation of the ER on critical residues of Ser 118 and Tyr 537 (10,16,18). We have previously demonstrated that in MCF-7 cells, insulin up-regulates ER content and ER binding capacity, which is blocked in the presence of tyrosine kinase inhibitor, genistein (9). The intracellular signaling mechanism by which insulin regulates ER in breast cancer cells is not known. One of the major signaling pathways of the IR involves a substrate, IRS-1(19). IRS-1 is a docking protein which becomes phosphorylated on multiple tyrosine residues immediately upon insulin binding to the IR. Tyrosine phosphorylated IRS-1 associates with different SH2-domain containing proteins activating a spectrum of downstream signaling pathways, such as Ras/MAP or PI-3 kinase pathways (19). The critical role of IRS-1 signaling in metabolic and mitogenic action of insulin has been well established in many cellular systems (19-21). In MCF-7 cells, IRS-1 is required for monolayer and anchorage-independent growth, and is critical for transmitting signals controlling cell survival (21, 22). In this study, we examined the role of IRS-1 in IR-ER cross-talk.

MATERIALS AND METHODS

Cell Lines. MCF-7/anti-IRS-1 clones 2 and 9 have been generated by stable transfection of MCF-7 cells with an expression plasmid encoding antisense IRS-1 RNA. The IRS-1 protein levels in MCF-7/anti-IRS-1 clone 2 and 9 have been down-regulated by approximately 80% and 85%, respectively. The other characteristics of these cells have been previously described in Ref. 22. In all experiments, the parental MCF-7 cells were used as a

control. The clone overexpressing IRS-1 (MCF-7/IRS-1, clone 3) was obtained by stable transfection of MCF-7 cells with the CMV-IRS-1 plasmid that contains a mouse IRS-1 cDNA cloned into the Hind III site of the pRC/CMV mammalian expression vector (Invitrogen) (21) . The resulting plasmid also confers neomicin resistance (inherent in pRC/CMV).

Routine Cell Culture. MCF-7 cells were grown in DMEM:F12 supplemented with 5% calf serum (CS); MCF-7/anti-IRS-1 cells were cultured in DMEM:F12 plus 5% CS plus 200 mg/ml G418 (22).

Experimental Culture Conditions. The cells cultured in growth medium were trypsinized and plated in phenol red-free (PRF) DMEM:F12 supplemented with 5% dextran-coated charcoal stripped CS (DCC-CS medium). After 24h, this medium was changed to PRF-DMEM:F12 for another 24 h. Next, the cells synchronized in PRF-DMEM:F12 (day 0) were treated for 1, 2, or 96 h with PRF-DMEM:F12 containing 10% of dithiotreitol treated DCC-CS (DCC-SH-CS medium) supplemented with 20 ng/ml of insulin or 1 nM E2. DCC-CS and DCC-SH-CS were prepared as previously described (23, 24).

DNA Content. Cells were plated in 24-well plates at a density of 1.5×10^4 cells/cm^2 in DCC-CS medium and then shifted to PRF-DMEM:F12 and DCC-SH-CS, as described above. At different times of treatment, cellular DNA content per well was assessed by fluorescent staining with Hoechst 33258, as described previously (9).

Estrogen Receptor Binding Assay. ER binding sites in cytosol and nuclear fractions were determined by Scatchard analysis with ^{3}H-E2, as previously described (9). In brief, the cells were seeded at a density of 1.13×10^4/cm^2 in 100 mm culture plates in growth medium, then shifted to DCC-CS, PRF-DMEM:F12 and next to DCC-SH-CS, as described above. The synchronized cells were treated with mitogens for different times. Cytosol fractions were obtained by harvesting and sonicating the cells in a cytosol buffer (9). The sonicate was sedimented at 15,000 g for 30 min at 4^{o}C. The supernatant contained the cytosol fraction, whereas the pellet contained cell nuclei.

Cytosol ER Content. 50 ml of the cytosol fraction were incubated for 18 h at 4°C with 0.125-4 nM ^{3}H-E2. Non-specific binding was determined by incubating the cells with ^{3}H-E2 in the presence of 500-fold molar excess of diethylstilbestrol (DES). Bound and free E2 were separated by absorbing free hormones on DCC (100 µl) at 4°C for 15 min. The radioactivity of the bound hormone was determined in a scintillator counter.

Nuclear ER Content. The nuclear pellet (obtained as described above) was resuspended in 0.6 M KCl for 1 h at 4°C, and then centrifuged at 15,000 g for 30 min at 4°C. 50µl of the supernatant (nuclear fraction) was incubated with 2-64 nM of ^{3}H-E2 for 18 h at 4°C. The non-specific binding was determined with a 250-fold molar excess of DES.

Western Blotting and Immunoprecipitation. The protein levels and tyrosine phosphorylation of the ER were measured by immunoprecipitation (IP) followed by Western Blotting (WB), as previously described (7, 9). Following treatment, the cells were lysed in ice-cold lysis buffer (9). ER protein levels were determined by IP 500 ug of protein lysate with an anti-ER 304 antibody (Ab) (Neo Marker, Freemont USA), followed by WB using an anti-ER 311 Ab (Neo Marker, Freemont USA). CHO (ER-negative) cells were used as a negative control. Tyrosine phosphorylation of the ER was detected by immunoprecipitating 500 ug of protein lysate with an anti-ER 304 Ab (Neo Marker, Freemont USA), followed by immunoblotting with an anti-phosphotyrosine monoclonal Ab (mAb) PY20 (Santa Cruz Biotechnology, CA)

Statistical Analysis. Data were analyzed using analysis of variance (ANOVA) or paired t-test, where applicable.

RESULTS

Down-regulation of IRS-1 inhibits growth-promoting effects of serum and insulin but not that of E2. The requirement for IRS-1 in IR-induced effects on the ER was studied using two MCF-7/anti-IRS-1 clones (2 and 9), in which the levels of IRS-1 were down-regulated by 80% and 85%, respectively. First, we investigated how decreased

levels of IRS-1 affect growth response (measured as an increase of DNA content) to different mitogens. Figure 1 A and B illustrates growth-promoting effects of 10% CS and 20 ng/ml insulin in MCF-7 and MCF-7/anti-IRS-1 cells synchronized in PRF-DMEM:F12. The mitogenic response to 10% CS in MCF-7/anti-IRS-1 clone 2 and 9, was suppressed by 55 (±SE 0.0) % and 57(±SE 0.0)%, respectively, compared that in the parental cells (Fig. 1A). Importantly, 20 ng/ml insulin did not produce any significant increase in DNA content in both MCF-7/anti-IRS-1 clones, whereas it stimulated DNA synthesis in the parental cells [40 (±SE 9.0) % ; $p<0.05$; Figure 1B].

In contrast, the growth-promoting effect of 1 nM E2 in all cell lines was similar (Figure 1B; variation among the cell lines: p= NS). In particular, in MCF-7 cells, E2 stimulation resulted in a 55 (±SE 11.1) % augmentation in DNA content over the basal level, and in MCF-7/anti-IRS-1, clones 2 and 9, the increase was 56 (±SE 15.3) % and 56 (± SE 11.2) %, respectively (Fig. 1B).

The stimulation of DNA synthesis by a combination E2 plus insulin was synergistic in all cell lines, but quantitatively smaller in MCF-7/anti-IRS-1 cells ($p<0.05$, Fig. 1B) Specifically, whereas under the treatment, in MCF-7 cells DNA content increased 187 (± SE 40.0) %, in MCF-7/anti-IRS-1 clone 2 and 9, the stimulation was 142 (±SE 39.0) % and 88 (± SE18.2) %, respectively.

Down-regulation of IRS-1 in MCF-7 cells is accompanied by the increase in both ER protein levels and ER binding capacity. We tested ER protein expression and binding capacity in MCF-7/anti-IRS-1 cells and, for comparison, in MCF-7/IRS-1 clone 3. All these cell lines express a neomycin resistance gene thus allowing us to test the eventual interfering effect of this gene on the ER. The basal content of the ER was clearly enhanced in both MCF-7/anti-IRS-1 clones with respect to MCF-7 cells. Interestingly, a 9-fold overexpression of IRS-1 in MCF-7/IRS-1, clone 3 did not significantely modulate ER expression (Fig. 2 A and B). Scatchard analysis of E2 binding sites upon insulin stimulation

confirmed significant upregulation of ER content in MCF-7/anti-IRS-1 clones compared with MCF-7 and MCF-7/IRS-1cells (Fig. 3).

IRS-1 levels impact the regulation of basal and insulin-induced tyrosine phosphorylation of the ER. The regulation of the ER protein expression and tyrosine phosphorylation by insulin and E2 was determined in MCF-7 cells and in MCF-7/anti-IRS-1, clone 2 (Fig. 4). In the parental cells, the expression of the ER was elevated with both mitogens at 12 h and 96 h of treatment. In contratst, MCF-7/anti-IRS-1cells expressed high basal levels of the ER that appeared refractory to insulin regulation, and were reduced by E2.

The basal ER tyrosine phosphorytation at 12 h was significantely elevated in MCF-7/anti-IRS-1 cells, compared with that seen in MCF-7 cells. Moreover, whereas in MCF-7 cells, insulin and E2 up-regulated ER phosphorylation, in MCF-7/anti-IRS-1 cells, these traetments had no effect on the ER phosphorylation status (Fig. 4B). At 96 h, basal ER tyrosine phosphorylation was similar in both cells lines, but it was clearly down-regulated by insulin in MCF-7 cells and not affected in MCF-7/anti-IRS-1 cells. E2 at 96 h induced the phosphorylation of the ER in both cell lines.

DISCUSSION

Cross-talk between signaling pathways of the IR or the IGF-IR and the ER is a powerful mechanism controlling the growth of many hormone-responsive breast cancer cells. It is thought that deregulation of this cross-talk may lead to the development of hormone-independence and antiestrogen-resistance (1, 3, 4). The molecular basis of growth factor-steroid communication are not entirely clear. Although considerable knowledge exists about the effects of estrogens on IGF or insulin systems, the role of these growth factors on ER expression and function is less known. In this context, of particular interest are recent data demonstrating that IGF or insulin are able to up-regulate E2 binding sites and stimulate the transcription of E2-responsive DNA, even in the absence of E2 (8, 9, 11, 17). In the latter case, growth factors appear to induce phosphorylation of the unligated ER on Ser 118 (via

MAP kinase pathway), and possibly on Tyr 537, in consequence enhancing ER transcriptional activity (10, 18, 25). These observations, together with our present results, provide the evidence that the IR modulates ER function on at least three different molecular levels: a) ER protein expression; b) ER binding capacity; and c) ER phosphorylation. The post-receptor events involved in the control of the ER by insulin are still poorly characterized. Here, we examined the role of IRS-1, a principal substrate of the IR that is critical for its metabolic and mitogenic action, in the modulation of ER expression and tyrosine phosphorylation.

First, IRS-1 was not critical for the stimulation of ER binding capacity by insulin, since in MCF-7 cells with significantly (~80%) decreased levels of IRS-1, insulin normally up-regulated E2 binding sites (Fig. 3), whereas its growth-promoting action was inhibited under the same conditions (Fig. 1B). The possibility that other IR-dependent signaling pathways, such as SHC or PI-3 kinase pathways, are responsible for stimulating E2 binding sites in MCF-7 cells is currently under investigation.

Second, IRS-1signaling may contribute to a physiological down-regulation of ER protein levels in MCF-7 cells, as the reduction of IRS-1 levels was clearly paralleled by an increase of ER expression and binding capacity (Fig. 1B, 2, and 4). However, overexpression of IRS-1 did not reduce ER levels, which suggest that the regulation of the ER depends on some other signaling pathways. Why lower levels of IRS-1 trigger ER overexpression is not known. Perhaps, when IR-dependent mitogenicity is compromised, as occurred in MCF-7/anti-IRS-1 cells, a compensatory mechanism stimulates an overexpression of the ER. Interestingly, however, this ER overexpression in MCF-7/anti-IRS-1 clones was not reflected by an increased mitogenic response to E2 (Fig. 1B), indicating that stimulation of cell growth by E2 is a saturable process, possbly controlled by a negative effect of estradiol on its own receptor (Ref. 26 and Fig. 4A).

Third, our studies suggest that IRS-1 is important for the regulation of ER tyrosine phosphorylation, at least in cells exposed to insulin for 96h. Specifically, such a long-term

insulin traetment evidently reduced the ER phosphorylation in the parental cells, but it produced no effect in MCF-7/anti-IRS-1 cells.

The biological relevance of ER tyrosine phosphorylation is still under debate. It is possible that overall tyrosine phosphorylation of the ER is not directly related to E2 trascriptional and growth effects, as already suggestred by other investgators ; for example ER phosphoryation has been shown to be induced by both estrogen and antiestrogens (11). The concept that in our system, impaired IRS-1 signaling affected phosphorylation of the ER on Ser 118, in consequence reducing ER transcriptional activity is currently under investigation.

In summary, IRS-1 pathway appears to be required for IR-dependent proliferation in MCF-7 cells, but not for E2-stimulated growth. In addition, the data suggest that IRS-1 may contribute to the process of physiologic downregulation of ER expression and function.

ACKNOWLEDGMENTS

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REFERENCES

1. Dickson, R., and Lippman, M. E. (1966) in Diseases of the Breast (Harris, J. R., Lippmann, M. E., Morrow, M., and Hellman, S., Eds.), pp. 272-283, Lippincott-Raven P, Philadelphia, New York.

2. Dickson, R., and Lippman, M. E. (1995) *Endocrine Rev.* **16,** 559-589.

3. Lee, A. V., and Yee, D. (1995) *Biomed. Pharmacother.* **49,** 415-421.

4. Surmacz, E., Guvakova, M., Nolan, M., Nicosia, R., and Sciacca, L. (1997) *Breast Cancer Res. Treatm.* ,in press.

5. Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E., and Auricchio, F. (1996) *EMBO J.* **15,** 1292-1300.

6. Auricchio, F., Migliaccio, A., Castoria, G., Di Domenico, M., Bilancio, A., and Rotondi, A, (1996) *Ann. N.Y. Acad. Sci.* **784,** 149-172.

7. Guvakova, M. A., and Surmacz, E. (1997) *Cancer Res* . **57**, 2606-2610.

8. Katzenellenbogen, B.S. (1996) *Biol. Reprod.* **54,** 287-293

9. Panno, M-L., Salerno, M., Pezzi, V., Sisci, D., Maggiolini, M., Mauro, L., Morrone, E. G., and Ando, S. (1996) *J. Cancer Res. Clin.* **122,** 745-749.

10. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawshima, H., Metzger, D., and Chambon, P. (1995) *Science* **270,** 1491-1494.

11. Aronica, S. M., and Katzenellenbogen, B. S. (1993) *Mol. Endocrin.* **7,** 743-752.

12.Patrone,C., Ma, Z. Q., Pollio, G., Agrati, P., Parker, M. G, and Maggi, A. (1996) *Mol. Endocrin.* **10**, 499-507.

13. Ignar-Trowbridge, D. M., Pimentel, M., Parker, M. G., McLachlan, J. A., and Korach, K. S. (1996) *Endocrinol.* **137**, 1735-1744.

14. Ignar-Trowbridge, D. M., Pimentel, M., Teng, C. T., Korach, K. S., and McLachlan, J. A. (1995) *Environ. Health Perspect.* **103**, 35-38.

15. Mattioni, T., Mayer, B. J., and Picard, D. (1996) *FEBS Lett.* **390**, 170-174.

16. Bunone, G., Briand, P. A., Miksicek, R. J., and Picard, D. (1996) *EMBO J.* **15,** 2174-2183.

17. Lee, A. V., Weng, C-N., Jackson, J. G., and Yee, D. (1997) *J. Endocrin.* **152,** 39-47.

18. Arnold, S. F., Melamed, M., Vorojeikina, D. P., Notides, A. C., and Sasson, S. (1997) *Mol. Endocrinol.* **11,** 48-53.

19. Cheatham, B., and Kahn, C. R. (1995) *Endocr. Rev.* **16,** 117-142.

20. D'Ambrosio, C., Keller, S. R., Morrione, A., Lienhard, G., Baserga, R., and Surmacz, E. (1995) *Cell Growth Differen* . **6,** 557-562.

21. Surmacz, E., and Burgaud, J. L. (1995) *Clin. Cancer Res.* **1,** 1429-1436.

22. Nolan, M., Jankowska, L., Prisco, M., Xu, S., Guvakova, M., and Surmacz, E. (1997) *Int. J. Cancer* **72,** 828-834.

23. Van der Burg, B., Rutterman, G. R., Blankenstein, M. A., de Laat, S. W., van Zoelen, E. J. (1988) *J. Cell. Physiol.* **123,** 101-110.

24. Van Zoelen, E. J. J., Oostward, T. M., van der Saag, P. T., de Laat, S. W. (1985) *J. Cell.Physiol.* **123,** 151-160.

25. Arnold, S. F., Obourn, J. D., Jaffe, H., Notides, A. C. (1995) *Mol. Endocrinol.* **9,** 24-33

26. Saceda, M., Lippman, M. E., Chambon, P., Lindsey, R.L., Ponglikitmongkol, M., Puente, M., and Martin, M. B. (1988) *Mol. Endocrinol.* **2,** 1157-1162

FIGURE LEGENDS

Fig. 1. Mitogenic effects of **A)** 10% CS on MCF-7 and **B)** Estradiol (E2), insulin or a combination of E2 and insulin on MCF-7 and MCF-7/anti-IRS-1 cells. The cells were synchronized in DCC-SH-CS medium (Day 0) and cultured in the presence of either 10% CS, 1 nM E2, 20 ng/ml insulin or 1nM E2 plus 20 ng/ml insulin for 2-12 days.Cell DNA content was determined as described in Materials and Methods. *$p<0.05$; **$p<0.01$ versus control (DNA content at day 0).

Fig. 2. ER protein content in growing MCF-7 cells, MCF-7/anti-IRS-1, clones 2 and 9, and in MCF-7/IRS-1, clone 3. ER protein content was determined in whole cell lysates (a) by WB or by IP followed by WB with specific anti-ER Ab (b) as described in Materials and Methods.

Fig. 3. Binding capacity of cytosolic, nuclear and total ER under basal conditions and upon insulin (20 ng/ml) stimulation in MCF-7 cells, MCF-7/anti-IRS-1 clones 2 and 9, and in MCF-7/IRS-1, clone 3. * $p<0.05$; ** $p<0.01$ versus control .

Fig. 4. A) E2 protein content (ER) in MCF-7 and in MCF/anti-IRS-1, clone 2. The levels of ER in cells untreated or treated with either insulin (20 ng/ml) or estradiol (1 nM) were determined by IP and WB at 12 and 96 h. **B)** Tyrosine phosphorylation of the ER (ER-P-Tyr) in MCF-7 and MCF-7/anti-IRS-1 clone 2 in cells untreated or treated with either insulin (20 ng/ml) or E2 (1 nM) for 12 or 96 h. ER content and ER tyrosine phosphorylation were determined by stripping the blots from Fig. 4A and reprobing with an anti-phosphotyrosine mAb as described under Materials and Methods.

FIGURE 1A

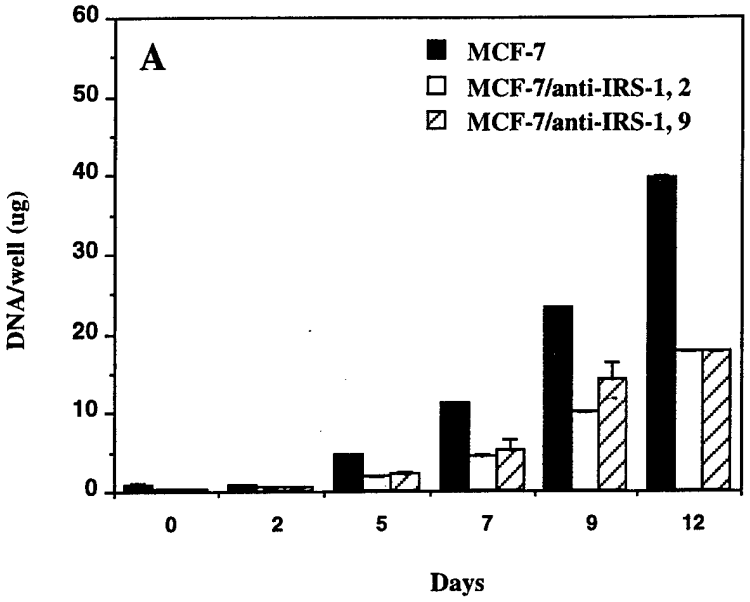


FIGURE 1B

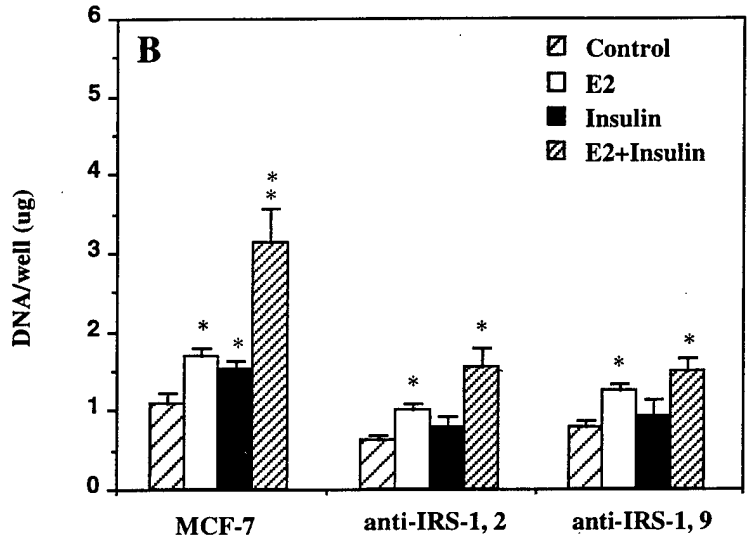
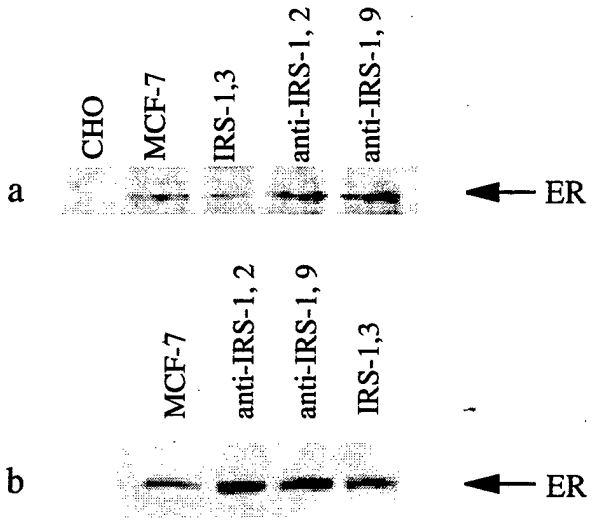


FIGURE 2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CHO
MCF-7
IRS-1,3
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b
MCF-7
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anti-IRS-1, 9
IRS-1,3
ER

FIGURE 3

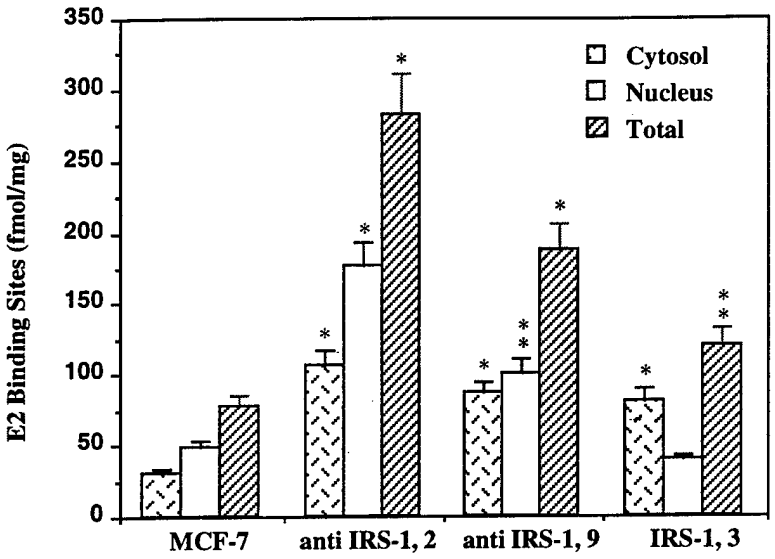
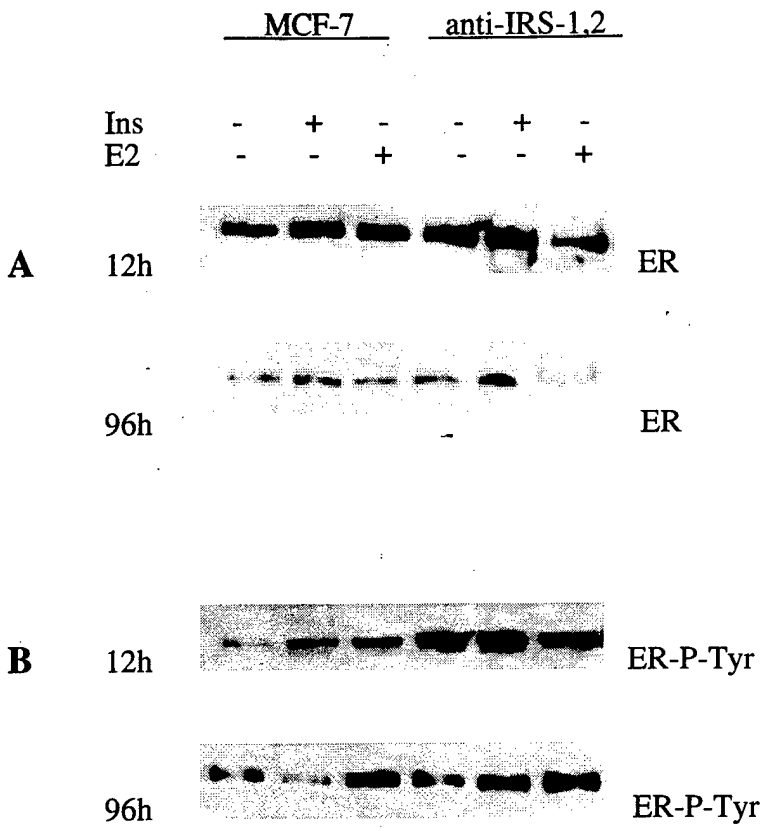

E2 Binding Sites (fmol/mg)
350
300
250
200
150
100
50
0
Cytosol
Nucleus
Total
MCF-7
anti IRS-1, 2
anti IRS-1, 9
IRS-1, 3

FIGURE 4



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Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

DIFFERENTIAL ROLES OF IRS-1 AND SHC SIGNALING PATHWAYS IN BREAST CANCER CELLS

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Several polypeptide growth factors stimulate breast cancer growth and may be involved in tumor progression. However, the relative importance of diverse growth factor signaling pathways in the development and maintenance of the neoplastic phenotype is largely unknown. The activation of such growth factor receptors as the insulin-like growth factor I receptor (IGF-I R), erbB-type receptors (erbB Rs) and FGF receptors (FGF Rs) controls the phenotype of a model breast cancer cell line MCF-7. To evaluate the function of 2 post-receptor signaling molecules, insulin receptor substrate-1 (IRS-1) (a major substrate of the IGF-IR) and SHC (a common substrate of tyrosine kinase receptors), we developed several MCF-7-derived cell clones in which the synthesis of either IRS-1 or SHC was blocked by antisense RNA. In MCF-7 cells, down-regulation of IRS-1 by 80–85% strongly suppressed anchorage-dependent and -independent growth and induced apoptotic cell death under growth factor- and estrogen-reduced conditions. The reduction of SHC levels by approximately 50% resulted in the inhibition of monolayer and anchorage-independent growth but did not decrease cell survival. Importantly, cell aggregation and the ability of cells to survive on the extracellular matrix were inhibited in MCF-7/anti-SHC clones, but not in MCF-7/anti-IRS-1 clones. Cell motility toward IGF was not attenuated in any of the tested cell lines, but motility toward EGF was decreased in MCF-7/anti-SHC clones. Our results suggest that in MCF-7 cells: 1) both IRS-1 and SHC are implicated in the control of monolayer and anchorage-independent growth; 2) IRS-1 is critical to support cell survival; 3) SHC is involved in EGF-dependent motility; and 4) normal levels of SHC, but not IRS-1, are necessary for the formation and maintenance of cell-cell interactions. *Int. J. Cancer* 72:828–834, 1997.
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Several polypeptide growth factors such as the insulin-like growth factors I and II (IGFs), the ligands of the erbB family of receptors (erbB Rs) and fibroblast growth factors (FGFs) regulate breast cancer growth and may be involved in breast cancer progression (Dickson and Lippman, 1995). The impact of these factors on the phenotype of breast cancer cells depends on the level and activity of their cognate membrane receptors. The growth of a model breast cancer cell line, MCF-7, is greatly stimulated by activation of the insulin-like growth factor I receptor (IGF-IR) and the epidermal growth factor receptor (EGFR) (Dickson and Lippman, 1995; Van der Burg *et al.,* 1988; Karey and Sibrascu, 1988).

The signal transduction pathways of the IGF-IR and the EGFR share several common substrates; one, for example, is SHC (Giorgetti *et al.,* 1994; Pelicci *et al.,* 1992). SHC proteins (p66, p52, p47) bind to the IGF-I or EGF receptors through a PTB or an SH2 domain (Tartare-Deckert *et al.,* 1995; Kavanaugh and Williams, 1994; Pelicci *et al.,* 1992). This association results in tyrosine phosphorylation of SHC proteins, which are then able to recruit other signaling molecules, for instance GRB-type adapters, and activate downstream signaling pathways, such as Ras/MAP kinase cascade (Giorgetti *et al.,* 1994; Skolnik *et al.,* 1993; Pelicci *et al.,* 1992).

The transmission of the IGF signal involves insulin receptor substrate 1 (IRS-1), which is not implicated in EGF signaling (Myers *et al.,* 1994; Rubin and Baserga, 1995). IRS-1 is a docking protein containing multiple tyrosine residues, which become rapidly phosphorylated upon receptor activation. This allows association of IRS-1 with different SH2-domain containing proteins and induction of various signaling pathways, such as Ras/MAP kinase (through an adapter GRB2), PI-3 kinase (through a p85 regulatory subunit) or SHPTP2 protein tyrosine phosphatase (Myers *et al.,* 1994). Ultimately, some of the signals generated by growth factors stimulate nuclear events (Myers *et al.,* 1994; Dickson and Lippman, 1995), while others are involved in the reorganization of cell morphology (Joneson *et al.,* 1996).

The significance of IRS-1- and SHC-dependent signaling in the biology of breast tumor cells is not clear. Preliminary data suggest that IRS-1 may regulate the proliferation of tumor cells. In MCF-7 cells, overexpression of IRS-1 enhanced monolayer and anchorage-independent growth and reduced growth requirements for estrogen (E2) (Surmacz and Burgaud, 1995). In primary breast tumors, a correlation has been reported between IRS-1 levels and recurrence of the disease (Rocha *et al.,* 1995). GRB2, an adapter linking IRS-1 and SHC to Ras/MAP kinase, is often overexpressed in breast cancer cell lines (Daly *et al.,* 1994). GRB7, a different adapter of SHC, is overexpressed and co-amplified with erbB2 in breast tumors (Stein *et al.,* 1994). The status of SHC proteins in breast cancer cell lines or tumor samples remains unknown.

Here we evaluated the roles of SHC and IRS-1 in growth, survival, transformation, migration toward chemo-attractants and cell-cell aggregation in MCF-7 breast cancer cells.

MATERIAL AND METHODS

Expression plasmids

To generate the sense and antisense SHC expression plasmids, a 287 bp fragment of a human SHC cDNA (from nt 55 to nt 342) was amplified by PCR using the pMJ/SHC plasmid (a kind gift of Dr. J. Schlessinger, New York, NY) as a template and oligonucleotides 5′-GTG CGG AGA CTC CAT GAG-3′ and 5′-CTC ACA CAC CAG ACT GAT G-3′, as the upstream and downstream primers, respectively. The amplified SHC DNA fragment was cloned into the pCR3 expression plasmid (Invitrogen, San Diego, CA) in either a 5′-3′ or 3′-5′ orientation to produce sense-SHC or antisense-SHC expression vectors (respectively). In the resulting expression vectors, transcription of sense or antisense-SHC RNA was driven by the CMV promoter. The expression plasmids also encoded neomycin resistance to allow for selection in G418.

The antisense- and sense-IRS-1 expression plasmids have been described previously (D'Ambrosio *et al.,* 1995). The plasmids contain the entire sequence of mouse IRS-1 cDNA cloned in either the sense or antisense direction in pRc/CMV expression vector (Invitrogen).

Cell lines and cell culture conditions

MCF-7/antisense-SHC (anti-SHC) and MCF-7/antisense-IRS-1 (anti-IRS-1) clones were generated by stable transfection using the

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calcium phosphate precipitation method. The clones were selected in 2 mg/ml G418, and the integration of transfected plasmids into genomic DNA was confirmed by PCR. In all cases, a "T7 primer" 5′-CGA CTC ACT ATA GG-3′ (located in the T7 promoter of all expression plasmids) was used as an upstream primer. The following downstream primers were used: for sense IRS-1 clones: 5′-GGC TTC TCA GAC GTG CGC AAG-3′; for antisense IRS-1 clones: 5′-GAT AAC TGC TAG GAG ACC-3′; for sense SHC clones: 5′-CTC ACA CAC CAG ACT GAT G-3′; and for antisense SHC clones: 5′-CTG CGG AGA CTC CAT GAG-3′. From each transfection, 13 PCR-positive clones were tested for the levels of target protein by Western immunoblotting (see below).

MCF-7/anti-IRS-1 and MCF-7/anti-SHC cells were maintained in DMEM: F12 supplemented with 5% calf serum (CS) containing 200 μg/ml G418. In the experiments requiring growth factor- and estrogen-reduced conditions, we used DMEM without phenol red (PRF-DMEM) with 0.5 mg/ml BSA, 1 μM $FeSO_4$ and 2 mM L-glutamine (PRF-serum free media, PRF-SFM).

Western blotting

The reduction of SHC and IRS-1 protein levels in MCF-7 clones was confirmed by Western immunoblotting. In MCF-7/anti-IRS-1 clones, cell lysates (1.5 mg) were immunoprecipitated with an anti-IRS-1 antibody (UBI, Lake Placid, NY) and probed with another anti-IRS-1 antibody (obtained from Dr. M. Myers, Boston, MA). The same method was used to assess the levels of IRS-1 in MCF-7/anti-SHC clones, except that 500 μg of protein lysate were used for immunoprecipitation.

In MCF-7/anti-SHC and MCF-7/anti-IRS-1 clones, SHC proteins were immunodetected with an anti-SHC monoclonal antibody (Transduction Labs, Lexington, KY) in 50 μg of total cell lysate. The levels of IRS-1 and SHC proteins were approximated by laser densitometry reading.

Anchorage-dependent growth assay

Cells were plated at a concentration of 1×10^5/30 mm well in DMEM:F12 supplemented with 5% CS. After 24 hr, the cells were washed 3 times with PRF-DMEM, and the medium was replaced with either PRF-SFM, PRF-SFM containing 20 ng/ml IGF-I or PRF-SFM with 5 ng/ml EGF. At days 0 (media change) and 2, the number of cells was determined by direct cell counting with the Trypan blue exclusion test.

Anchorage-independent growth assay

This assay was performed as previously described (Sell *et al.*, 1993). Briefly, the cells were plated at a concentration of 5×10^3/30 mm plate in DMEM with 10% FBS solidified with 0.2% agarose. DMEM with 10% FBS plus 0.4% agarose was used as an underlay. Colonies greater than 150 μm were scored after 3 weeks.

Apoptosis analysis

Flow cytometry cell sorting (FACS). At time 0, or after a 24 hr incubation in PRF-SFM media, cells were washed with cold PBS and fixed in 70% ice-cold ethanol. Following another wash in PBS, the cells were treated with RNase (75 μg/ml) for 30 min at 37°C, washed again in PBS and then resuspended in PBS containing 15 μg/ml propidium iodide. A minimum of 2×10^4 cells was analyzed by FACS with a Coulter Epics Profile II (Hialeah, FL).

In situ *detection of apoptosis.* Apoptotic cells were identified with the TACS/Blue Label *in situ* apoptotic detection kit (Trevigen, Gaithersburg, MD) following the manufacturer's protocol. Briefly, the cells were plated on glass slides in 100 mm plates and grown until 70% confluence. Then the cultures were washed 3 times with PRF-DMEM and shifted to PRF-SFM for 24 hr. Next, the cells were fixed in 3.7% paraformaldehyde and treated first with protease and then with H_2O_2 (to remove exogenous peroxidase). *In situ* labeling of fragmented DNA was performed with Klenow enzyme in the presence of labeled oligodeoxynucleotides. Labeled DNA was visualized with Blue Label, followed by counterstaining with Red Counterstain B. For each experimental condition, at least 1×10^3 cells were counted, and apoptosis was determined based on specific staining and cell morphology.

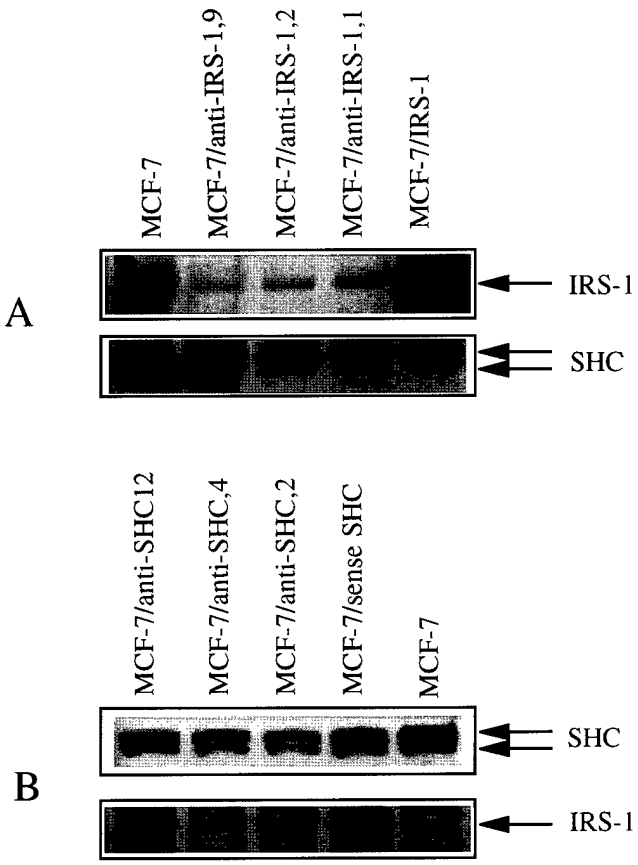

FIGURE 1 – Levels of IRS-1 and SHC in the developed clones. The levels of target proteins in MCF-7/anti-IRS-1 cells *(a)* and MCF-7/anti-SHC cells *(b)* and control cell lines were immunodetected as described in Material and Methods.

Cell aggregation assay

This assay has been performed as described before (Guvakova and Surmacz, 1997). Briefly, Matrigel (extracellular matrix) (Biocoat/Becton Dickinson, Bedford, MA) was reconstituted according to the manufacturer's instruction. Cells were plated at a concentration 2×10^4 cells/well in 24-well plates coated with 200 μl of Matrigel. After 6 days, the number and size of spheroids (aggregates) were counted and measured, and the cultures were photographed. To determine the number of viable cells, the spheroids were dissociated from the matrix during a 2 hr incubation in Dispase (Biocoat/Becton Dickinson) at 37°C, and the cells were counted with the Trypan blue exclusion test.

Cell motility assay

Cell motility was tested using Transwell polycarbonate membrane inserts with a 0.8 μm pore size (Corning/Cambridge, MA), as previously described (Doerr and Jones, 1996). The cells were plated in DMEM:F12 plus 5% CS at a concentration of 2×10^4 cells/insert. The inserts were placed in wells containing either DMEM:F12 plus 5% CS (controls), or DMEM:F12 plus 5% CS supplemented with either 20 ng/ml IGF-I or 5 ng/ml EGF. After a 16 hr incubation, the cells that traversed through the pores and attached to the underside of the insert were stained with Coomassie blue. The number of cells was determined by direct cell counting.

RESULTS

Development of MCF-7/anti-IRS-1 and MCF-7/anti-SHC clones

To investigate the importance of IRS-1- and SHC-dependent signaling pathways in MCF-7 breast cancer cells, we developed, by stable transfection and selection in G418, several MCF-7-derived clones expressing antisense RNA to either IRS-1 or SHC. Ninety-five percent of the G418-resistant clones were PCR positive for plasmid integration; among these clones, approximately 25% exhibited an evident down-regulation of target protein. In MCF-7/anti-IRS-1 clones, the level of IRS-1 was reduced up to 85%, whereas in MCF-7/anti-SHC clones, up to 55% inhibition of SHC protein expression was observed. Interestingly, in both cases, we did not obtain clones with an intermediate (approx. 25–40%) degree of reduction. The levels of IRS-1 and SHC in several clones with the best inhibition of target protein expression are shown in Figure 1. In MCF-7/anti-IRS-1 clones 9, 2 and 1, the amounts of IRS-1 were reduced by 85%, 80% and 70%, respectively (Fig. 1*a*). In MCF-7/anti-SHC clones 12, 4 and 2, SHC expression (both p47 and p52) was inhibited by 47%, 50% and 55%, respectively (Fig. 1*b*). Notably, p66 SHC was undetectable in all MCF-7-derived cell lines, which confirmed our previous findings (Guvakova and Surmacz, 1997).

To control for specificity of antisense RNA activity, we measured the amounts of IRS-1 in MCF-7/anti-SHC clones and, conversely, the levels of SHC in MCF-7/anti-IRS-1 clones. The amounts of IRS-1 in all MCF-7/anti-SHC clones and MCF-7 cells were similar, with a variation of ±12%. Also, the levels of SHC were comparable in MCF-7/anti-IRS-1 clones and MCF-7 cells, with a variation of ±15% (Fig. 1*a,b,* lower panels).

The clones MCF-7/anti-IRS-1 2 and 9 and MCF-7/antisense SHC 2 and 4, exhibiting the best inhibition of target protein expression, were selected for further experiments.

MCF-7 cells with reduced levels of IRS-1 or SHC exhibit inhibition of monolayer growth

The ability of MCF-7/anti-SHC and MCF-7/anti-IRS-1 clones to grow in monolayer culture was tested under 4 different conditions: DMEM:F12 plus 5% CS, PRF-SFM, PRF-SFM plus 20 ng/ml IGF-I, or PRF-SFM plus 5 ng/ml EGF (Fig. 2). The treatments with IGF-I and EGF were chosen because, of the many growth factors tested, these were the best mitogens for MCF-7 cells cultured in our laboratory (data not shown). Several control cell lines were used in this experiment: the parental MCF-7 cells, MCF-7/pc4 cells transfected with an empty vector (Guvakova and Surmacz, 1997) and MCF-7/IRS-1 cells characterized by overexpression of IRS-1 and amplification of IGF signaling (Surmacz and Burgaud, 1995). In a 2-day experiment, the growth of MCF-7 cells increased 50%, 70%, 54% and 80% in PRF-SFM, and PRF-SFM supplemented with IGF-I, EGF and CS, respectively. The increase in the number of MCF-7 cells under given condition was taken as 100%; the increase of the number of tested cells was calculated relative to MCF-7 cells. The growth was defined as increase in the number of viable cells. It should be noted that MCF-7 cells secrete IGF-like mitogens (Surmacz and Burgaud, 1995); therefore all experimental conditions included additional IGF-like autocrine factors.

In medium containing 5% CS (Fig. 2), the proliferation of MCF-7/anti-SHC clones was significantly inhibited. Specifically, relative to the parental cells, the growth was reduced by 55% (clone 2) and 27% (clone 4). Similarly, in MCF-7/anti-IRS-1 cells, the viable cell number was decreased by 61% (clone 2) and 57% (clone 9).

In PRF-SFM (Fig. 2), despite the presence of IGF-like autocrine factors, a large population of MCF-7/anti-IRS-1 cells was dying. In fact, compared with MCF-7 cells, the viable cell number was decreased by 142% (clone 2) and 130% (clone 9). Under the same conditions, MCF-7/anti-SHC clones survived better, although their growth was inhibited by 60% (clone 2) and 93% (clone 4).

Similar results were obtained in PRF-SFM supplemented with 20 ng/ml IGF (Fig. 2). Here, the growth of MCF-7/anti-SHC clones was inhibited by 67% (clone 2) and 83% (clone 4). Under these conditions, MCF-7/anti-IRS-1 cells were massively dying; relative to MCF-7 cells, a 145% (clone 2) and 148% (clone 9) decrease in cell number was noted.

In PRF-SFM supplemented with 5 ng/ml EGF (Fig. 2*d*), MCF-7/anti-SHC clones were inhibited by 82% (clone 2) and 74% (clone 4), while in MCF-7/anti-IRS-1 clones a 75% (clone 2) and 41% (clone 9) growth decrease was obtained.

The control cells, MCF-7/sense-SHC and MCF-7/pc4, grew like MCF-7 cells under all tested conditions. MCF-7/IRS-1 cells exhibited increased responsiveness to IGF-I and EGF, compared with the parental cell line, consistent with the previously published data (Surmacz and Burgaud, 1995).

All developed cell lines retained responsiveness to E2. In all tested cell lines, a 2-day stimulation with E2 alone caused an

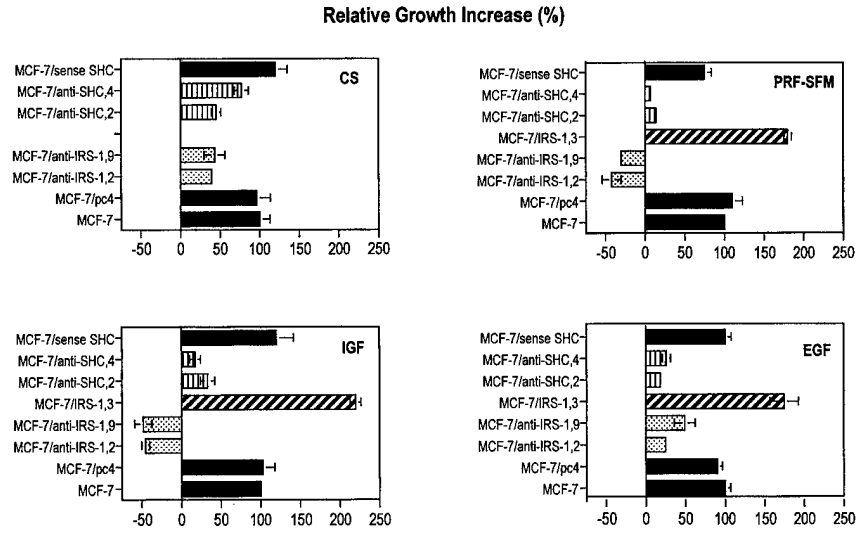

FIGURE 2 – Anchorage-dependent growth. Ordinate: relative percent increase in cell number, with the increase of MCF-7 cells taken as 100%. Abscissa: cell lines tested.

approximately 30% growth increase relative to the cell number at day 0 (data not shown).

MCF-7/anti-IRS-1 cells undergo apoptosis under serum-free conditions

To determine the mechanism of cell death apparent in monolayer growth in PRF-SFM and PRF-SFM plus IGF-I, the clones were analyzed for evidence of apoptosis. Two independent methods were employed, *in situ* detection of fragmented DNA and FACS analysis.

In growing cells (time 0), apoptosis was identified in a small fraction of all tested cell lines (Table I). In contrast, after 24 hr in culture of growth factor- and estrogen-reduced conditions, the rate of apoptosis considerably increased in MCF-7/anti-IRS-1 cells, up to 39.6%, but not in MCF-7/anti-SHC cells or other cell lines. Similar results were obtained after a 48 hr culture in PRF-SFM (data not shown). The higher incidence of apoptotic cell death in MCF-7/anti-IRS-1 clones was confirmed with FACS analysis, in which a pre-G_1 peak, possibly representing the subfraction of apoptotic cells, was observed (Fig. 3). In contrast, such a subfraction was undetectable in MCF-7 cells (Fig. 3) and MCF-7/anti-SHC clones (data not shown).

Anchorage-independent growth is blocked in MCF-7/anti-IRS-1 and MCF-7/anti-SHC cells

The overexpression of IRS-1 has been shown to enhance anchorage-independent growth in MCF-7 cells (Surmacz and Burgaud, 1995). Amplification of SHC promoted transforming abilities in fibroblasts (Pelicci *et al.,* 1992). Here, we tested anchorage-independent growth (colony formation in soft agar) of MCF-7/anti-IRS-1 and MCF-7/anti-SHC clones (Table II). In both cases, we found that colony formation was similarly inhibited, by at least 72%, when compared with MCF-7 cells. The anchorage-independent growth of control cell lines was comparable to that of MCF-7 cells.

MCF-7/anti-SHC clones exhibit impaired aggregation and survival on the extracellular matrix

Our previous results indicated that overexpression of the IGF-IR in MCF-7 cells markedly increased the ability of cells to aggregate on the extracellular matrix (Matrigel) (Guvakova and Surmacz, 1997). Moreover, the formation of multiple cell-cell contacts supported proliferation of clustered cells and decreased the rate of cell death. Here we studied whether reduced levels of IRS-1 or SHC (and, presumably, impaired signaling depending on these substrates) would affect cell-cell interaction on Matrigel.

The experiments demonstrated that while MCF-7/anti-IRS-1 clones were able to aggregate on ECM to a similar extent as control cell lines (MCF-7 and MCF-7/sense SHC cells), the aggregation of both MCF-7/anti-SHC clones was clearly inhibited (Fig. 4). Specifically, MCF-7, MCF-7/sense SHC cells and MCF-7/anti-IRS-1 clones produced large spheroids ranging in size from 230 to 300 μm, whereas MCF-7/anti-SHC clones formed small aggregates (approximately 50 μm in diameter). Furthermore, the clones that formed large spheroids (MCF-7, MCF-7/sense SHC and MCF-7/anti-IRS-1 cells) were also able to survive on ECM up to 7 days. In contrast, the population of viable MCF-7/anti-SHC cells was reduced by at least 50% during this period of time (Table III).

EGF-dependent cell motility is affected by the reduction of SHC levels in MCF-7 cells

The IGF-IR has been shown to mediate motility in breast cancer cells (Doerr and Jones, 1996). We studied the ability of MCF-7/anti-IRS-1 and MCF-7/anti-SHC cells to migrate toward a chemo-attractant, IGF or EGF (Table IV). Both growth factors stimulated the motility of all studied cell lines. The tendencies to migrate toward IGF were similar for all clones; however, some clonal variations were observed (64–95% increase over basal migration in growth medium). When EGF was used as an chemo-attractant, in MCF-7 cells, MCF-7/IRS-1 and MCF-7/sense-SHC clones, as well as in both MCF-7/anti-IRS-1 clones, migration increased by 28–56% over that stimulated by IGF (Table IV). In contrast, migration of MCF-7/anti-SHC clones toward EGF was decreased by 32% (clone 2) and 70% (clone 4) compared with IGF stimulation. In all cell lines tested, the differences between IGF-I and EGF chemo-attraction were statistically significant ($p < 0.05$, by ANOVA).

DISCUSSION

Although it is known that polypeptide growth factors, such as the IGFs and the ligands of the erbB family of receptors, play an important role in the regulation of breast cancer growth and progression, the functions of their different signaling pathways in the development of a neoplastic phenotype have not been elucidated. We have investigated the role of 2 signaling elements, IRS-1, a major substrate of the IGF-IR (but also involved in insulin and IL-4 signaling; Myers *et al.,* 1994) and SHC, an important substrate of different tyrosine kinase receptors, *e.g.,* the IGF-IR and erbB-type Rs (Sepp-Lorenzino *et al.,* 1996; Giorgetti *et al.,* 1994; Pelicci *et al.,* 1992). Since previous studies have demonstrated growth inhibition in MCF-7 cells stably expressing an IGF-IR antisense RNA (Neuenschwander *et al.,* 1995), we have used an antisense RNA approach to generate MCF-7 cell lines expressing reduced levels of either IRS-1 or SHC. The developed antisense clones were tested for their ability to grow under anchorage-dependent and -independent conditions, to survive in estrogen- and growth factor-reduced media, to migrate toward chemo-attractants and to develop and maintain cell-cell interactions on the extracellular matrix.

The major findings of this work are: 1) In MCF-7 cells, IRS-1 and SHC are involved in the regulation of monolayer and anchorage-independent growth; 2) significant reduction of IRS-1 levels is accompanied by cell death; 3) down-regulation of SHC levels affects cell-cell interactions on extracellular matrix; and 4) decrease of SHC levels impairs EGF-, but not IGF-I-stimulated migration of MCF-7 cells.

The most striking differences between MCF-7/anti-IRS-1 and MCF-7/anti-SHC clones were seen in cell aggregation on Matrigel. The results suggested that normal amounts of SHC are required for the formation and maintenance of cell-cell contacts. We have demonstrated previously that in MCF-7 cells, E-cadherin-dependent cell-cell adhesion is significantly enhanced by the overexpression of the IGF-IR. Moreover, the IGF-IR and its substrates, IRS-1 and SHC, are able to associate with the E-cadherin complex (Guvakova and Surmacz, 1997). The mechanism of IGF-I-stimulated adhesion in breast epithelial cells remains unclear (Guvakova and Surmacz, 1997; Bracke *et al.,* 1993), but based on the present work, SHC signaling could be a contributing factor. The involvement of SHC in cell-cell interactions is also supported by the finding of a direct association of SHC and N-cadherin *in vitro* (Xu *et al.,* 1996).

TABLE I – APOPTOSIS IN MCF-7/ANTI/IRS-1 AND MCF-7/ANTI-SHC CELLS

Cell line	Apoptotic cells (%)[1]	
	0 hr	24 hr
MCF-7	0.8 ± 0.5	3.0 ± 0.8
MCF-7/pc4	0.5 ± 0.1	1.9 ± 0.1
MCF-7/IRS	1.2 ± 0.7	3.7 ± 1.1
MCF-7/anti-IRS-1, 2	1.9 ± 0.8	22.5 ± 2.3
MCF-7/anti-IRS-1, 9	4.3 ± 1.2	39.6 ± 1.4
MCF-7/anti-SHC, 2	1.2 ± 0.8	1.5 ± 0.3
MCF-7/anti-SHC, 4	0.8 ± 0.1	3.6 ± 0.5
MCF-7/sense-SHC	2.0 ± 0.2	2.3 ± 0.0

[1]DNA fragmentation *in situ* was detected using a Trevigen *in situ* detection kit following the manufacturer's methodology, as described in Material and Methods. The results shown are means ± SD from at least 3 independent experiments.

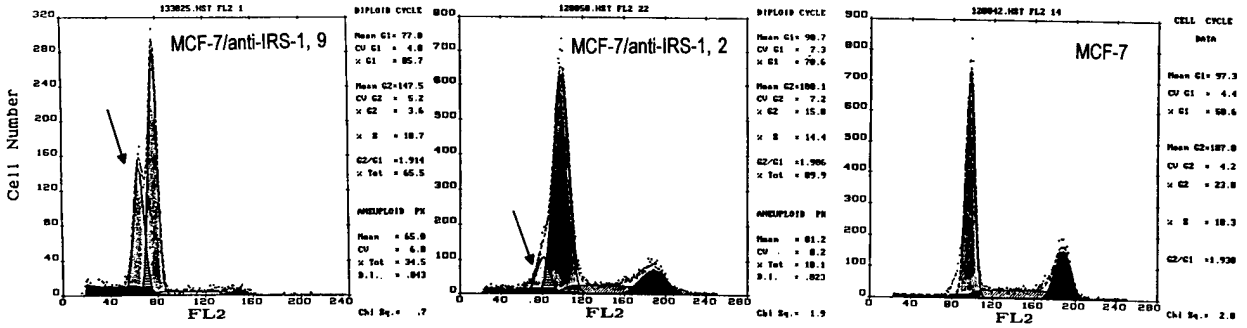

FIGURE 3 – Apoptosis analysis. To identify apoptosis in MCF-7 cells and MCF-7/anti-IRS-1 clones 2 and 9, FACS analysis was performed with a Coulter Epics Profile II as described in Material and Methods. The arrows indicate pre-G_1 peaks, representing apoptotic cell fractions in MCF-7/anti-IRS-1, 9 and MCF-7/anti-IRS-1, 2 clones. Subsequent to pre-G_1 (left to right), are the peaks representing G_1, S, and G_2 cell subpopulations.

TABLE II – ANCHORAGE-INDEPENDENT GROWTH OF MCF-7/ANTI-IRS-1 AND MCF-7/ANTI-SHC CELLS

Cell line	Number of colonies[1]	Inhibition (%)
MCF-7	101 ± 5.9	—
MCF-7/pc4	98 ± 1.0	3
MCF-7/anti-IRS1, 2	14 ± 6.7	86
MCF-7/anti-IRS-1, 9	28 ± 4.5	72
MCF-7/anti-SHC, 2	12 ± 3.5	88
MCF-7/anti-SHC, 4	28 ± 6.0	72
MCF-7/sense SHC	103 ± 9.4	—

[1]Cells were plated in soft agar in 10% FBS at 5×10^5 cells/plate. Colonies greater than 150 μm were counted after 3 weeks. The data are means ± SD from 3 independent experiments.

Remarkably, in MCF-7 cells, down-regulation of IRS-1 levels, which was correlated with the inhibition of the growth in monolayer culture and in soft agar, did not affect cell aggregation, and only moderately (20%) inhibited cell survival on Matrigel. A limited role of IRS-1 in cell-cell adhesion is also suggested by the fact that overexpression of this molecule does not improve aggregation in MCF-7/IRS-1 cells; however, it does prolong cell survival on Matrigel (data not shown). The latter suggest a role of IRS-1 in protection from cell death. This function of IRS-1 has also been demonstrated in the present work; in particular, MCF-7/anti-IRS-1 cells cultured as monolayer in PRF-SFM and PRF-SFM with IGF-I, were massively dying. This suggested that other pathways activated under these conditions, for instance SHC, did not provide sufficient signal for survival and could not compensate for IRS-1 loss. Importantly, in anti-IRS-1 clones, cell death was executed through apoptosis. Apoptosis was detected by FACS and *in situ* labeling, the methods of choice for breast epithelial cells in which a classical apoptotic DNA ladder is usually undetectable (Wilson *et al.*, 1995). Apoptosis was not identified in cells with normal IRS-1 amounts, for instance, in MCF-7 cells or in anti-SHC clones growing in the presence of IGF (autocrine or exogenous). Interestingly, when anti-IRS-1 clones were cultured in media supplemented with CS or EGF, the cells were able to survive, possibly due to the activation of some IRS-1-independent anti-apoptotic mechanisms. For example, a PI-3 kinase pathway (which can be activated directly by the EGFR) has been found to control cell survival (Parrizas *et al.*, 1997).

The role of SHC in survival of MCF-7 cells is difficult to evaluate, partly because in our model, reduction of SHC levels was not as great as that of IRS-1. The fact that the inhibition of SHC by approximately 60% was not sufficient to induce cell death in monolayer culture, even in PRF-SFM, indicates that normal amounts of SHC were not essential for survival under these conditions. However, the survival of cells on Matrigel (in the presence of different growth factors) was inhibited in MCF-7/anti-SHC clones. We speculate that this phenomenon represented a secondary effect to the impaired cell aggregation in these cells, since aggregation itself has been shown to promote survival on Matrigel (Guvakova and Surmacz, 1997).

The studies on anchorage-dependent growth also suggested an important function of both SHC and IRS-1 in cell proliferation. In MCF-7/anti-IRS-1 and MCF-7/anti-SHC clones, cell growth was blocked even in medium containing CS. This reflected mostly the inhibition of proliferation, since, even in MCF-7/anti-IRS-1 cells, only minimal cell death was observed (Table I and data not shown). Similar results were obtained in medium supplemented with EGF (naturally containing autocrine IGF-like factors and possibly other unidentified mitogens) (Fig. 2). The greatest extent of growth reduction, for both MCF-7/anti-IRS-1 and MCF-7/anti-SHC, was seen in SFM containing only IGF (autocrine or exogenous). The results suggested that normal levels of either IRS-1 or SHC were not sufficient to sustain growth in IGF when the other pathway was (presumably) impaired. Therefore, both substrates must control IGF-I-dependent monolayer growth of MCF-7 cells.

Both SHC and IRS-1 also appear to be critical in the maintenance of anchorage-independent growth since colony formation in both MCF-7/anti-SHC and MCF-7/anti-IRS-1 clones was significantly (by at least 70%) inhibited, compared with control cell lines expressing normal amounts of both substrates (Table II).

It has been shown that in different breast cancer cell lines, motility is stimulated by the activation of the IGF-IR (Doerr and Jones, 1996). Our results did confirm that IGF-I stimulates migration of MCF-7 cells. We also found that in MCF-7 cells, migration was stimulated by EGF. Contrary to Doerr and Jones (1996), in our hands EGF was a significantly stronger chemoattractant for the cells studied than IGF. The reason for this discrepancy is unclear. It is possible that the subline of MCF-7 cells cultured in our laboratory differs from the one described by others; in particular, our MCF-7 cells were able to traverse only uncoated membranes, whereas the cells described by Doerr and Jones (1996) invaded through either gelatin, laminin or collagen.

Under our experimental conditions, IGF-I-dependent migration was similar in all tested cell lines and was not significantly inhibited in either MCF-7/anti-SHC or MCF-7/anti-IRS-1 clones. It is possible that the IGF-IR activated other pathways providing sufficient signal for migration, or, alternatively, the extent of the inhibition of either IRS-1 or SHC signaling was insufficient to inhibit migration. It is noteworthy that the EGF-stimulated motility was significantly blocked in MCF-7/anti-SHC clones, suggesting that SHC may act as a critical signaling substrate of the EGFR-regulated migration.

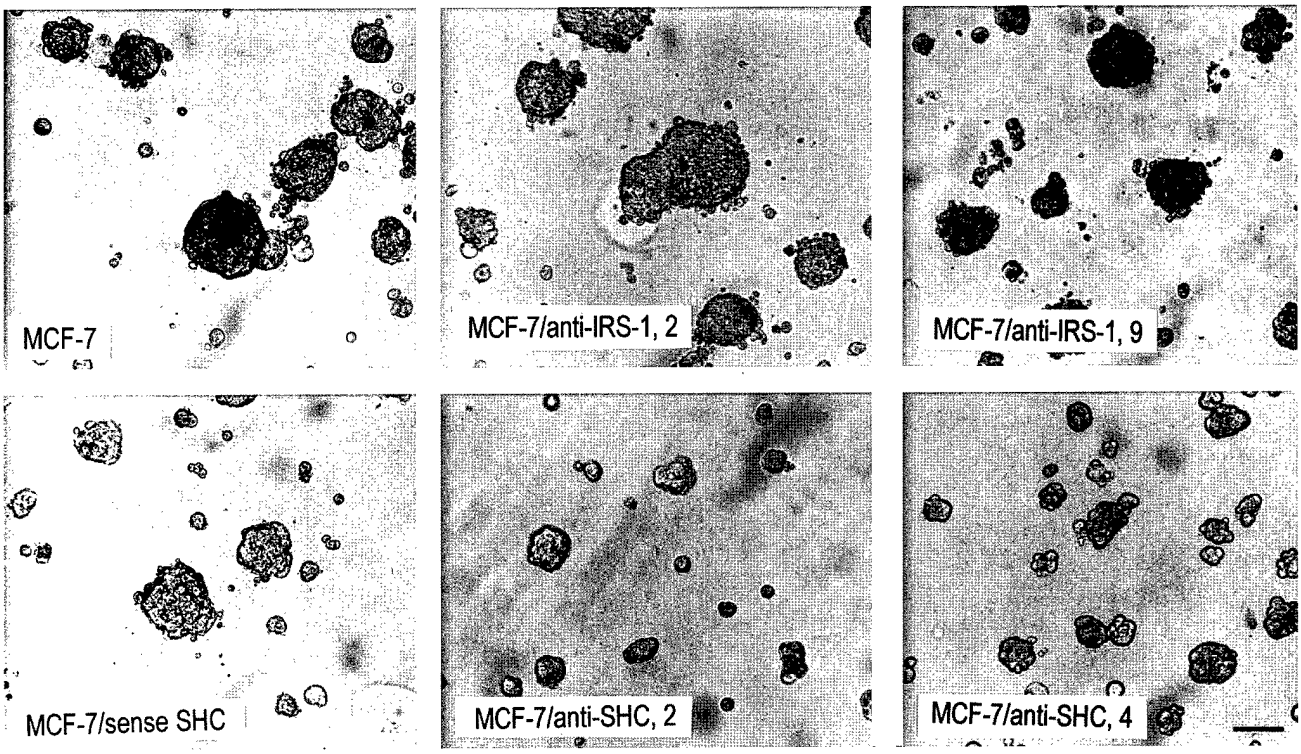

FIGURE 4 – Cell aggregation on Matrigel. Representative photographs of cell aggregation in cell lines tested. The aggregation was tested as described in Material and Methods; cells were photographed with 100× magnification on day 5 of the experiment. Scale bar = 100 μm.

TABLE III – SURVIVAL OF MCF-7/ANTI-IRS-1 AND MCF-7/ANTI-SHC CLONES ON MATRIGEL

Cell lines	Number of cells at day 6[1]
MCF-7	19,300 ± 818
MCF-7/pc4	17,266 ± 1,010
MCF-7/anti-IRS-1, 2	18,233 ± 709
MCF-7/anti-IRS-1, 9	18,366 ± 1,517
MCF-7/anti-SHC, 2	6,000 ± 1,000
MCF-7/anti-SHC, 4	9,500 ± 1,040
MCF-7/sense SHC	19,333 ± 1,527

[1]Cells were plated at 2×10^4/well in 24-well plates on Matrigel Matrix (Biocoat/Fisher). On day 6, the number of cells was determined by direct cell counting with Trypan blue exclusion after dissociation of aggregates by Dispase at 37°C for 2 hr. The data are means ± SD from at least 3 independent experiments.

TABLE IV – MOTILITY OF MCF-7/ANTI-IRS-1 AND MCF-7/ANTI-SHC CELLS

Cell line	Chemo-attraction (% over basal)[1]	
	IGF	EGF
MCF-7	195 ± 7.2	245 ± 7.1
MCF-7/IRS-1	186 ± 6.1	217 ± 2.5
MCF-7/sense SHC	167 ± 6.1	212 ± 10.0
MCF-7/anti-IRS-1, 2	164 ± 3.0	194 ± 2.6
MCF-7/anti-IRS-1, 9	165 ± 5.0	195 ± 9.0
MCF-7/anti-SHC, 2	175 ± 2.0	148 ± 5.9
MCF-7/anti-SHC, 4	195 ± 8.6	128 ± 7.0

[1]Cells (2×10^4) suspended in growth medium were plated in Transwell inserts, and the migration toward IGF or EGF was evaluated as described in Material and Methods. Migration toward growth medium was taken as basal. The data are average ± SD from at least 3 independent experiments.

In summary, our results point to the importance of 2 post-receptor signaling molecules, IRS-1 and SHC, in the maintenance of the neoplastic phenotype in breast epithelial cells; the results also suggested that these substrates may have distinct functions in breast cancer cell biology.

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REFERENCES

BRACKE, M.E., VYNCKE, B.M., BRUYNEEL, E.A., VERMEULEN, S.J., DE BRUYNE, G.K., VAN LAREBEKE, N.A., VLEMINCK, K., VAN ROY, F.M. and MAREEL, M.M., Insulin-like growth factor I activates the invasion suppressor function of E-cadherin in MCF-7 human mammary carcinoma cells *in vitro*. *Brit. J. Cancer,* **68,** 282–289 (1993).

DALY, R.J., BINDER, M.D. and SUTHERLAND, R.L., Overexpression of GRB2 gene in human breast cancer cell lines. *Oncogene,* **9,** 2723–2727 (1994).

D'AMBROSIO, C., KELLER, S.R., MORRIONE, A., LIENHARD, G.E., BASERGA, R. and SURMACZ, E., Transforming potential of the insulin receptor substrate 1. *Cell Growth Differentiation,* **6,** 557–562 (1995).

DICKSON, R.B. and LIPPMAN, M.E., Growth factors in breast cancer. *Endocrine Rev.,* **16,** 559–589 (1995).

DOERR, M. and JONES, J., The roles of integrins and extracellular matrix proteins in the IGF-I-stimulated chemotaxis of human breast cancer cells. *J. biol. Chem.,* **271,** 2443–2447 (1996).

GIORGETTI, S., PELICCI, P.G., PELICCI, G. and VAN OBBERGHEN, E., Involve-

ment of Src-homology/collagen (SHC) proteins in signaling through the insulin receptor and the insulin-like growth factor-I receptor. *Europ. J. Bioch.,* **223,** 195–202 (1994).

GUVAKOVA, M. and SURMACZ, E., Overexpressed IGF-IR reduce IGF-I and estrogen growth requirements, enhance survival and promote E-cadherin-mediated cell-cell adhesion in human breast cancer cells. *Exp. Cell Res.,* **231,** 149–162 (1997).

JONESON, T., WHITE, M.A., WIGLER, M.H. and BAR-SAGI, D., Stimulation of membrane ruffling and MAP kinase activation by distinct effectors of RAS. *Science,* **271,** 810–812 (1996).

KAREY, K.P. and SIBRASCU, P.A., Differential responsiveness of human breast cancer cell lines MCF-7 and T47 D to growth factors and 17-beta estradiol. *Cancer Res.,* **98,** 4089–4092 (1988).

KAVANAUGH, W.M. and WILLIAMS, L.T., An alternative to Sh2 domains for binding tyrosine-phosphorylated proteins. *Science,* **266,** 1862–1864 (1994).

MYERS, M., SUN, X.J. and WHITE, M., The IRS-1 signaling system. *Trends biochem. Sci.,* **19,** 289–293 (1994).

NEUENSCHWANDER, S., ROBERTS, C.T. and LEROITH, D., Growth inhibition of MCF-7 breast cancer cells by stable expression of an insulin-like growth factor I receptor antisense ribonucleic acid. *Endocrinology,* **136,** 4298–42303 (1995).

PARRIZAS, M., SALTIEL, A. and LEROITH, D., IGF-I inhibits apoptosis using the PI-3 kinase pathway and the Map kinase pathway. *J. biol. Chem.,* **272,** 154–161 (1997).

PELICCI, G., LANFRANCONE, L., GRIGNANI, F., MCGLADE, J., CAVALLO, F., FORNI, G., NICOLETTI, I., GRIGNANI, F., PAWSON, T. and PELICCI, P.G., A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell,* **70,** 93–104 (1992).

ROCHA, R.L., HILSENBECK, S.G., JACKSON, J.G. and YEE, D., Insulin-like growth factor binding protein-3 (IGFBP3) and insulin receptor substrate 1 (IRS1) in primary breast cancer: large tumors have higher BP3 levels and higher levels of IRS-1 are associated with lower disease-free survival (DFS) rate. *Breast Cancer Res. Treat. (Suppl.),* **37,** 55 (1995).

RUBIN, R. and BASERGA, R., Biology of disease. IGF-IR. Its role in cell proliferation, apoptosis and tumorgenicity. *Lab Invest.,* **73,** 311–331 (1995).

SELL, C., RUBINI, R., LIU, J.P., EFSTRATIADIS, A. and BASERGA, R., Simian virus 40 large tumor antigen is unable to transform mouse embyonic fibroblasts lacking type-1 IGF receptor. *Proc. nat. Acad. Sci. (Wash.),* **90,** 11217–11221 (1993).

SEPP-LORENZINO, L., EBERHARD, I., MA, Z., CHO, C., SERVE, H., LIU, F., ROSEN, N. and LUPU, R., Signal transduction pathways induced by heregulin in MDA-MB-453 breast cancer cells. *Oncogene,* **12,** 1679–1687 (1996).

SKOLNIK, E.Y., LEE, C.H., BATZER, A., VICENTINI, L.M., ZHOU, M., DALY, R., MYERS, M.J., BACKER, J.M., ULRICH, A., WHITE, M.F. and SCHLESSINGER, J., The SH2/SH3 domain containing protein GRB2 interacts with tyrosine-phosphorylation IRS-1 and SHC: implications for control of ras signaling. *EMBO J.,* **12,** 1929–1936 (1993).

STEIN, D., WU, J., FUQUA, S.A., ROONPRAPUNT, C., YAJNIK, V., D'EUSTACHIO, P., MOSKOW, J.J., BUCHBERG, A.M., OSBORNE, C.K. and MARGOLIS, B., The SH2 domain protein GRB-7 is co-amplified, overexpressed and in a tight complex with HER2 in breast cancer. *EMBO J.,* **13,** 1331–1340 (1994).

SURMACZ, E. and BURGAUD, J.L., Overexpression of IRS-1 in the human breast cancer cell line MCF-7 induces loss of estrogen requirements for growth and transformation. *Clin. Cancer Res.,* **1,** 1429–1436 (1995).

TARTARE-DECKERT, S., SAWKA-VERHELLE, D., MURDACA, J. and VAN OBBERGHEN, E., Evidence for differential interaction of SHC and the IRS-1 with the IGF-IR in the yeast two-hybrid system. *J. biol. Chem.,* **270,** 23456–23460 (1995).

VAN DER BURG, B., RUTTEMAN, G.R., BLANKENSTEIN, M.A., DE LAAT, S.W. and VAN ZOELEN, E.J., Mitogenic stimulation of human breast cancer cells in growth factor-defined medium: synergistic action of insulin and estrogen. *J. Cell Physiol.,* **134,** 101–108 (1988).

WILSON, W.W., WAKELING, A.E., MORRIS, I.D., HICKMAN, J.A. and DIVE, C., MCF-7 human mammary adenocarcinoma cell death *in vitro* in response to hormone-withdrawal and DNA damage. *Int. J. Cancer,* **61,** 502–508 (1995).

XU, Y., GUO, D.F., DAVIDSON, M., INAGAMI, T. and CARPENTER, G., *In vitro* interaction of adapter protein SHC with cell adhesion molecule cadherin. *Mol. Biol. Cell.,* **7,** 342a (1996).

ROLE OF A SIGNALING PROTEIN SHC IN BREAST CANCER CELL ADHESION AND MOTILITY

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Running title: SHC regulates adhesion and motility

Key words: SHC, alpha 5 beta 1 integrin, motility, adhesion, breast cancer

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Summary

The oncogenic SHC proteins are signaling substrates for most receptor and cytoplasmic tyrosine kinases. SHC is known to transmit signals important for anchorage-dependent and -independent growth as well as modulate growth-unrelated processes such cell-substrate adhesion and motility. SHC is overexpressed in different tumor cells, including different breast cancer cell lines. The biological consequences of SHC amplification in breast cancer cells are not known. Here, we addressed this question using MCF-7-derived cell lines with ectopic SHC expression (MCF-7/SHC cells). The 3-8-fold overexpression of SHC produced a moderately increased responsiveness to IGF-I and EGF (20-70%) but did not significantly modulate MAP kinase activity in response to growth factors or the rate of proliferation in serum-containing medium. Similarly, high levels of SHC did not improve the ability of cells to grow under anchorage-independent conditions. SHC, however, was found involved in breast cancer cell adhesion and motlity.

In MCF-7/SHC cells, the amount of SHC associated with alpha 5 beta 1 integrin, a fibronectin (FN) receptor, was ~ 6-fold greater that in the parental cells or other 4 cell lines overexpressing the IGF-IR or its substrate IRS-1. When plated on FN, MCF-7/SHC cells attached faster than other tested cell lines (1 h vs. 2-3 h). This fast attachment was accompanied by earlier decline of adhesion-induced MAP kinase activity. The attachment of cells to FN was associated with decreased binding of p47 SHC to alpha 5 beta 1 integrin. Conversely, addition of EGF caused partlal detachment of cells from FN and stimulated cell motility,which was associated with increased binding of p47 to alpha 5 beta 1 integrin. The motility of MCF-7/SHC cells tested in FN-coated inserts was inhibited compared with that of the other cell lines. However, in the presence of EGF or IGF, the motility of SHC overexpressing cells was greatly (up to 300 %) increased, whereas it was only minimally altered in other cell lines (from -20 to + 40%). These data suggest that SHC is involved in dynamic regulation of cell adhesion and motility in breast cancer cells.

Figure Legends

Fig.1. MCF-7/SHC cells. A. SHC expression and tyrosine phosphorylation. The levels and tyrosine phosphorylation of SHC in two selected MCF-7/SHC clones 1 and 9 were determined by IP and WB with specific antibodies as detailed under Materials and Methods. **B. Growth response of MCF-7/SHC cells to EGF, IGF and serum.** The cells were synchronized in PRF-SFM and stimulated with mitogens for 4 days as described in Materials and Methods. The results are average of 4 experiments.

Fig. 2. SHC associates with alpha 5 beta 1 integrin. (a) The amounts of SHC associated with alpha 5 beta 1 in MCF-7/SHC cells, MCF-7 cells and several control clones without SHC overexpression (MCF-7/IRS-1, clones 3 and 18 and MCF-7/IGF-IR, clone 17) were determined by IP with an anti-alpha 5 beta 1 pAb and WB with an anti-SHC pAb. **(b)** The expression of alpha 5 beta 1 integrin in the tested cells was determined after stripping the blot (a) and reprobing it with the above anti-integrin pAb.

(Fig. 3, in preparation)
Fig. 3. Adhesion of MCF-7/SHC clones on FN and COL. MCF-7/SHC clones, MCF-7 cells and other cell lines with normal SHC levels (MCF-7/IRS-1, clone 3 and MCF-7/IGF-IR, clone 17) synchronized for 24h in PRF-SFM were plated in plates coated with either FN or COL IV. The cells were photographed at times 0 (floating cells), 1 h, and 2 h after plating. The role of alpha 5 beta 1 integrin in MCF-7/SHC adhesion was assessed by blocking the FN receptor with a specific antibody 30 min before cell plating, as described in Materials and Methods.

Fig. 4. Adhesion and EGF-dependent ERK 1 and ERK 2 activity in MCF-7/SHC cells. (a) MCF-7/SHC, clone 1 and MCF-7 cells were plated on plastic, COL IV or FN. The cells were lysed at the indicated times after plating. The tyrosine phosphorylated forms of ERK 1 and ERK 2 were determined as described under Materials and Methods.**(b)** MCF-7 cells and MCF-7/SHC, clone 1 were plated on plastic, COL IV or FN, allowed to attach for 1h , and then treated with 5 ng/ml EGF. The cells were lysed at different times (0-24h) of the treatment.

(Fig. 5, in preparation)
Fig. 5. Motility of MCF-7/SHC cells. (a) Basal motility in COL IV- or FN-coated inserts. The motility of MCF-7/SHC cells and several control cell lines without SHC overexpression was tested as described under Materials and Methods. The upper and lower chambers contained PRF-SFM. The percentage of cells that migrated to the undersite of inserts (ralative to the number of cells plated) is designated as % Motility. **(b)** Growth factor-induced motility. The were suspended in PRF-SFM and plated cells plated in FN or COL IV coated inserts. The lower chamber contained PRF-SFM with 1 or 5 ng/ml EGF or 20 ng/ml IGF-I.

(Fig. 6, in preparation)
Fig. 6. SHC/alpha 5 beta 1 association is modulated during attachement to FN. (a) The association of SHC with alpha 5 beta 1 integrin was determined by IP with an anti-alpha 5 beta 1 pAb and by WB with an anti-SHC pAb in 1 mg of protein lysate of MCF-7/SHC, clone 1. The cells were either floating, attached to FN, or treated with 5 ng/ml EGF. **(b)** Tyrosine phosphorylation of SHC was detected in the same blot (a) after stripping and reprobing with an anti-phosphotyrosine antibody PY20.

FIGURE 1A

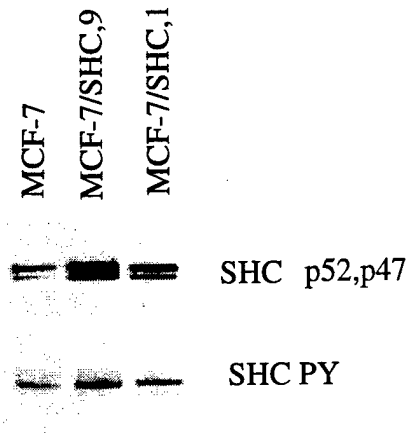


FIGURE 1B

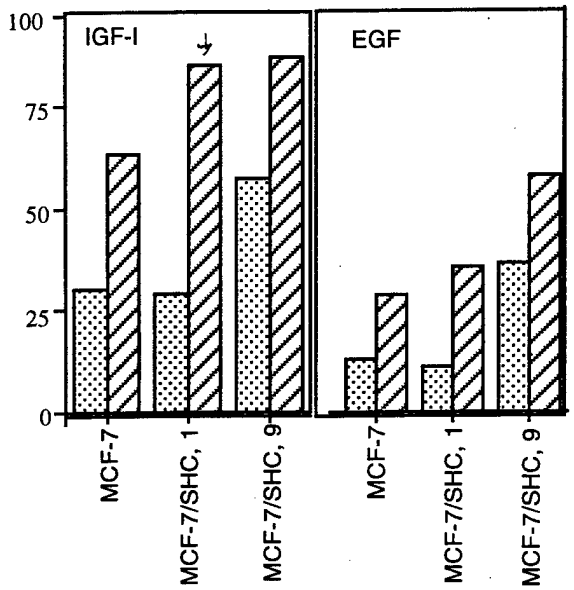
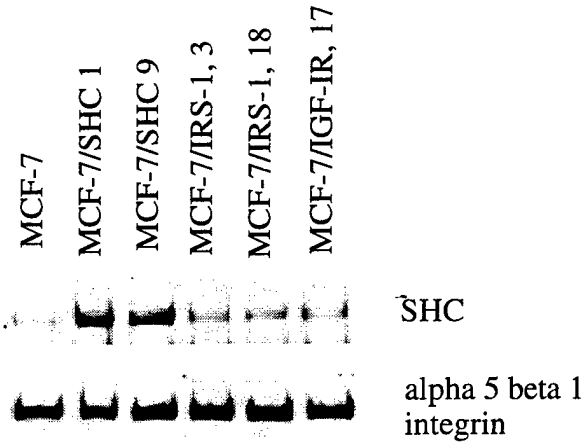


FIGURE 2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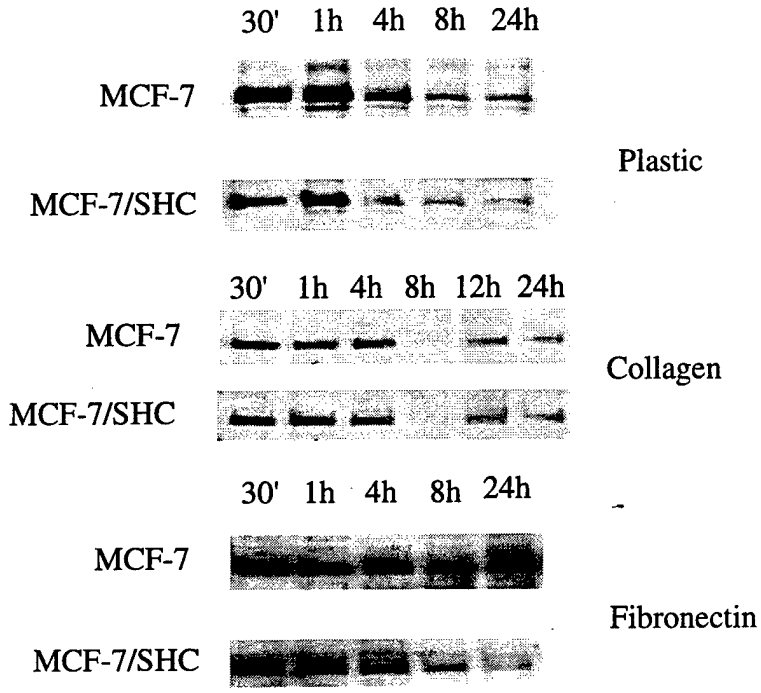
SHC
alpha 5 beta 1 integrin

FIGURE 4A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Plastic
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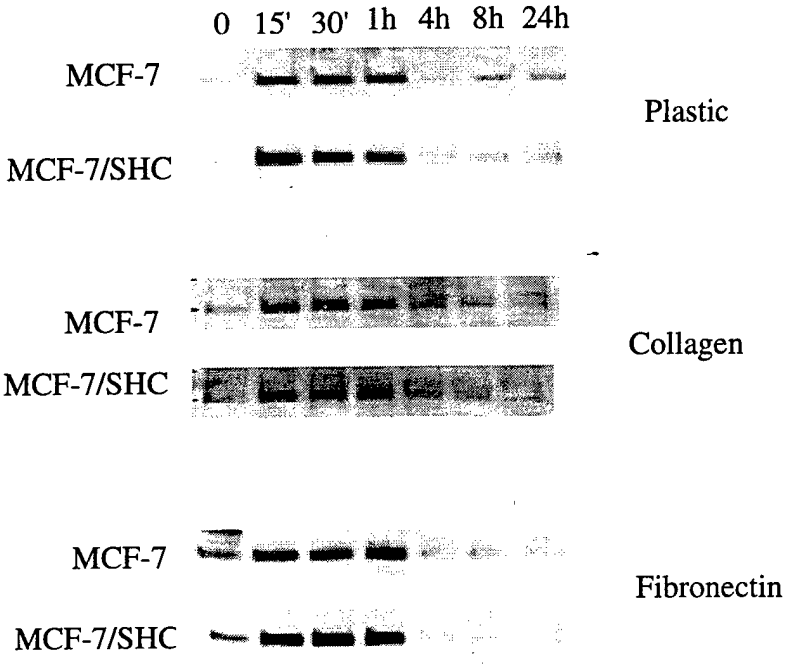
Collagen
30' 1h 4h 8h 24h
MCF-7
MCF-7/SHC
Fibronectin

FIGURE 4B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Collagen
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Fibronectin