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13. ABSTRACT ***(Maximum 200 Words)***

The insulin-like growth factor I receptor (IGF-IR) is a multifunctional tyrosine kinase that has been implicated in breast cancer. Current evidence suggests an important role of the IGF-IR in the growth and survival of ER-positive primary breast tumors. However, the function of the IGF-IR in breast cancer metastasis is unknown. Previously we have shown that IGF-I modulates cell-cell and cell-substrate adhesion in breast cancer cells. Consequently, we proposed to investigate the molecular mechanism of IGF-IR-stimulated cell adhesion, and the role of IGF-IR overexpression in breast metastasis.

We demonstrated that the IGF-IR promotes E-cadherin-dependent cell-cell adhesion and interacts with the elements of E-cadherin adhesion complex. IGF-IR overexpression or stimulation of cells with IGF-I improved the expression of one of the E-cadherin-associated proteins, ZO-1. This was paralleled by augmented binding of the IGF-IR to ZO-1 and to ZO-1-associated-alpha-catenin and actin.

In parallel, we pursued IGF-IR function in E-cadherin negative metastatic breast cancer cells. We demonstrated that in this cellular context, IGF-I has no growth or adhesion function, but it is necessary to stimulate cell motility. The IGF-I-induced motility was mediated through PI-3 and p38 kinases, and was inhibited by the activation of ERK1/ERK2 kinases.

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INTRODUCTION

The insulin-like growth factor I (IGF-I) receptor (IGF-IR) is a ubiquitous multifuctional tyrosine kinase. The IGF-IR regulates normal breast development; however, hyperactivation of the same receptor has been implicated in breast cancer (1). In particular, overexpression of either the IGF-IR or its major signaling substrate IRS-1 in estrogen receptor (ER)-positive breast tumors has been linked with cancer recurrence at the primary site. Furthermore, high circulating levels of IGF-I (an IGF-IR ligand) have been associated with increased breast cancer risk in premenopausal women (1).

Although current evidence suggests that abnormal activation of the IGF-IR may contribute to the autonomous growth and increased survival of primary ER-positive breast tumors, the function of this receptor in breast cancer metastasis is not clear. For instance, some small clinical studies demonstrated a correlation between IGF-IR expression in node-positive tumors and worse prognosis. Other data linked IGF-IR expression with better clinical outcome, as the IGF-IR was predominantly expressed in a subset of breast tumors with good prognostic characteristics (1). In the experimental setting, anti-IGF-IR strategies were successfully applied to inhibit the growth and spread of human breast cancer xenografts, which implicated the role of the IGF-IR in metastasis (1). Thus, understanding whether hyperactivation of the IGF-IR is a factor promoting breast cancer metastasis is be of great importance.

Our previous data demonstrated that overexpressed IGF-IRs not only promote breast cancer cells growth, but also activate aggregation and prolong survival of detached cells (2). This process required E-cadherin (E-cad)—a major cell-cell adhesion protein expressed by epithelial cells. The molecular mechanism of IGF-I-induced E-cad-dependent adhesion is unknown and its significance in the process of metastasis is equally obscure. Consequently, our first goal was to study the effects of IGF-I on the expression of E-cad and E-cad-associated proteins, and to determine the interactions of the IGF-IR with these adhesion proteins.

In parallel, we begun to study the function of the IGF-IR in metastatic breast cancer cells that have lost E-cad expression.

TECHNICAL REPORT

During this reporting performance period, the experiments proceeded according to the SOW.

Project I: IGF-IR and E-cad complex.

We confirmed our previous observation that MCF-7 breast cancer cells overexpressing the IGF-IR exhibit increased aggregation in 3-dimensional (3-D) culture. The cells were tested in Matrigel (extracellular matrix) culture (as published in Ref. 2) or in suspension culture in agar-coated plates *(Fig. 1, Appendix)*. The optimized suspension culture conditions allowed for accumulation of large number of 3-D spheroids necessary for biochemical studies.

Our previous studies implicated that IGF-IR-induced adhesion requires a functional E-cad complex. Consequently, we tested how overexpression of the IGF-IR in MCF-7 cells can modulate the levels of E-cad and associated adhesion proteins. We found that the levels of E-

cad, alpha-, and beta-catenin were not affected by IGF-IR overexpression. However, the levels of ZO-1, a scaffolding protein linking E-cadherin through alpha-catenin to the actin cytoskeleton (3, 4), were significantly increased in cells overexpressing the IGF-IR (MCF-7/IGF-IR clones 17, 15, and 12) *(Fig. 2 and 3, Appendix)*. Subsequent analysis demonstrated that ZO-1 mRNA was overexpressed in MCF-7/IGF-IR cells relative to MCF-7 cells *(Fig. 4, Appendix)*. The analysis of interactions of the IGF-IR with adhesion proteins revealed that the IGF-IR co-precipitates with E-cadherin and ZO-1 *(Fig. 5, Appendix)*. The amounts of the IGF-IR found in E-cad and ZO-1 immunoprecipitates were greater in IGF-IR overexpressing cells *(Fig. 5, Appendix)*. IGF-IR and ZO-1 also associated with alpha-catenin, with greater amounts of these complexes found in MCF-7/IGF-IR cells *(Fig. 5, Appendix)*. In addition, we noted greater binding of ZO-1 to actin in MCF-7/IGF-IR cells *(Fig. 6, Appendix)*. This all suggested that one of the mechanisms by which the IGF-IR stimulates adhesion relies on the overexpression of ZO-1. Future experiments confirming the importance of ZO-1 in IGF-I-induced adhesion will involve the use of anti-ZO-1 antisense RNA. This reagent is now being generated in the laboratory.

Project II: IGF-IR function in E-cadherin-negative cells.

Using MDA-MB-231 cells, a well-described metastatic cell line lacking E-cad expression, we generated several clones expressing different levels of the IGF-IR. The clones were obtained by stable calcium phosphate transfection with a pcDNA3/IGF-IR expression vector (encoding neomycin-G418 resistance), as described before (3). Forty-five G418 resistant clones were analyzed by FACS (fluorescence-assisted cell sorting) with an anti-IGF-IR alpha-subunit antibody to select the clones with different IGF-IR expression levels. Based on this analysis, we selected MDA-MB-231/IGF-IR clones 2, 21, 31 expressing ~$1.4x10^4$, $3x10^4$, $2.5x10^5$ receptors/cell. To confirm differential IGF-IR expression, we analyzed the clones by Western blotting with a specific anti-IGF-IR beta subunit antibody *(Fig. 1, Bartucci et al., Appendix)*.

Cell-cell adhesion of MDA-MB-231/IGF-IR cells was similar to that of the parental MDA-MB-231 cells, confirming that E-cad is required to mediate the adhesion function of the IGF-IR .

Since IGF-I is an important growth and antiapoptotic factor for breast cancer cells, we next characterized growth and survival properties of MDA-MB-231/IGF-IR clones. Interestingly, we found that IGF-I was not a mitogen or a survival factor for these ER-negative metastatic cells, whereas the same factor exerted growth-promoting and anti-apoptotic action in ER-positive MCF-7 cells *(Tab. 1 and Fig. 2 and 3, Bartucci et al., Appendix)*. Notably, the lack of growth-related properties of IGF-I in MDA-MB-231 cells was evident in all clones derived from this cell line, regardless of IGF-IR expression levels.

To investigate why the IGF-IR was not inducing mitogenic signals in MDA-MB-231 cells, we studied IGF-IR activation and intracellular signaling. We focused on two IGF-IR pathways known to control growth and survival of ER-positive breast cancer cells, namely IGF-IR/IRS-1/PI-3K/Akt and MAPK pathways (1). We found that in MDA-MB-231 cells, the IGF-IR/IRS-1 signal was normal, however the cells were not able to sustain the downstream PI-3K/Akt activation. We hypothesized that this feature may contribute to the lack of mitogenic/survival response to IGF in metastatic cells *(Tab. 1 and Figs 4 and 5, Bartucci et al., Appendix)*. Indeed, upon re-activation of the Akt pathway by transfection of a constitutively

active Akt mutant, we noticed improved survival of MDA-MB-231 cells *(Fig.6, Bartucci at al., Appendix).*

Considering that the IGF-IR was not mitogenic in ER-negative cells, we asked whether this receptor can transmit other signals critical for tumor progression. We focused on growth-unrelated functions such as adhesion and migration. We found that unlike with growth and survival, the IGF-IR was able to induce migration in MDA-MB-231 cells and MDA-MB-231/IGF-IR clones. The induction of migration was proportional to the levels of the IGF-IR *(Tab.3, Bartucci et al., Appendix).* Subsequent experiments suggested that IGF-I-induced migration was transmitted through p38 kinase and PI-3 kinase pathways and inhibited by ERK1/2 MAP kinases *(Tab.4 , Bartucci et al., Appendix).*

Key Research Accomplishments:

- Demonstrated that the activation of the IGF-IR induces cell-cell adhesion, which coincides with overexpression of ZO-1, one of the adhesion proteins;
- Determined that the IGF-IR interacts with several proteins of the E-cadherin cell-cell adhesion complex;
- Demonstrated that higher levels of ZO-1 are associated with improved ZO-1/alpha-catenin/actin binding;
- Demonstrated that activation of the IGF-IR in E-cadherin-negative ZO-1-negative MDA-MB-231 cells does not induce cell-cell adhesion, but it regulates cell migration.
- Determined that IGF-I-dependent migration in both cell types is mediated through p38 kinase and PI-3K pathways, and is inhibited by ERK1/ERK2 pathways.

Reportable Outcomes:

1. Manuscripts, abstracts and scientific presentations:

Manuscripts:

1. Bartucci, M., Morelli, C., Mauro, L., Ando', S., Surmacz, E. IGF-I receptor signaling and function are different in non-metastatic and metastatic breast cancer cells. 2000. Submitted.
2. Surmacz, E. Function of the IGF-IR in breast cancer. J. Mammary Gland Biol. Neopl., 5: 95-105, 2000.

Abstracts (Posters or Oral Presentations):

1. Morelli, C., Bartucci, M., Mauro, L., Ando' S., Surmacz, E. Insulin-like growth factor I receptor (IGF-IR) signaling in metastatic breast cancer cells. The Endocrine Society Annual Meeting, Toronto, Canada, June 21-24, 2000.
2. Bartucci, M., Mauro, L., Salerno, M., Morelli, C., Ando', Surmacz, E. Function of the insulin-like growth factor I receptor in metastatic breast cancer cells. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Atlanta, June 8-11, 2000.
3. Bartucci, M., Mauro, L., Salerno, M., Morelli, C., Ando', S., Surmacz, E. Function of the insulin-like growth factor I receptor in metastatic breast cancer cells. 22nd Annual Breast Cancer Symposium. San Antonio, TX, December 8-11, 1999

4. Surmacz, E, Mauro, L., Ando', S. New insights into IGF-I-dependent regulation of cell-cell adhesion in breast cancer cells. 5th International Symposium on IGFs. Brighton, U.K., October 31-November 4, 1999.

Invited Talks:

1. Surmacz, E. Differential IGF-IR function in ER-positive and ER-negative breast cancer cells. Astra Zeneca, Macclesfield, U.K., September 18, 2000.
2. Surmacz, E. Evolution of IGF-IR signaling during breast cancer progression. "IGFs and Cancer" International Symposium, Halle, Germany, September 14-17, 2000.
3. Surmacz E. Is IGF-IR involved in breast cancer? Lankenau Cancer Research Center, Wynnewood, PA, February 3, 2000.
4. Surmacz E. Diabetes, insulin treatment and breast cancer. Novo Nordisk Pharmaceuticals. Princeton, NJ., January 25, 2000.
5. Surmacz, E. IGF-IR and breast cancer. Department of Cellular Biology, University of Calabria, Cosenza, Italy, December 9, 1999.
6. Surmacz, E. IGF-IR signaling in breast cancer. University of Gent, Gent, Belgium, September 29, 1999.

2. Patents and licenses: None

3. Degrees: N/A

4. Development of biologic reagents:

- metastatic breast cancer cell lines overexpressing IGF-IR: MDA-MB-231/IGF-IR cells

5. Databases: None

6. Funding applied for:

1. Evolution of IGF-IR signaling in breast cancer progression. IDEA Award DOD, pending
2. Evolution of IGF-IR signaling during breast cancer progression, PA Dept. of Health, pending

7. Employment applied for: None

Conclusions

The IGF-IR regulates cell-cell adhesion in ER-positive cells. The mechanism of this phenomenon may involve overexpression of one of the adhesion proteins—ZO-1, and improved ZO-1/alpha-catenin/actin binding.

The IGF-IR has different functions in E-cad-negative cells, where it does not induce cell-cell adhesion, but stimulates cell migration.

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1. Surmacz, E. Function of the IGF-IR in breast cancer. J. Mammary Gland Biol. Neopl., *5*: 95-105, 2000.

2. Guvakova, M. A., and 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.

3. Rajasekaran, A.K., Hojo, M., Huima, T., Rodriguez-Boulan, E. Catenins and zonula occludens-1 form a complex during early stages in the assembly of tight junctions. J. Cell Biol. 132: 451-463, 1996.

4. Itoh, M., Nagafuchi, A., Moroi, S., Tsukita, S. Involvement of ZO-1 in cadherin-based cell adhesion through its direct binding to alpha catenin and actin filaments. J. Cell Biol. 138: 181-192, 1997.

Figure Legends

Fig. 1. Overexpression of IGF-IR increases intercellular adhesion and survival of MCF-7 cells in 3-D culture.
MCF-7 and MCF-7/IGF-IR cells were plated in 2% agarose-coated plates (to prevent cell adhesion and spreading) as a singe cell suspension at 20,000 cells/well in DMEM+5% calf serum and rotated for 4 hr in the incubator. During this time, the cells formed spheroids. MCF-7 cell aggregated in small clusters (~50 um in diameter) which survived for ~10 days and then disaggregated and died. MCF-7/IGF-IR cells formed large spheroids (~200-300 um in diameter) surviving for up to 20 days. The cells were photographed at days 5 and 12 after plating [8].

Fig. 2. Abundance of different adhesion proteins in MCF-7 and MCF-7/IGF-IR cells.
The expression of E-cad, alpha-, beta-, and ZO-1 was assessed in 50 ug of whole cell lysate obtained from 3-D cultures of MCF-7 and MCF-7/IGF-IR cells. The expression of adhesion proteins was detected by WB with specific antibodies: anti-E-cad pAb (Santa Cruz), anti-alpha-catenin pAb (Sigma), anti-beta pAb (Sigma), anti-gamma pAb (Sigma), anti-ZO-1 pAb (Zymed).

Fig. 3. Abundance of ZO-1 in MCF-7-derived cell lines overexpressing IGF-IR or IRS-1.
ZO-1 was immunoprecipitated with anti-ZO-1 pAb (Zymed) from 500 ug of Triton-soluble cell lysate, and detected with the same Ab by WB. Compared with MCF-7 cells , ZO-1 was significantly overexpressed in MCF-7/IGF-IGF-IR, clones 17, 15, and 12 and slightly elevated in MCF-7/IRS-1 clones 3 and 18.

Fig. 4. Regulation of ZO-1 expression in by IGF-I. ZO-1 mRNA expression.
A 70% confluent culture of MCF-7 and MCF-7/IGF-IRmix cells was synchronized in PRF-SFM for 24h and then treated with 50 ng/ml IGF-I for 2, 4, 8, 24, and 72h ZO-1 mRNA was detected by Northern blotting using a 959 bp ZO-1 cDNA fragment (obtained by BamH-1 digestion) labeled with 32-P. 25ug of total RNA was loaded in each lane. Ribosomal RNA is shown as a control of loading.

Fig. 5. Association between IGF-IR and adhesion proteins.
500 ug of total cell lysate form 3-D cultures of MCF-7 or MCF-7/IGF-IR cells were immunoprecipitated with either anti-E-cad mAb (Zymed), anti-IGF-IR mAb (Calbiochem), or anti-ZO-1 pAb (Zymed). IGF-IR and alpha-catenin were detected by WB in the same filter after stripping and reprobing with specific Abs (described under Fig. 2.

Fig. 6. ZO-1/actin interaction in MCF-7 and MCF-7/IGF-IR cells.
ZO-1 was immunoprecipitated from 500 ug of total cell lysates obtained from 3-D cultures and then detected by WB, as described under Fig. 2. Actin complexed with ZO-1 was probed by WB (in the same filter) with anti-actin mAb (Sigma).

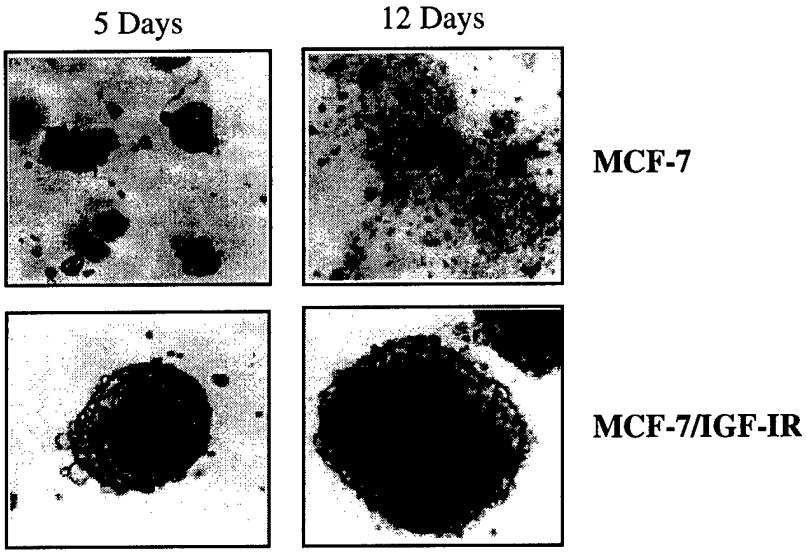
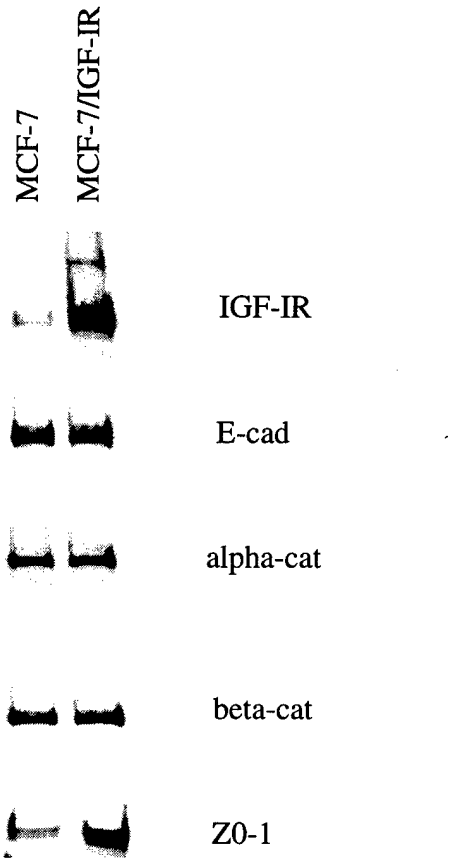


FIGURE 1



Expression of adhesion proteins in spheroids (whole lysates)

FIGURE 2


MCF-7
MCF-7/IGF-IR,17
MCF-7/IGF-IR,15
MCF-7/IGF-IR,12
MCF-7/IRS-1,18
MCF-7/IRS-1,3
ZO-1

FIGURE 3

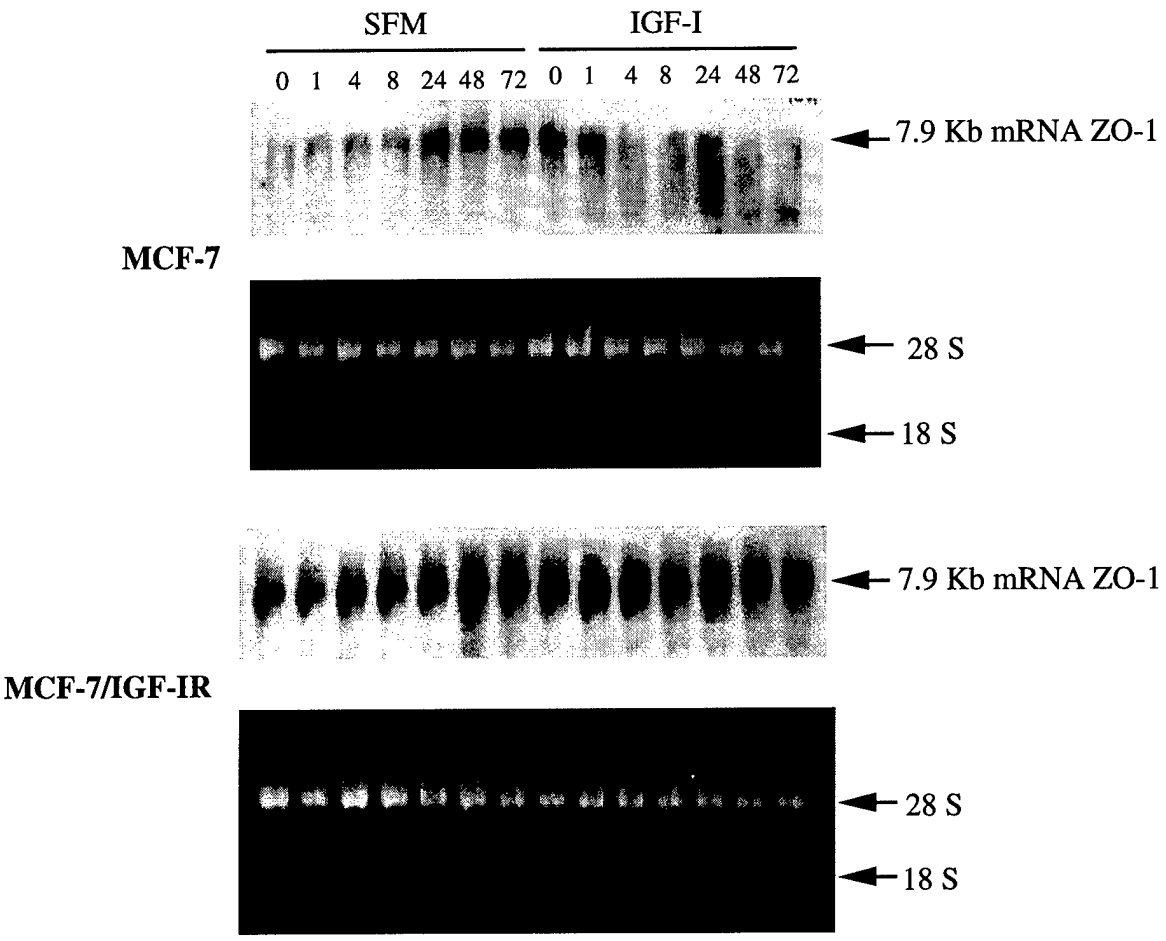

SFM
IGF-I
0 1 4 8 24 48 72 0 1 4 8 24 48 72
7.9 Kb mRNA ZO-1
MCF-7
28 S
18 S
7.9 Kb mRNA ZO-1
MCF-7/IGF-IR
28 S
18 S

FIGURE 4

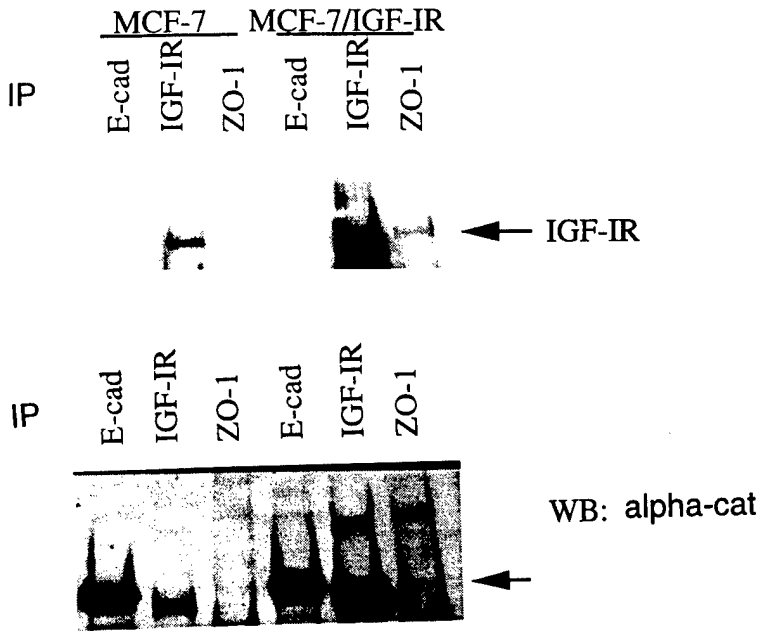

MCF-7
MCF-7/IGF-IR
IP
E-cad
IGF-IR
ZO-1
E-cad
IGF-IR
ZO-1
IGF-IR
IP
E-cad
IGF-IR
ZO-1
E-cad
IGF-IR
ZO-1
WB: alpha-cat

FIGURE 5

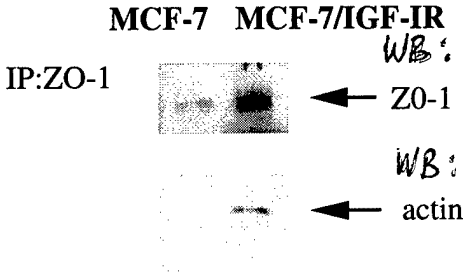


FIGURE 6

Insulin-like Growth Factor I Receptor (IGF-IR) Signaling in Metastatic Breast Cancer Cells. Catia Morelli, Monica Bartucci, Loredana Mauro, Sebastiano Ando, Eva Surmacz, [1]*Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA;* [2]*University of Calabria, Cosenza, Italy*

High levels of IGF-IR in primary breast tumors correlate with tumor recurrence and radio-resistance. However, the role of IGF-IR in metastatic breast cancer is not clear. Highly aggressive, ER-negative breast cancer cell lines express low levels of IGF-IR and do not respond to IGF-I with mitogenesis. Despite this, inhibition of IGF-IR in invasive breast cancer cells reduced their metastatic potential suggesting that IGF-IR may be required in advanced stages of the disease. In breast tumors, decreased IGF-IR expression has been linked with bad prognosis.

To examine the function of IGF-IR in metastatic breast cancer, we expressed IGF-IR in invasive MDA-MB-321 cells which normally contain low levels of IGF-IR (~5,000 receptors/cell) and do not grow in IGF-I. Increasing IGF-IR content to ~ 50,000 or ~200,000 receptors/cell improved cell motility towards IGF-I (50 ng/ml) by 20 and 63%, respectively, and significantly enhanced anchorage-independent growth in serum-containing medium. However, high expression of IGF-IR did not enhance monolayer growth or decrease apoptosis (determined by TUNEL) in serum-free medium (SFM) supplemented with 1-100 ng/ml IGF-I. To explain the latter phenomenon, we examined IGF-I-induced signal in MDA-MB-231 and MDA-MB-231/IGF-IR cells. At 15 min after IGF-I stimulation, the IGF-IR and its major substrate IRS-1 were tyrosine-phosphorylated, and downstream effectors: PI-3, Akt, GSK-3 and MAP kinases were activated in a manner reflecting the IGF-IR content. In contrast, at 2 days in SFM with 50 ng/ml IGF-I, IGF-IR and IRS-1 were still active but the stimulation of MAP and Akt kinases substantially declined. Under the same conditions, control MCF-7 cells and MCF-7 clones expressing equivalent levels of the IGF-IR proliferated, and all IGF-I pathways studied were induced. Importantly, an increase of the cellular activity of Akt (through transient expression of constitutively active mutants Myr-Akt or Akt-E40K) did not restore the growth or survival of MDA-MD-231 cells.

We conclude that in metastatic MDA-MB-231 breast cancer cells, IGF-IR controls migration and affects anchorage-independent growth, but does not promote proliferation. This lack of mitogenic response may be caused by a block in IGF-IR signaling that affects MAP and Akt kinases, and occurs downstream of IRS-1.

463 FUNCTION OF THE INSULIN-LIKE GROWTH FACTOR RECEPTOR I IN METASTATIC BREAST CANCER CELLS Bartucci, M., Mauro, L., Salerno, M., Morelli, C., Ando, S., Surmacz, E.* Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA and Department of Cellular Biology, University of Calabria, Cosenza, Italy

The type I insulin-like growth factor receptor (IGF-IR) is a multifunctional tyrosine kinase regulating processes such as proliferation, survival, transformation, as well as cell-cell and cell-substrate interactions. In primary breast cancer, the IGF-IR is overexpressed and hyperphosphorylated, and high levels of the IGF-IR correlate with tumor radio-resistance and recurrence, and predict shorter disease-free survival. The role of the IGF-IR in metastatic breast cancer is not clear. Highly aggressive, metastatic breast cancer cell lines express low levels of the IGF-IR and often do not respond to IGF-IR with mitogenesis. In agreement with this, clinical studies linked low IGF-IR expression with worse prognosis. On the other hand, inhibition of the IGF-IR in invasive cells reduced their metastatic potential.

To examine the function of the IGF-IR in metastatic breast cancer, we expressed the IGF-IR in invasive MDA-MB-321 cells. These cells contain low levels of the IGF-IR (~5,000 molecules/cell) and do not grow in IGF-I. We found that increasing IGF-IR content to ~50,000 and ~100,000 receptors/cell improved cell motility towards IGF-I (50 ng/ml) by 30 and 41%, respectively, and significantly enhanced anchorage-independent growth in serum-containing medium (more than 10-fold in respect to the parental cells). However, high expression of the IGF-IR did not alter monolayer growth and survival in serum-free medium (SFM) supplemented with 1-100 ng/ml IGF-I. To address the latter phenomenon, we examined IGF-I-induced signal in MDA-MB-231 and MBA-MD-231/IGF-IR cells. At 15 min after IGF-I stimulation the IGF-IR and its major substrate IRS-1 were tyrosine-phosphorylated, and downstream effectors PI-3, Akt and MAP kinases were activated in a manner reflecting the IGF-IR content. In contrast, at 2 days in SFM with 50 ng/ml IGF-I, the IGF-IR and IRS-1 were still active but the stimulation of MAP and Akt kinases dramatically declined. Under the same conditions, control MCF-7 cells and MCF-7 clones expressing equivalent levels of the IGF-IR proliferated and all IGF-I pathways were induced.

We conclude that in metastatic MDA-MB-231 breast cancer cells, the IGF-IR controls migration and anchorage-independent growth, but does not promote proliferation. This lack of mitogenic response may be caused by a block in IGF-IR signaling that affects MAP and Akt kinases, and occurs downstream of IRS-1.

FUNCTION OF THE INSULIN-LIKE GROWTH FACTOR RECEPTOR I IN METASTATIC BREAST CANCER CELLS

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The type I insulin-like growth factor receptor (IGF-IR) is a multifunctional tyrosine kinase regulating processes such as proliferation, survival, transformation, as well as cell-cell and cell-substrate interactions.

In primary ER-positive breast cancer, IGF-IR is often overexpressed and hyperphosphorylated, and high levels of IGF-IR correlate with tumor radio-resistance and recurrence, and predict shorter disease-free survival.

The role of IGF-IR in metastatic breast cancer is not clear. Highly aggressive, ER-negative breast cancer cell lines express low levels of IGF-IR and often do not respond to IGF-I with mitogenesis. In agreement with these findings, clinical studies linked low IGF-IR expression with worse prognosis and high IGF-IR expression with better outcome. Interestingly, however, inhibition of IGF-IR in invasive breast cancer cells reduced their metastatic potential suggesting that IGF-IR may also control advanced stages of the disease.

To examine the function of IGF-IR in metastatic breast cancer, we expressed IGF-IR in invasive MDA-MB-321 cells. These cells contain low levels of IGF-IR (~5,000 receptors/cell) and do not grow in IGF-I. We found that increasing IGF-IR content to ~ 50,000 or ~200,000 receptors/cell improved cell motility towards IGF-I (50 ng/ml) by 20 and 63%, respectively, and significantly enhanced anchorage-independent growth in serum-containing medium. However, high expression of IGF-IR did not enhance monolayer growth and survival in serum-free medium (SFM) supplemented with 1-100 ng/ml IGF-I. To address the latter phenomenon, we examined IGF-I-induced signal in MDA-MB-231 and MDA-MB-231/IGF-IR cells. At 15 min after IGF-I stimulation, the IGF-IR and its major substrate IRS-1 were tyrosine-phosphorylated, and downstream effectors: PI-3, Akt, GSK-3 and MAP kinases were activated in a manner reflecting the IGF-IR content. In contrast, at 2 days in SFM with 50 ng/ml IGF-I, IGF-IR and IRS-1 were still active but the stimulation of MAP and Akt kinases substantially declined. Under the same conditions, control MCF-7 cells and MCF-7 clones expressing equivalent levels of the IGF-IR proliferated and all IGF-I pathways were induced

We conclude that in metastatic MDA-MB-231 breast cancer cells, IGF-IR controls migration and anchorage-independent growth, but does not promote proliferation. This lack of mitogenic response may be caused by a block in IGF-IR signaling that affects MAP and Akt kinases, and occurs downstream of IRS-1.

The U.S. Army Medical Research and Materiel Command under DAMD 17-97-1-7211 supported this work.

INS VNTR class I allele size with respect to BMI in 2331 men, using a new development of our microplate array diagonal gel electrophoresis (MADGE) system. A strong association was found; each extra tandem repeat (14 bp) is associated with a 0.33% (95% CI = 0.13%, 0.50%) increase in BMI ($P < 0.0001$). The association is evident in each of the three *Igf2 ApaI* genotype groups, but there appears to be no single haplotype with distinctive weight effect. However, we have now genotyped the tyrosine hydroxylase (TH) HUMTH01 tetranucleotide repeat polymorphism using MADGE and it is apparent that a haplotype containing HUMTH01 allele 9, which is in strong linkage disequilibrium with the INS VNTR class I small subclass alleles, and with which *Igf2 ApaI* alleles are preferentially associated, may be important.

At least 1.1% of total population BMI variance in this sample is accounted for by variation at the INS VNTR and *Igf2 ApaI* sites. Given a mean estimate of the genetic contribution to weight variance of 50%, identification and mechanistic understanding of 50 or so such genomic regions could enable the future recognition of subpopulations with differential functional susceptibilities to obesity.

OC16.1 NEW INSIGHTS INTO IGF-IR-DEPENDENT REGULATION OF CELL–CELL ADHESION IN BREAST CANCER

E Surmacz[1], L Mauro[1,2], S Ando[2]. [1]Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, USA; [2]Department of Cellular Biology, University of Calabria, Cosenza, Italy

The IGF-I receptor (IGF-IR) is overexpressed and hyperphosphorylated in a large fraction of primary breast tumors. The enhanced activation of IGF-IR is regarded as a factor contributing to breast cancer growth, survival, radio- and drug-resistance.

We have previously found that IGF-I treatment or overexpression of IGF-IR significantly stimulates cell–cell adhesion and in consequence improves survival of breast cancer cells grown in 3-dimensional (3-D) culture. Here we pursued the mechanism underlying this phenomenon focusing on the major epithelial adherens complex organized around a transmembrane protein E-cadherin. The extracellular portion of E-cadherin serves as a link between two cells, while its intracellular tail connects cell membrane with actin cytoskeleton through several intermediates, i.e. different catenins (α-, β-, and γ), and ZO-1.

MCF-7 human breast cancer cells and MCF-7 cells overexpressing IGF-IR (MCF-7/IGF-IR) were grown as 3-D spheroids and the expression of adherens junction proteins as well as co-associations between these proteins and IGF-IR were determined by Western blotting. IGF-IR-dependent upregulation of cell–cell adhesion correlated with significantly augmented expression of ZO-1 (mRNA and protein), but did not affect the abundance of other elements of the complex. IGF-IR immunoprecipitated with E-cadherin, α-, and β-catenin, and ZO-1 in both studied cell lines. Overexpression of ZO-1 in MCF-7/IGF-IR cells was paralleled by the enhanced ZO-1/α-catenin, IGF-IR/α-catenin, and α-catenin/F-actin binding. Since ZO-1 links E-cadherin through α-catenin to the actin cytoskeleton, we speculate that high levels of ZO-1 and enhanced ZO-1/α-catenin/actin connection may strengthen intercellular adhesion. Indeed, we found by pulse-chase labeling that the stability of the adhesion complex (precipitated by α-catenin antibody) was increased in MCF-7/IGF-IR cells relative to that in MCF-7 cells.

In conclusion, the mechanism by which IGF-IR regulates cell–cell adhesion may be associated with upregulation of ZO-1 expression and subsequent strengthening of α-catenin-F actin connections.

OC16.2 REGULATION OF THE CATENIN-CADHERIN COMPLEX BY INSULIN-LIKE GROWTH FACTOR-I (IGF-I)

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We are studying the link between IGF signalling and cadherin/catenin function in human cancer. In colorectal cancer, approximately 80% of tumours exhibit APC mutations, whereas 10% show β-catenin mutations. The two are mutually exclusive. c10 is a recently derived human colorectal cancer cell line which we have found to express the highest levels of cell surface receptor expression (approximately 30 000 IGF-1 receptors/cell), the highest amongst a panel of 20 tested and at least twice that of all others examined. c10 cells appeared to wild-type for APC, p53, β-catenin and E-cadherin. These IGF1R were functional as shown by a mitogenic response to exogenous IGF-I, and growth inhibition in the presence of the blocking anti-IGF1R antibody αIR3. In c10 cells we could detect no direct interaction between β catenin and IGF1R or its principal substrate IRS-1. However, upon IGF-I stimulation we observed a significant increase in tyrosine phosphorylation of two proteins of approximately 90 and 170 kDa which co-immunoprecipitated with E-cadherin. These proteins were identified by Western blotting as β-catenin and IRS-1. Furthermore, IGF-I-induced tyrosine phosphorylation of β-catenin appeared to reduce the amount of β-catenin coprecipitating with E-cadherin. Western blot analysis suggested that this may be associated with an inhibition of degradation of β-catenin. We have performed *in vitro* kinase assays showing that IGF-I stimulation leads to phosphorylation of Glycogen Synthase Kinase (GSK3) N-terminal serine residues. This process is thought to be responsible for inhibition of GSK3 activity, resulting in the prevention of β-catenin degradation. We have also overexpressed the IGF1R in the immortalized breast epithelial cell line HBL100. High IGF1R expressing clones show an increase in the detergent insoluble levels of β-catenin, suggesting possible nuclear translocation. We plan to follow this up by assessing the effects of IGF1R activation on β-catenin localization and half-life. These results suggest that IGF-I stimulation can modulate the interaction between β-catenin and E-cadherin, and may therefore influence cell–cell interactions and β-catenin function in colorectal cancer cells.

OC16.3 THE ROLE OF THE B3 SUBUNIT OF THE α V β 3 INTEGRIN RECEPTOR IN IGF-I SIGNALING

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Our previous studies have shown that ligand occupancy of the α v β 3 integrin receptor is necessary for porcine smooth muscle cells (pSMCs) to respond to IGF-I. The aim of this study was to investigate the role of the β3 cytoplasmic domain of the α v β 3 integrin receptor in IGF-I signaling. Two mutant forms of the β 3 receptor, termination at amino acid residue 715, resulting in deletion of the cytoplasmic domain (WK) and termination at amino acid residue 731, resulting in loss of two potential sites of tyrosine phosphorylation (E), were stably expressed in pSMCs.

Basal migration of cells expressing either form of the truncated receptor on vitronectin (Vn) coated plates (α v β 3 ligand) was reduced (WK = 9.5 ± 0.5 cells/mm^2 and E = 8 ± 1.0 cells /mm^2) when compared with cells expressing the full length receptor (WT) (15 ± 0.3 cells/mm^2). Following incubation with IGF-I (100 ng/ml) there was a >100% increase in migration of WT cells (35 ± 4.8 cells/mm^2), however, cells expressing either of the two truncated receptors showed no increase in migration in response to IGF-I. In cells expressing either of the two truncated receptors the addition of 10^{-9} M echistatin (α v β 3 antagonist) resulted in a significant reduction in the number of cells remaining attached to Vn coated plates (WK = 54%; E = 48%). This concentration of echistatin had no significant effect on attachment of WT cells. However, when the cells expressing the truncated receptors were incubated with echistatin in the presence of IGF-I (100 ng/ml) the reduction in cells remaining attached was markedly attenuated (WK = 98%; E = 80%). The presence of a cell-permeable peptide homologous to the carboxyl-terminal region of the β3 receptor (amino acids 747–762) reduced IGF-I stimulated migration of non-transfected pSMCs by 36 ± 5% and reduced IGF-I stimulated receptor phosphorylation by 20 ± 5%. In conclusion, these studies suggest that the carboxyl-terminal

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Function of the IGF-I Receptor in Breast Cancer

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The insulin-like growth factor-I receptor (IGF-IR)[3] is a transmembrane tyrosine kinase regulating various biological processes such as proliferation, survival, transformation, differentiation, cell-cell and cell-substrate interactions. Different signaling pathways may underlie these pleiotropic effects. The specific pathways engaged depend on the number of activated IGF-IRs, availability of intracellular signal transducers, the action of negative regulators, and is influenced by extracellular modulators. Experimental and clinical data implicate the IGF-IR in breast cancer etiology. There is strong evidence linking hyperactivation of the IGF-IR with the early stages of breast cancer. In primary breast tumors, the IGF-IR is overexpressed and hyperphosphorylated, which correlates with radio-resistance and tumor recurrence. *In vitro,* the IGF-IR is often required for mitogenesis and transformation, and its overexpression or activation counteract effects of various pro-apoptotic treatments. In hormone-responsive breast cancer cells, IGF-IR function is strongly linked with estrogen receptor (ER) action. The IGF-IR and the ER are co-expressed in breast tumors. Moreover, estrogens stimulate the expression of the IGF-IR and its major signaling substrate IRS-1, while antiestrogens downregulate IGF-IR signaling, mainly by decreasing IRS-1 expression and function. On the other hand, overexpression of IRS-1 promotes estrogen-independence for growth and transformation. In ER-negative breast cancer cells, usually displaying a more aggressive phenotype, the levels of the IGF-IR and IRS-1 are often low and IGF is not mitogenic, yet the IGF-IR is still required for metastatic spread. Consequently, IGF-IR function in the late stages of breast cancer remains one of the most important questions to be addressed before rational anti-IGF-IR therapies are developed.

KEY WORDS: Breast cancer; insulin-like growth factor I receptor; IRS-1; estrogen-independence; antiestrogen; metastasis.

INTRODUCTION

The insulin-like growth factors I and II (IGFs) act as endocrine, paracrine or autocrine regulators of various biological processes in normal and neoplastic cells. The actions of IGF-I in the adult are mediated primarily by the type I insulin-like growth factor receptor (IGF-IR), while IGF-II stimulates both the IGF-IR and the insulin receptor (IR) (1,2). It has been well established that in many cell types, activation of the IGF-IR is essential for cell survival, transformation, and hormone-independence—the processes that pro-

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[3] Abbreviations: three-dimensional (3-D); amino acid; (aa); disease-free survival (DFS); E-cadherin (E-cad); 17-beta estradiol (E2); extracellular matrix (ECM); epidermal growth factor receptor (EGFR); estrogen receptor (ER); histidine (His); insulin-like growth factor (IGF); IGF-I receptor (IGF-IR); insulin receptor (IR); insulin receptor substrate (IRS); lysine (Lys); overall survival (OS); phosphatidylinositol-3 kinase (PI-3K); progesterone receptor (PgR); protein kinase C (PKC); phosphotyrosine binding domain (PTB); MCF-7 cells expressing anti-IRS-1 and anti-SHC RNA, respectively (MCF-7/anti-IRS-1 and MCF-7/anti-SHC); MCF-7 cells overexpressing the IGF-IR, IRS-1, and SHC, respectively (MCF-7/IGF-IR, MCF-7/IRS-1, and MCF-7/SHC); serine (Ser); src-homology 2 domain (SH2); src/collagen homology proteins (SHC); Tamoxifen (Tam).

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mote tumorigenesis (3–6). During the past several years, the impact of the IGF-IR on breast cancer development and progression has also been recognized, providing a new direction for the design of anti-growth factor compounds for breast cancer therapy.

IGF-IR EXPRESSION AND STRUCTURE

Almost all cell types, except hepatocytes and T-lymphocytes, express the IGF-IR (1,3). The IGF-IR is encoded by a 100 kb gene containing 21 exons located on the distal arm of chromosome 15 (1,7). The IGF-IR promoter region is GC-rich, and similar to other housekeeping genes lacks TATA or CCAAT boxes, but contains several sites for binding transcriptional factors such as SP-1, E2F, and early growth response (EGR) proteins (5,8,9). The expression of the IGF-IR is regulated by different physiologic stimuli and may be altered in certain pathologies (e.g., diabetes, cancer). For instance, IGF-IR mRNA is enhanced by growth hormone, follicle stimulating hormone, luteinizing hormone, thyroid hormones, glucocorticoids, and estrogens (9). Moreover, different mitogens (e.g., platelet-derived growth factor, fibroblast growth factor) or oncoproteins (e.g., c-myb, hepatitis B Hbx) can induce IGF-IR transcription. Conversely, IGF-IR expression is downregulated by high concentrations of IGF-II, interferon, antiestrogens, and tumor supressors (e.g., Wilms' tumor or p53 proteins) (4,8,9).

The major IGF-IR 11 kb transcript is translated into a single 1,367 amino acid (aa) (180 kDa) precursor protein, which is then cleaved to form 135 kDa alpha and 90 kDa beta subunits. A mature IGF-IR is a heterotetramer composed of two alpha and two beta subunits linked by disulfide bonds (Fig. 1). The extracellular alpha subunits are responsible for ligand binding. IGF-IR beta subunits, which contain short transmembrane and large intracellular segments, transmit ligand-induced signal (1,7,9). Within the beta subunit, three major domains have been recognized: a tyrosine kinase domain, a juxtamembrane part, and the C-terminus, each containing residues essential for different IGF-IR functions (Fig. 1). Specifically, in the kinase domain, the ATP binding site containing lysine (Lys) 1003 as well as the tyrosine (Tyr) cluster (Tyr 1131, 1135, 1136) are critical for the catalytic activity of the receptor (5,9,10). In the juxtamembrane domain, Tyr 950 flanked by the NPEY motif is required for recruiting major signaling substrates such as insulin receptor substrates (IRS) 1-4 and src/collagen-homology (SHC) proteins (5,6,9,10). The C-terminus contains several residues essential for IGF-I signaling, including Tyr 1250, Tyr 1251, a stretch of serines (Ser) 1280-1283, histidine (His) 1293, Lys 1294, and Tyr 1316. In particular, the region between residues 1229 and 1245 has been found necessary for the association of an adapter GRB10, Tyr 1251 is required for binding a putative substrate p28, Ser 1280-1283 are necessary to sequester an adapter 14-3-3 epsilon, and Tyr 1316 is capable of recruiting either p85 subunit of phosphatidyl inositol-3 kinase (PI-3K) or SHPTP2 phosphatase (5,9,10, Baserga *et al.*, unpublished data). According to recent evidence, Tyr 1251 also appears to be indirectly involved in binding of SHC to the IGF-IR (11).

The IGF-IR shares significant structural homology with the IR. The kinase domains of these receptors are 80–90% identical. Also, Tyr 950 of the IGF-IR has its equivalent, Tyr 960, in the juxtamembrane domain of the IR (7). Importantly, the C-terminal regions of the receptors are quite different, sharing only approximately 40% homology. The equivalents of Tyr 1250 and 1251, Ser 1280–1283, and aa 1293–1301 are not present in the IR. Consequently, it is believed that the differences between biological responses of the IGF-IR and IR are associated with the induction of specialized signaling pathways arising from the C-terminus (1,7,10).

IGF-IR SIGNALING

Upon ligand binding, IGF-IRs cluster and tyrosine kinase is activated leading to autophosphorylation and transphosphorylation of beta subunits (1). Phosphorylation of specific Tyr and Ser residues creates binding sites for IGF-IR signaling substrates. The best known substrates are docking proteins IRS-1 and SHC. Both bind Tyr 950 through their phosphotyrosine binding (PTB) domain (4,9).

IRS-1 is a remarkable effector of the IGF-IR, capable of amplification and diversification of the signal because it can recruit various signaling molecules and induce numerous cellular responses. IRS-1 contains about 20 tyrosine phosphorylation sites which can directly bind signaling molecules equipped with phosphotyrosine binding domains, such as src-homology 2 (SH2) domains. For instance, there are nine YMXM motifs in IRS-1 that can attract the p85 subunit of PI-3K via SH2-type interactions and other domains recruiting SH2-containing adapters GRB2, Nck, and Crk, SHP2 phosphotyrosine phosphatase, and Fyn

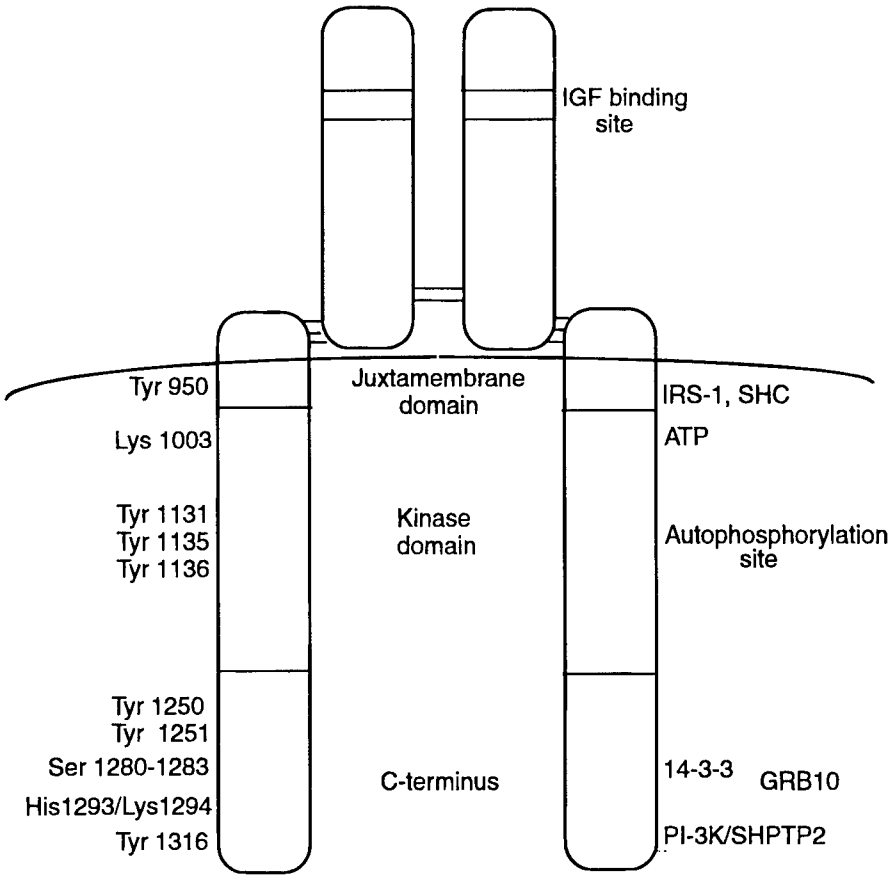

Fig. 1. Structure of the IGF-IR. The key residues involved in IGF-IR signaling are indicated on the left; the signaling elements binding to these regions of the IGF-IR are listed on the right.

kinase. Other partners of IRS-1, such as integrin $\alpha v\beta 3$ or the adapter 14-3-3, associate with the substrate through unknown mechanisms (12).

The major pathway induced by tyrosine phosphorylation of IRS-1 involves PI-3K, whose downstream effectors are Ser/threonine kinases Akt, $p70^{S6}$, and some isoforms of protein kinase C (PKC) (13). PI-3K is involved in the regulation of mitogenesis, metabolism, and actin cytoskeleton rearrangements, and the PI-3K/Akt pathway has recently been recognized as one of the most important signals ensuring cell survival. One of the cellular targets of Akt is a pro-apoptotic protein BAD, which induces cell death when bound to anti-apoptotic proteins Bcl2 or Bcl_{XL}. The phosphorylation of BAD by Akt facilitates its sequestration by 14-3-3 adapters and prevents apoptosis. Another effector of Akt is $p70^{S6}$ kinase which activates expression of cyclin D1 initiating cell cycle progression (13,14).

Association between IRS-1 and the GRB2/SOS complex leads to the stimulation of the classic Ras/MAP cascade of kinases, the pathway that is implicated in a broad array of biological responses, including cell growth and differentiation (12,15). The Ras/MAP pathway can also be induced through Nck or Crk adapters binding to IRS-1 or by GRB2 binding to IGF-IR-associated SHC proteins (6,12,15).

Three additional IRS proteins (IRS-2, -3, and -4) exhibiting different degrees of structural homology to IRS-1 have been cloned. The activities of IRS-1 and IRS-2 appear to partially overlap; for instance, in IRS-1-knock-out mice, IRS-2 substituted for IRS-1 function in stimulating PI-3K and activating glucose metabolism. However, in IRS-1-deficient fibroblasts, only IRS-1, but not IRS-2, reconstituted cell cycle progression (12,13). The functions of IRS-3 and -4 are not well understood. Similarly, the pathways initiated by

the binding of GRB10 or 14-3-3 to the IGF-IR are still obscure.

IGF-IR SIGNAL SPECIFICITY

Because the IGF-IR may regulate many, often contradictory (e.g., growth vs. differentiation), processes, it is of great importance to understand how signal specificity is achieved. Here, I will focus on IGF-IR-dependent survival, mitogenesis, and anchorage-independent growth, the most studied IGF effects. The current view is that the IGF-I response is dictated by the engagement of different sets of intracellular pathways. Which pathways are stimulated depends on (i) the number of activated receptors on the cell surface; (ii) the availability of signaling substrates and receptor-substrate binding sites; (iii) the abundance and activity of negative regulators such as phosphatases; and (iv) extracellular context, e.g., ligand availability or extracellular matrix (ECM) components and their interaction with cells.

The first point is best illustrated by the work of Rubini *et al.* (16) and Reiss *et al.* (17), who analyzed the relationship between the number of IGF-IRs expressed on the cell and IGF-I-induced biological response. While activation of $2–10 \times 10^3$ receptors stimulates tyrosine phosphorylation of IRS-1 as well as activation of an early response gene *c-myc*, it is not sufficient for SHC phosphorylation or the entry of cells into the cell cycle. With 1.5×10^4 receptors, the cells progress through the S phase, but they are not able to complete cell division, and their survival ability under anchorage-independence is minimal. The increase of receptor expression to $2.2–6.0 \times 10^4$ receptors/cell ensures phosphorylation of the major substrates, full mitogenic response, and good survival, but produces only a weak transforming activity (measured by growth in soft agar). Activation of more than 1×10^5 IGF-IRs provides signal strong enough to activate both IRS-1 and SHC signaling pathways, stimulate cell division, and support robust transformation (16,17). A direct relationship between the number of stimulated IGF-IRs and cell survival and/or tumorigenesis in animal models has been documented by the Baserga and LeRoith laboratories (18,19). For instance, NIH 3T3 mouse fibroblasts expressing 1.9×10^5 IGF-IR/cell form tumors in nude mice, while fibroblasts with lower IGF-IR levels (1.6×10^4) are nontumorigenic. In addition, the latency of tumor formation *in vivo* was reduced with high doses (4–10 mg/kg) of endocrine IGF-I, suggesting that chronic stimulation of a high number of IGF-IRs was critical for the onset of tumorigenesis (19).

A mutational analysis has been performed to determine whether various functions of the IGF-IR are induced by overlapping or distinct pathways. In the studies of Baserga *et al.*, different mutant IGF-IRs have been expressed in R-minus cells (derived from IGF-IR knock-out mice), which allowed the analysis of signaling pathways of the mutants without interference from the endogenous wild-type IGF-IRs (10). This work was complemented by O'Connor *et al.* who studied mutant receptors expressed in either hematopoietic IRS-1-negative FL5.12 cells or apoptosis-prone Rat-1/Myc fibroblasts, and by LeRoith *et al.* who used NIH 3T3 fibroblasts for the analysis (5,6). All studies demonstrated that a mutation in the ATP binding site produced "dead" receptors incapable of signal transmission. Replacement of all three Tyr 1131, 1135, and 1136, or Tyr 1136 alone, with phenylalanine produced a receptor that was not mitogenic or transforming, but it still induced an efficient survival signal. Mutations in either Tyr 1131 or Tyr 1135 downregulated transformation without reducing cell growth. Tyr 950 in the IGF-IR juxtamembrane domain was found necessary for IRS and SHC association, and for induction of mitogenic and transforming activity. Interestingly, however, the IGF-IR/Tyr 950 mutant transmitted anti-apoptotic signaling. This finding indicates that in addition to the classic IRS-1-dependent PI-3K/Akt pathway, other survival pathway(s) emanate from the IGF-IR (5,6,9,10).

Deletion of the entire C-terminus at aa 1229 produced a receptor that retained normal mitogenic function but was totally lacking transforming potential (20). Subsequent detailed studies with mutant IGF-IRs expressed in R-minus cells mapped the "transforming domain" between residues 1245 and 1310, with Tyr 1251, Ser 1280–1283, His 1293, and Lys 1294 required for transformation (10). Importantly, this region does not have an exact counterpart in the IR. Indeed, the expression of the IR or a chimeric IGF-IR containing an IR C-terminus did not support soft agar growth of R-minus cells (10). Notably, the IGF-IR transforming signal appears to be truly unique, at least in mouse fibroblasts, as overexpression of various growth factor receptors, signaling molecules or oncogenes (except for v-src), singly or in combination, did not restore transformation in R-minus cells, while the IGF-IR did (3,21). The mediators of the IGF-IR transforming pathway are not yet known, but the adapters 14-3-3 and

GRB10, which bind to the C-terminus, could be involved.

Interestingly, the IGF-IR C-terminus also appears to play a unique role in survival signaling. Mutants with a deleted C-terminus (at residues 1229 or 1245) retained or even amplified anti-apoptotic function, while single mutations in Tyr 1251, His 1293, and Lys 1294 reduced survival (5). Consequently, it has been suggested that the C-terminus is an intrinsic inhibitory domain of the IGF-IR, while the residues Tyr 1251, His 1293, Lys 1294 act as neutralizers of this pro-apoptotic function. Indeed, expression of the C-terminal 108 aa as a membrane-targeted protein resulted in induction of apoptosis, and mutations in Tyr 1250/1251 and His 1293/Lys 1294 abrogated this cytotoxic activity (5).

To summarize, IGF-IR signals required for mitogenesis, transformation, and survival are distinct but partially overlap. For instance, no transforming activity is seen in the absence of mitogenic activity. Transformation also seems to have some common pathways with IGF-dependent survival. However, cell survival can be induced by a weak signal which is not sufficient for mitogenesis or transformation, while transformation requires strong IGF-IR activation and induction of specific signals originating at the C-terminus.

The pathways mediating non-growth IGF-I responses such as cell-cell or cell-substrate interactions are less well characterized. Our preliminary data indicate that in epithelial cells, intercellular adhesion requires the tyrosine kinase domain as well as the C-terminus of the IGF-IR, and depends on SHC but not on IRS-1 signaling (4,22, Surmacz *et al.*, unpublished data). IGF-I-induced motility and reorganization of actin cytoskeleton involves PI-3K and SHC activities, and modification of proteins associated with focal adhesions (22,23).

It is known that the IGF-IR response may be cell-type specific (24). One mechanism ensuring such specificity is the availability of intracellular signaling intermediates. For instance, with the same cellular content of the IGF-IR, downregulation of IRS-1 expression inhibits cell growth, transformation, and results in cell death, while amplification of IRS-1 sensitizes cells to low concentrations of IGF-I and enhances anchorage-independent growth (22,25,26). On the other hand, overexpression of SHC does not improve IGF-I-dependent growth, but inhibition of SHC expression inhibits cell growth, transformation, and to a lesser extent, cell survival (22,27).

Finally, the extracellular context plays a role in IGF-I response, for instance, survival and growth of cells adhering to a proper substrate is mediated through the IRS-1 pathway, while the same pathway is much less important in IGF-I-dependent protection from apoptosis due to anchorage-independence (22,28,29).

Requirement for IGF-IR in Proliferation, Transformation, and Survival of Breast Cancer Cells

The critical role of the IGF-IR in breast cancer growth, survival, and transformation has been well documented *in vitro* and in animal models (Table I) (4). Reducing ligand availability by excess IGF-BP1 or exposure to suramin blocked IGF-IR activation and limited breast cancer cell proliferation. Furthermore, inhibiting the expression of the IGF-IR with an antisense-IGF-IR RNA, or its function with anti-IGF-IR antibodies or dominant-negative mutants, resulted in growth inhibition and reduced transforming potential (4). Our studies with MCF-7 breast cancer cell lines expressing antisense-IRS-1 or antisense-SHC RNAs (MCF-7/anti-IRS-1 or MCF-7/anti-SHC cells) demonstrated that both IRS-1- and SHC-dependent signals are necessary for cell proliferation and transformation (22). The critical role of IRS-1 (but not IRS-2) and IRS-1 downstream pathways—Ras/MAP and PI-3K in the growth of estrogen receptor (ER)-positive breast cancer cells has recently been confirmed by the Yee laboratory (30). Using dominant-negative IGF-IRs lacking the C-terminus, we demonstrated that in breast tumor cells, as in fibroblasts, the C-terminal portion is essential for transformation *in vitro* and tumorigenesis *in vivo* (4).

Dunn *et al.* have shown that activation of the IGF-IR protects breast cancer cells from apoptosis induced by various therapeutic agents, serum deprivation and irradiation (31). Our results with MCF-7 cells in which IRS-1 has been downregulated by either antisense-IRS-1 oligonucleotides, expression of antisense-IRS-1 RNA, or antiestrogen treatment suggest that the IRS-1/PI-3K signal is required for IGF-IR-induced survival (22,25,32).

Amplification of IGF-IR Signaling and Anchorage-Dependent and -Independent Growth of Breast Cancer Cells

Further understanding of IGF-IR function in breast cancer pathobiology stemmed from studies of

Table I. IGF-IR Function in Breast Cancer

Signaling molecule	Function in breast cancer	
	Experimental models	Tumors
IGF-IR	elevated in ER-positive breast cancer cells; stimulates proliferation; counteracts apoptotic effects of anti-tumor drugs; improves 3-D growth and survival; regulates cell-substrate connections; required for anchorage-independent growth *in vitro* and tumorigenesis and metastasis in animal models.	correlates with the ER status; elevated in primary tumors; high levels correlate with radio-resistance and recurrence at the primary site; usually co-expressed with markers of better overall prognosis.
IRS-1	elevated in ER-positive breast cancer cells; required for anchorage-dependent and independent growth; critical for survival; high levels induce estrogen-independence and antiestrogen-resistance.	correlates with shorter DFS in ER-positive primary tumors.
SHC	required for proliferation, anchorage-independent growth, migration, and cell-cell adhesion; high levels improve adhesion to fibronectin.	?

cells with amplified IGF-IR signaling. In order to correlate the strength of the IGF signal with the progression towards a more neoplastic phenotype, we developed a series of MCF-7-derived cell lines overexpressing different levels of either the IGF-IR (MCF-7/IGF-IR cells), IRS-1 (MCF-7/IRS-1 cells), or SHC (MCF-7/SHC cells) (25,27,29).

Overexpression of the IGF-IR (8–50-fold) was paralleled by enhanced IGF-IR tyrosine kinase activity and hyperphosphorylation of IRS-1, even in the absence of exogenous IGF-I. Compared to the parental cells, all MCF-7/IGF-IR clones exhibited enhanced autocrine growth in serum-free medium and improved growth responsiveness to low concentrations of IGF (0.1–1.0 ng/ml), especially in the presence of 10 nM estradiol (E2). With higher doses of IGF-I (4–50 ng/ml), the synergistic effect was not seen and the maximal mitogenic effect was achieved with IGF-I alone (29). Similar results were described by Daws *et al.*, who independently developed IGF-IR overexpressing MCF-7 clones (33). Interestingly, we as well as others noticed that high doses of IGF-I (20–50 ng/ml) combined with 10 nM E2 inhibited MCF-7/IGF-IR cell growth, especially in the clones with the highest IGF-IR levels (29,33).

Anchorage-independent growth of MCF-7/IGF-IR cells treated with E2 was slightly elevated relative to the parental cells, but this effect of IGF-IR overexpression was not present in cells treated with both E2 and IGF-I or cultured in serum-containing medium (29,33).

In contrast with the modest effects of amplified IGF-IR, overexpression of IRS-1 (1.5–9-fold) produced marked changes in the growth phenotype (25). In MCF-7/IRS-1 cells, proliferation was enhanced under all conditions studied (serum-free and serum-containing medium, or serum-free medium with 20 ng/ml IGF); the addition of E2 never inhibited the growth. Also, MCF-7/IRS-1 cells exhibited greatly a enhanced potential for growth in soft agar, especially in the presence of high (200–400 ng/ml) doses of IGF-I. Remarkably, this IGF-dependent transformation was further potentiated with E2. The above effects were correlated with the cellular levels of IRS-1 and the extent of IRS-1 tyrosine phosphorylation (25).

Amplification of SHC in MCF-7 cells (two–sevenfold) did not alter growth properties under standard monolayer or anchorage-independent conditions, but it amplified cell-substrate interactions on fibronectin (27).

IGF-IR/ER Cross-Talk

In hormone-dependent breast cancer cells, ER and the IGF-IR are co-expressed and E2 acts in synergy with IGF-I to stimulate proliferation (4). The effects of E2 are mediated in part via sensitization of cells to IGF action. E2 treatment up-regulates IGF-IR mRNA and protein levels by two–tenfold, reflected in enhanced IGF-IR tyrosine phosphorylation (4,34,35). Furthermore, E2 significantly (two–fivefold) stimulates the expression of IRS-1 in different ER-positive cell lines, and the extent of this stimulation depends on the cellular ER content (35, Surmacz *et al.*, unpublished data). Of note, E2 action appears to be at least partially specific to the IGF-IR/IRS-1 pathway since it does not modulate SHC levels (36).

Importantly, various antiestrogens such as Tamoxifen (Tam) and its derivatives, droloxifene, and

pure antiestrogens ICI 164,384 and ICI 182,780 inhibit IGF-IR-dependent proliferation (4,32,36–38). We demonstrated that at the molecular level, the anti-IGF-IR actions of Tam and ICI 182,780 are accomplished by downregulation of IRS-1/PI-3 kinase signaling (32,36). Specifically, growth arrest and apoptosis resulting from antiestrogen treatment were associated with continuos suppression of IRS-1 mRNA and protein expression, reflected by reduced IRS-1 tyrosine phosphorylation, decreased IRS-1/PI-3K binding and reduced PI-3K activity (32,36). These anti-IRS-1 effects of ER antagonists were partially reversed in the presence of IGF-I (36).

Antiestrogens also inhibit IGF-IR expression and tyrosine phosphorylation (by 30–50%) but only in the presence of IGF-I (32,36). In the absence of IGF-I, Tam and ICI 182,780 enhance IGF-IR phosphorylation, which suggest that the drugs may act through modulation of IGF-I-dependent phosphatases. Indeed, the involvement of tyrosine phosphatases LAR and FAP-1 in antiestrogen inhibition of IGF-dependent growth has been demonstrated by the Vignon laboratory (39). Interestingly, in different antiestrogen-treated cell lines, SHC expression or signaling were not altered, while SHC tyrosine phosphorylation was increased in Tam- but not in ICI 182,780-arrested cells (32,36).

Because E2 upregulates IGF-IR signaling, it has been postulated that amplification of the IGF-IR or its key signaling substrates may lead to estrogen-independence. In agreement with this hypothesis, MCF-7/IRS-1 cells exhibited reduced estrogen requirements for growth and transformation, and were not inhibited by E2 alone or in combination with of IGF-I (25). Interestingly, however, such estrogen-independence has not been detected in MCF-7/IGF-IR cells (29,33). These cells still appear to remain under ER control as their growth is restrained by high doses of E2 in the presence or absence of IGF-I. This finding suggests the existence of a negative growth regulatory loop which is not operative in MCF-7/IRS-1 cells and may be triggered by hyperactivation of IGF-I signaling pathways not involving IRS-1.

The role of amplified IGF-IR signaling in the development of antiestrogen-resistance is of particular interest. We and others have shown that overexpression of different IGF-IR signaling elements did not affect ER content (25,29,33). However, MCF-7/IRS-1 clones with very high IRS-1 levels (9 or 12-fold overexpression with respect to MCF-7 cells) exhibited resistance to ICI 182,780, confirming that the IRS-1 pathway is an essential target for antiestrogens and suggesting that overexpression of IRS-1 in tumors may hinder antiestrogen therapy (36). Interestingly, in contrast with IRS-1, overexpressed IGF-IRs (50-fold) or SHC (fivefold) did not alter antiestrogen sensitivity in MCF-7 cells (36).

IGF-IR-DEPENDENT CELL-CELL AND CELL-SUBSTRATE INTERACTIONS IN BREAST CANCER CELLS

Breast cancer cells, like other polarized epithelial cells, are governed by cell-cell and cell-substrate interactions. The regulation of these processes by growth factors is now being increasingly recognized. We studied intercellular interactions of MCF-7 cells and their derivatives with modified IGF-IR signaling. We found that overexpression of the IGF-IR greatly enhanced aggregation of cells in three-dimensional (3-D) culture (29). Specifically, when plated on Matrigel, MCF-7/IGF-IR cells formed large spheroids (150–300 μm in diameter) surviving or even proliferating for up to 20 days, while the parental MCF-7 cells formed smaller clusters (50 μm) which disaggregated and died after 7 days of culture. Similar stimulation of cell-cell adhesion has been described in IGF-I treated MCF-7 and MCF-7/6 cells as well as in MCF-7 cells constitutively secreting IGF-I (4). Our subsequent research demonstrated that enhanced cell-cell adhesion is IGF-I-specific as it cannot be induced by physiologic concentrations of EGF, IGF-II or insulin (4). The mechanism of this phenomenon is still not clear. We have shown that the IGF-IR co-localizes with an adherens junction protein E-cadherin and co-precipitates with E-cadherin, α-catenin, and β-catenin (4,29). In addition, we obtained preliminary data suggesting that the IGF-IR stimulates the expression of a junction protein ZO-1, thereby strenghtening the α-catenin/ZO-1/F-actin connections (Surmacz *et al.*, in preparation). The signals required for cell-cell adhesion depend on IGF-IR tyrosine kinase activity and the presence of the C-terminus (4, Surmacz *et al.*, in preparation). We also observed that cell-cell adhesion is reduced in MCF-7/anti-SHC, but not MCF-7/anti-IRS-1 cells, which points to SHC as a putative mediator of IGF-induced aggregation (22).

The enhanced intercellular connections and improved survival of IGF-IR overexpressing cells may contribute to their tumorigenic activity *in vivo*. Indeed, when MCF-7/IGF-IR cells were injected into mam-

mary fat pad of nude mice, they formed tumors after 8 weeks, while the parental MCF-7 cells or MCF-7 clones expressing an IGF-IR with a C-terminal truncation were non-tumorigenic (Surmacz *et al.*, in preparation).

Cell-substrate adhesion and migration of epithelial cells is also regulated by IGF-I. For instance, depolarization of MCF-7 and MCF-7/IGF-IR cells and induction of cell migration can be achieved with a 4 hour treatment with 50 ng/ml IGF-I. The initial stages of this process are associated with transient dephosphorylation of the focal adhesion proteins FAK, paxillin and p130 Cas (23). IGF-IR pathways involved in the regulation of breast cancer cell motility are still quite obscure, but we observed reduced migration of MCF-7 cells with impaired SHC or PI-3K signaling (22,23).

Obviously, extracellular cell context may dictate whether cellular response to IGF-I involves increased cell-cell adhesion or enhanced migration. Increased intercellular adhesion may be seen in cells expressing low levels of integrins necessary for attachment to a given ECM substrate, whereas enhanced migration occurs when the cells interact well with a substrate, or produce sufficient amounts of their own ECM (23,28).

IGF-IR Signaling in Breast Tumors

Studies on IGF-IR expression in breast tumors and its correlation with other host or tumor parameters are very limited (4). Moreover, the interpretation of the available data is complicated by the fact that different techniques were used to assess the IGF-IR levels. The most frequently performed IGF-I binding assay is inherently inaccurate due to the interaction of IGF with membrane IGFBPs, often resulting in overestimation of the number of the IGF-IR (4). To circumvent this problem, the expression of IGF-IR in tumor samples was examined with anti-IGF-IR antibody-based techniques (radioimmunoassay or immunocytochemistry) (40,41). Despite the differences in experimental approach, in all large series studies (>100 cases) the IGF-IR has been detected in a majority of breast tumor samples (4,42,43).

Most important, IGF-IR levels have been found to be elevated (up to 14-fold) in primary breast cancer compared to non-malignant tumors or normal epithelium (40–43) (Table I). The mechanism of the common IGF-IR overexpression in breast cancer is not clear, but it does not appear to be associated with IGF-IR gene amplification since this event was reported in only 2% of cases analyzed (44). Recent data indicate that IGF-IR overexpression may be related to derepression of IGF-IR transcription due to aberrant expression of the tumor supressor protein p53 (40,45,46). Overexpression of the IGF-IR in tumors has been found to be associated with hyperactivation of the tyrosine kinase (up to sixfold), and correlated with radio-resistance and tumor recurrence at the primary site (40,41). High IGF-IR levels in primary tumors have been reported as predictors of shorter disease-free survival, but data on the prognostic value of the IGF-IR for overall survival are conflicting (4,41).

Importantly, not only the IGF-IR, but also IRS-1 has been found to be overexpressed in a fraction of primary breast tumors (35,47). High levels of IRS-1 correlated with shorter disease-free survival in ER-positive tumors (35). The mechanism of IRS-1 overexpression is not known, but it could be associated with E2 or IGF activity since both mitogens are known to stimulate IRS-1 transcription and both can be found (often at superphysiological concentrations) in breast tumors.

Attempts to correlate IGF-IR expression with other host or tumor variables showed a positive link between the IGF-IR and ER status (4,42,43). In addition, frequent co-expression of the IGF-IR and IR has been shown (42). Co-expression of these structurally homologous receptors leads to the formation of functional hybrids which bind IGF-I with high affinity, and thereby amplify the IGF-I signal (48).

In several large series analyses, no significant correlations were found between IGF-IR expression and menopausal status (42), body weight (42), tumor size (42,43), tumor grade (42,49,50), histological type (42,43), node status (42,43,49,50), or EGFR status (49), and the link with the progesterone receptor (PgR) status is uncertain (4,42, 43,49–51). However, because most of these associations were established based on IGF-I binding assays, they should be re-assessed using more accurate techniques of IGF-IR measurement before any firm conclusions can be drawn.

The expression of IRS-1 correlated with ER levels but not with other parameters such as age, tumor size, or PgR status (35,47). The levels of another IGF-IR substrate, SHC, are similar in aggressive and more differentiated breast cancer cell lines, but its activity (tyrosine phosphorylation) in cell lines and tumors reflects the levels of oncogenic kinases ERB2 or c-src

(27,52,53). SHC association with IGF-IR in breast tumors has not been studied.

The Unclear Role of the IGF-IR in Breast Cancer Progression

The experimental and clinical evidence points to the fact that the IGF-IR may be important in early steps of tumor development, promoting cell growth, survival, and resistance to therapeutic treatments. However, the function of the IGF-IR in the later stages, including metastasis, is still obscure.

Especially intriguing is the fact that, whereas the IGF-IR has been found to be overexpressed in primary tumors, its levels, like ER levels, appear to undergo reduction during the course of the disease. For instance, Pezzino *et al.* assessed IGF-IR status in two patient subgroups, representing either a low risk (ER- and PgR-positive, low mitotic index, diploid) or a high risk (ER- and PgR-negative, high mitotic index, aneuploid) population and found a highly significant correlation between IGF-IR expression and better prognosis (42). Similar conclusions were reached by Peyratt and Bonneterre (43). Therefore, it has been proposed that like the ER, the IGF-IR marks more differentiated tumors with better clinical outcome. However, it has also been argued that the IGF-IR may play a role in early steps of tumor spread since node-positive/IGF-IR-positive tumors appeared to have a worse prognosis than node-negative/IGF-IR-positive tumors (49). In addition, quite rare cases of ER-negative but IGF-IR-positive tumors are associated with shorter disease-free survival (48).

In breast cancer cell lines, a hormone-dependent and less aggressive phenotype correlates with a good expression of the IGF-IR and IRS-1 (29,35). In contrast, highly metastatic ER-negative breast cancer cell lines express low levels of the IGF-IR and often do not respond to IGF-I with growth (54,55). Similarly, IRS-1 levels are downregulated in a majority of these cell lines (35,55). Despite this "IGF-IR-reduced phenotype", metastatic cell lines appear to depend on the IGF-IR. For instance, blockade of the IGF-IR in MDA-MB-231 cells by anti-IGF-IR antibody reduced migration *in vitro* and tumorigenesis *in vivo*, and expression of a soluble IGF-IR in MDA-MB-435 cells impaired growth, tumorigenesis and metastasis in animal models (56–58). Whether this particular IGF-I-dependence of metastatic breast cancer cells relates to the survival function of the IGF-IR is under investigation in our laboratory.

CONCLUSIONS

Over the past few years much has been learned about the function of the IGF-IR in the process of tumorigenesis. Clearly, IGF-IR-mediated survival and transformation are key factors affecting tumor development. In primary breast cancer, high levels of the IGF-IR may promote survival and proliferation, counteracting cytotoxic or cytostatic effects of drugs or radiation. The mechanism of this IGF-I action includes strengthening intercellular connections, amplification of anti-apoptotic signals, and sensitization of cells to low concentrations of IGFs and E2. Therefore, targeting the IGF-IR, especially the IGF-IR/IRS-1 pathway, should help in eradicating primary tumor cells.

The importance of the IGF-IR in metastatic breast disease is still not clear. It is possible that the IGF-IR has a role in cell spread, functioning primarily as an anti-apoptotic, and possibly a motogenic factor. Unquestionably, further understanding of IGF-IR function in metastatic cells will be critical in creating successful anti-IGF-IR therapies for late stages of breast cancer.

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IGF-I Receptor Signaling and Function are Different in Non-Metastatic and Metastatic Breast Cancer Cells

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ABSTRACT

The insulin-like growth factor receptor (IGF-IR) is a ubiquitous and multifunctinal tyrosine kinase that has been implicated in breast cancer development. In ER-positive breast tumors, the levels of IGF-IR and its substrate IRS-1 are often elevated and these characteristics have been linked with increased radio-resistance and cancer recurrence. *In vitro*, activation of the IGF-IR/IRS-1 pathway in ER-positive cells improves growth and counteracts apoptosis induced by anticancer treatments.

The role of the IGF-IR in ER-negative metastatic breast cancer is not clear. Highly aggressive, ER-negative breast cancer cell lines express low levels of the IGF-IR and fail to respond to IGF-I with mitogenesis. On the other hand, inhibition of the IGF-IR reduces metastatic potential of these cells, suggesting a role of this receptor in late stages of the disease. Here we examined IGF-IR signaling and function in ER-negative metastatic breast cancer cells. Using MDA-MB-231 cells and their IGF-IR-overexpressing derivatives, we demonstrated that IGF-I acts as a chemoattractant for these cells. The extent of IGF-I-induced migration reflected IGF-IR levels and required the activation of PI-3K and p38 kinases. The same pathways promoted IGF-I-dependent motility in ER-positive MCF-7 cells.

In contrast with the positive effects on cell migration, IGF-I was unable to stimulate the growth or improve survival in MDA-MB-231 cells, while it induced mitogenic and anti-apoptotic effects in MCF-7 cells. Moreover, IGF-I counteracted the action of PI-3K and ERK1/ERK2 inhibitors in MCF-7 cells, while it had no protective effects in MDA-MB-231 cells. The impaired IGF-I growth response in ER-negative cells was not caused by the low IGF-IR expression, defective IGF-IR tyrosine phosphorylation, or improper tyrosine phosphorylation of IRS-1. Also, the acute (15 min) IGF-I activation of PI-3 and Akt kinases was similar in ER-negative and ER-positive cells. However, a long-term (2 days) IGF-I exposure induced the PI-3K/Akt pathway only in MCF-7 cells. The reactivation of this pathway in ER-negative cells by overexpression of constitutively active Akt mutants was not sufficient to improve proliferation or survival (with or without IGF-I), which indicated that other pathways are also required to support these functions.

Our results suggest that IGF-IR function undergoes evolution during breast cancer progression from the ER-positive to ER-negative phenotype: growth-related signaling becomes attenuated, while non-mitogenic processes, such as migration, still remain under IGF-IR control.

INTRODUCTION

The insulin-like growth factor I (IGF-I) receptor (IGF-IR) is a ubiquitous, transmembrane tyrosine kinase that has been implicated in different growth-related and – unrelated processes critical for the development and progression of malignant tumors, such as proliferation, survival, and anchorage-independent growth as well as cell adhesion, migration, and invasion (1, 2).

The IGF-IR is necessary for normal breast biology, but recent clinical and experimental data strongly suggest that the same receptor is involved in the development of breast cancer (1, 3). The IGF-IR is overexpressed (up to 14-fold) in estrogen receptor (ER)-positive breast cancer cells compared with its levels in normal epithelial cells (1, 4, 5). The elevated expression and hyperactivation of the IGF-IR has been linked with increased radio-resistance and cancer recurrence at the primary site (4). Similarly, high levels of insulin-receptor substrate 1 (IRS-1), a major signaling molecule of the IGF-IR, correlated with tumor size and shorter disease-free survival in ER-positive tumors (6, 7).

IGF-IR ligands, IGF-I and IGF-II, are strong mitogens for many hormone-dependent breast cancer cell lines and have been found in the epithelial and/or stromal component of breast tumors (1). Importantly, higher levels of circulating IGF-I predict increased breast cancer risk in premenopausal women (8). *In vitro*, activation of the IGF-IR, especially the IGF-IR/IRS-1/PI-3K pathway in ER-positive breast cancer cells, counteracts apoptosis induced by different anti-cancer treatments or low concentrations of hormones (1, 9-11). On the other hand, overexpression of either the IGF-IR or IRS-1 in ER-positive breast cancer cells improves responsiveness to IGF and, in consequence,

results in estrogen-independent proliferation (1, 12, 13). In agreement with these observations, blockade of IGF-IR activity with various reagents targeting the IGF-IR or its signaling through IRS-1/PI-3K reduced the growth of breast cancer cells *in vitro* and/or *in vivo* (1, 12, 14-17).

The requirement for the IGF-IR/IRS-1 pathway for growth and survival appears to be a characteristic of ER-positive, more differentiated, breast cancer cells. By contrast, ER-negative tumors and cell lines, which frequently exhibit a less differentiated, mesenchymal phenotype, express low levels of the IGF-IR and often decreased levels of IRS-1 (1, 17). Notably, these cells do not respond to IGF-I with growth (1, 18-21). Despite the lack of IGF-I mitogenic response, metastatic potential of ER-negative breast cancer cells can be effectively inhibited by different compounds targeting the IGF-IR. For instance, blockade of the IGF-IR in MDA-MB-231 cells by an anti-IGF-IR antibody reduced migration *in vitro* and tumorigenesis *in vivo*, and expression of a soluble IGF-IR in MDA-MB-435 cells inhibited adhesion on the extracellular matrix and impaired metastasis in animals (14, 16, 22). These observations suggested that some functions of the IGF-IR must be critical for metastatic cell spread. Here we addressed the possibility that in ER-negative metastatic breast cancer cells, the IGF-IR selectively promotes growth-unrelated processes, such as migration and invasion, but is not engaged in the transmission of growth and survival signals. Using ER-negative MDA-MB-231 breast cancer cells, we set about to delineate IGF-I-dependent pathways involved in migration, and to pinpoint the defects in IGF mitogenic signal. For comparison, the relevant IGF-I responses were analyzed in ER-positive MCF-7 cells.

MATERIALS AND METHODS

Plasmids. The pcDNA3-IGF-IR expression plasmid encoding the wild-type IGF-IR under the CMV promoter was described before (13). The expression plasmids encoding constitutively active forms of Akt kinase, myristylated Akt (MyrAkt) and Akt with an activating point mutation (AktE40K), were obtained from Drs. P. Tsichlis and T. Chan (Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA) and described were before (23). The Akt plasmids contain the HA-tag allowing for easy identification of Akt transfected cells.

Cell Lines. MDA-MB-231 cells were obtained from ATCC. MDA-MB-231/IGF-IR clones were generated by stable transfection of MDA-MB-231 cells with the plasmid pcDNA3-IGF-IR using a standard calcium phosphate precipitate procedure (13). Transfectants resistant to 1 mg/ml G418 were screened for IGF-IR expression by FACS (Fluorescence-assisted cell sorting) analysis using anti-IGF-IR mouse monoclonal antibody (mAb) alpha-IR3 10 ug/ml (Calbiochem) and fluorescein goat anti-mouse IgG 2 ug/ml (Calbiochem). Cells stained with the secondary antibody alone were used as a control. IGF-IR expression in transfectants was always analyzed in parallel with that in the parental MDA-MB-231 cells and in IGF-IR overexpressing cells MCF-7/IGF-IR clones 12 and 15 (13). IGF-IR levels in MDA-MB-213-derived clones and control cell lines were then confirmed by Western blotting (WB) with specific antibodies (listed below) and by binding assay with (^{125}-I-IGF-I) (as described before in Ref. 13). MCF-7 cells and MCF-7/IGF-IR clone 12 overexpressing the IGF-IR were described in detail previously (12).

Transient Transfection. 70% confluent cultures of MDA-MB-231 and MCF-7 cells were transiently transfected with Akt kinase expression plasmids using Fugene 6 (Roche). Transfection was carried out for 6h; the optimal plasmid/Fugene 6 ratio was 1 ug/3 ul. Upon transfection, the cells were shifted to PRF-SFM and the expression of total and active Akt kinase at 0 (media shift), 2, and 4 days post transfection was assessed by WB with specific antibodies (see below). In parallel, the efficiency of transfection and plasmid expression was monitored by measuring the cellular levels of HA-tag by WB (see below).

Cell Culture. MDA-MB-231 and MCF-7 cells were grown in DMEM:F12 (1:1) containing 5% calf serum (CS). MDA-MB-231- and MCF-7-derived clones overexpressing the IGF-IR were maintained in DMEM:F12 plus 5% CS plus 200 ug/ml G418. In the experiments requiring E2- and serum-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 (referred to as PRF-SFM) (13).

Growth Curves. To analyze the growth in serum-containing medium, the cells were plated in 6-well plates in DMEM:F12 (1:1) containing 5% CS at a concentration of 1.5-2.0x10^5 cells/plate; the number of cells was then assessed by direct counting at 1, 2, and 4 days after plating. To study IGF-I-dependent proliferation, the cells were plated in 6-well plates in the growth medium as above. The following day (day 0), the cells at approximately 50% confluence were shifted to PRF-SFM containing 20 ng/ml IGF-I. Cell number was determined at days 1, 2, and 4.

Apoptosis. The cells grown on coverslips in normal growth medium were shifted to PRF-SFM at 70% confluence and then cultured in the presence or absence of 20 ng/ml IGF-I for 0, 1, 2, and 4 days. Apoptosis in the cultures was determined with the In Situ Cell Death Detection kit, Fluorescein (Roche), following manufacturer instructions. The cells containing DNA strand breaks were stained with fluorescein-dUTP, and detected by fluorescence microscopy. Cells that detached during the experiment were spun on glass slides using cytospin and processed as above. Apoptotic index (% number of apoptotic cells/total cell number in a sample field) was determined for adherent and floating cell populations and the indices combined.

Immunoprecipitation and Western Blotting. 70% cultures were shifted to PRF-SFM for 24 h and then stimulated with 20 ng/ml IGF-I for 15 min, 1 day or 2 days. Proteins were obtained by lysing the cells with a buffer containing: 50 mM HEPES pH 7.5, 150 mM, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM EGTA, 10% glycerin, 0.2 mM Na_3VO_4, 1% PMSF, 1% aprotinin. The IGF-IR was immunoprecipitated (IP) from 500 ug of protein lysate with anti-IGF-IR mAb (Calbiochem), and subsequently detected by WB with anti-IGF-IR polyclonal Ab (pAb) (Santa Cruz). IRS-1 was precipitated from 500 ug of lysate with anti-IRS-1 pAb (UBI) and detected by WB using the same Ab. Tyrosine phosphorylation (PY) of immunoprecipitated IRS-1 or IGF-IR was assessed by WB with anti-phosphotyrosine mAb PY20 (Transduction Laboratories). IRS-1-associated p85 subunit of PI-3K was detected in IRS-1 immunoprecipitates by WB with anti-p85 mAb (Transduction Laboratories).

Akt, ERK1/ERK2 and p38 MAP kinases (active and total), and active GSK3 were assessed by WB in 50 ug of whole cell lysates with appropriate Abs from New England Biolabs. The expression of HA-tag was probed by WB in 50 ug of protein lysate with anti-HA mAb (Babco). The intensity of bands representing relevant proteins was measured by laser densitometry scanning.

IRS-1 Associated PI-3K Activity. PI-3K activity was determined in vitro, as described by us before (24). Briefly, 70 % cultures were synchronized in PRF-SFM for 24h and then stimulated with 20 ng IGF-I for 15 min or 2 days. Untreated cells were used as controls. IRS-1 was precipitated from 500 ug of cell lysates; IRS-1 IPs were then incubated in the presence of inositol and ^{32}P-ATP for 30 min at room temperature. The products of the kinase reaction were analyzed by thin-layer chromatography using TLC plates (Eastman Kodak). Radioactive spots representing PI-3P were visualized by autoradiography, quantified by laser densitometry (ULTRO Scan XL, Pharmacia), and than excised from the plates and counted in a beta-counter.

Motility Assay. Motility was tested in modified Boyden chambers containing porous (8 mm) polycarbonate membranes, as described by us before (10, 25). Briefly, $2x10^4$ cells synchronized in PRF-SFM for 24h were suspended in 200 ul of PRF-SFM and plated into upper chambers. Lower chambers contained 500 ul of PRF-SFM with IGF-I (20 ng/ml). After 24h, the cells in the upper chamber were removed, while the cells that migrated to the lower chamber were fixed and stained in Coomassie Blue solution (0.25g Coomassie blue/45 ml water/45ml methanol/10 ml glacial acetic acid) for 5 min. After that,

the chambers were washed 3 times with H_2O. The cells that migrated to the lower chamber were counted under the microscope.

Inhibitors of PI-3K and MAPK. LY294002 (Biomol Res. Labs) was used to specifically inhibit PI-3K (26). UO126 (Calbiochem) was used to block MEK 1/MEK 2 kinases, and subsequently inhibit ERK1 and ERK2 (27), and SB203580 (Calbiochem) was employed to downregulate p38 MAP kinase (28). To determine the optimal non-toxic concentrations of the compounds, different doses (1-100 uM) of the inhibitors were studied. Additionally, the efficacy of LY294002 and UO126 in inhibiting the phosphorylation of the relevant downstream substrates (Akt and ERK1/ ERK2 kinases, respectively) was determined by WB. This test was not used for SB203580, as its inhibitory action in intact cells is not associated with decreased tyrosine phosphorylation of p38 kinase (28). Ultimately, for both growth and migration experiments LY294002 was used at 50 uM, UO126 at 5 uM, and SB203580 at 10uM.

RESULTS

MDA-MB-231/IGF-IR cells. To study growth related and -unrelated effects of IGF-I in metastatic cells, we used an ER-negative, metastatic breast cancer cells MDA-MB-231. These cells express low levels of the IGF-IR and do not respond to IGF-I with growth (18, 21). Since it has been established that mitogenic response to IGF-I requires a threshold level of IGF-IR expression (e.g., in NIH 3T3-like fibroblasts, ~$1.5x10^4$ IGF-IRs) (29, 30), our first goal was to test whether increasing the cellular content of the IGF-IR would induce IGF-I-dependent growth in MDA-MB-231 cells. To this end, several MDA-

MB-231 clones overexpressing the IGF-IR (MDA-MB-231/IGF-IR cells) were generated by stable transfection, and the receptor content was analyzed by binding assay, FACS analysis (data not shown) and WB (Fig. 1). We determined that MDA-MB-231 clones 2, 21, and 31 express approximately $3x10^4$, $1.5x10^4$, and $2.5x10^5$ IGF-IRs/cell, respectively, while the parental MDA-MB-231 cells express approximately $7x10^3$ IGF-IRs/cell (18). For comparison, ~ $6x10^4$ IGF-IRs were found in ER-positive MCF-7 cells (Fig. 1) (13).

IGF-IR overexpression does not enhance the growth of MDA-MB-231/IGF-IR cells in serum-containing medium. Analysis of growth curves of different MDA-MB-231/IGF-IR clones indicated that overexpression of the IGF-IR never improved proliferation in normal growth medium, and in the case of clone 31 characterized by the highest IGF-IR content, an evident growth retardation at days 2 and 4 ($p<0.05$) was observed (Fig. 2A). By contrast, overexpression of the IGF-IR augmented proliferation of ER-positive MCF-7 cells (Fig. 2B).

IGF-IR overexpression does not promote IGF-I-dependent growth or survival of MDA-MB-321 cells. Subsequent studies established that increasing the levels of the IGF-IR from $7x10^3$ up to $2.5x10^5$ was not sufficient to induce IGF-I-dependent growth response in MDA-MB-231 cells. In fact, similar to the parental cells, all MDA-MB-231/IGF-IR clones were progressively dying in PRF-SFM supplemented with 20 ng/ml (Fig. 3A). Notably, in the clone 31 expressing $2.5x10^5$ IGF-IRs/cells, cell death rate in PRF-SFM with or without IGF-I exceeded that in the parental cells and in other clones with lower IGF-IR levels (Fig. 3A and data not shown). Conversely, in ER-positive cells, the IGF-IR was effectively transducing growth signals, and increasing receptor levels from

$6x10^4$ (MCF-7 cells) to $5x10^5$ (MCF-7/IGF-IR clone 12) significantly ($p<0,05$, day 4) promoted IGF-I-dependent proliferation (Fig. 3B).

The analysis of the anti-apoptotic effects of IGF-I in the above cell lines cultured for 48 h under PRF-SFM indicated that IGF-I reduced apoptosis, by ~3-fold, in ER-positive cells, but it was totally ineffective in MDA-MB-231 and MDA-MB-231/IGF-IR cells (Tab. 1).

IGF-IR signaling in MDA-MB-231 and MDA-MB-231/IGF-IR cells. Next, we investigated, on a molecular level, the basis underlying the lack of IGF-I growth response in ER-negative cells. IGF-I signaling was studied in MDA-MB-231 cells, MDA-MB-231, clone 31, and in parallel, in the control cell lines MCF-7 and MCF-7/IGF-IR clone 12. The experiments focused on IGF-IR tyrosine kinase activity and several postreceptor signaling pathways that are known to control the growth and survival in ER-positive breast cancer cells (and many other cell types), namely the IRS-1/PI-3K, Akt, and ERK1/ERK2 pathways (1, 17, 24, 31, 33). We also probed other IGF-I effectors that have been shown to contribute to non-mitogenic responses in ER-positive breast cancer cells, such as p38 kinase and SHC (10, 25, 34).

Because both acute and chronic effects may be important for biologic IGF-I response (35), we studied IGF-IR signaling at different times following stimulation: 15 min, 1h, 2 days and 4 days. In both ER-positive and -negative cell types, IGF-I signaling seen at 15 min was identical to that at 1h, while IGF-I response at 2 days was similar to that at 4 days. Thus, Fig. 4 demonstrates the representative results obtained with cells stimulated for 15 min and 2 days.

In MDA-MB-231 and MDA-MB-231/IGF-IR cells, IGF-IR and its major substrate, IRS-1, were tyrosine phosphorylated at both time-points in a manner roughly reflecting the receptor levels. The activation of both molecules was stronger just after stimulation and weaker at 2 days of treatment (Fig. 4A). Analogous IGF-I effects were seen in MCF-7 cells and their IGF-IR-overexpressing derivatives (Fig. 4B).

One of the major growth/survival pathways initiated at IRS-1 is the PI-3K pathway (31, 36). In all cell lines studied, the p85 regulatory subunit of PI-3K was found associated with IRS-1 at 15 min and 2 days (Fig. 4A and B), which suggested a continuos stimulation of PI-3K. However, subsequent measurements of IRS-1-associated PI-3K activity in vitro demonstrated that p85/IRS-1 binding at later time points is not a direct marker of enzyme stimulation. Specifically, at 15 min after IGF-I addition, PI-3K activity was similar in both cell types, but at 2 days, in MDA-MB-231 and MDA-MB-231/IGF-IR cells, IGF-I did not stimulate PI-3K through IRS-1, or induced it very weakly, while in MCF-7 and MCF-7/IGF-IR cells, a good level of PI-3K activation was observed (Fig. 5).

The in vitro activity of PI-3K was reflected by the stimulation of its downstream effector Akt kinase. At 15 min, Akt was upregulated in response to IGF-I an all cell lines, but at 2 days, no effects of IGF-I were seen in MDA-MB-231 and MDA-MB-231/IGF-IR cells, while upregulation of Akt was still evident in MCF-7 and MCF-7/IGF-IR cells (Fig. 4C and D). Akt is known to phosphorylate (on Ser9) and downregulate the glycogen synthase kinase GSK3-beta (23, 31, 33). We found that in both cell types, the phosphorylation of GSK-3 beta reflected the dynamics of Akt activity, with no induction of phosphorylation observed at 2 days in ER-negative cells (Fig. 4C) and IGF-I-stimulated

phosphorylation in MCF-7 and MCF-7/IGF-IR cells (by 40 and 120%, respectively) (Fig. 4D).

Another effector pathway of IGF-I that is important in growth and survival involves ERK1 and ERK2 kinases (1, 35, 37). This pathway was strongly upregulated at 15 min and weakly induced at 2 days in MCF-7 and MCF-7/IGF-IR cells. In MDA-MB-231 and MDA-MB-231/IGF-IR cells, the basal activation of ERK1/2 kinases was always high, and the addition of IGF-I only minimally (10-20%) induced the enzymes at 15 min, with no effects seen at 2 days (Fig. 4E and F).

p38, a stress-induced MAP kinase, and a known mediator of non-growth responses in breast cancer cells (34), was strongly stimulated by IGF-I in ER-negative cells at 15 min (Fig. 4E). By contrast, in ER-positive cells, the enzyme was induced only at 2 days (Fig. 4F). The stimulation of SHC, a substrate of the IGF-IR involved in migration and growth in ER-positive cells (10, 25), was weak in all cell types and no differences in the activation patterns were observed (data not shown).

Reactivation of Akt kinase in MDA-MB-231 cells. Previous results indicated that MDA-MB-231 and MDA-MB-231/IGF-IR cells are unable to sustain IGF-I-dependent activation of the PI-3K/Akt survival pathway when cultured in the absence of serum for 2-4 days. Consequently, we tested whether cell death under PRF-SFM conditions can be reversed by forced overexpression Akt kinase. Two different expression plasmids encoding constitutively active forms of Akt kinase, Myr-Akt and Akt/E40K, (23) were transiently transfected into MDA-MB-231 cells, and as a control, into MCF-7 cells. The increased expression of Akt, without any concomitant cytotoxicity, was evident in all

cases (Fig. 6A and data not shown), however; no significant improvement in the growth or survival with or without IGF-I in ER-negative cells was observed (Fig. 6B and data not shown). A tendency of MDA-MB-231 cells to survive better at 2 days post transfection (at the time of the greatest Akt activity) was noted, but the differences did not reach the statistical significance ($p>0.05$). This suggested that although improper Akt stimulation may be associated with the lack of IGF-I mitogenic response in ER-negative cells, some other pathways must also be responsible for growth and survival.

Inhibition of IGF-IR signaling pathways. To complement the above studies, we examined the importance of the PI-3K, ERK1/ERK2, and p38 kinase pathways in IGF-I-dependent growth and survival of ER-positive and ER-negative breast cancer cells using specific inhibitors (26-28). The efficacy of PI-3K and ERK1/ERK2 inhibitors was first tested by establishing their effects on the activity of target proteins (Fig. 7). Tab. 2 demonstrates that the inhibition of PI-3K with LY294002 reduced the growth of MCF-7 and MCF-7/IGF-IR cells, but did not have significant impact or had only minimal effects on MDA-MB-231 and MDA-MB-231/IGF-IR cells. Furthermore, the action of LY294002 was counteracted by IGF-I in ER-positive, but not in ER-negative cells.

The inhibition of ERK1/ERK2 with UO126, a compound targeting the upstream kinases MEK1/MEK2, affected the growth and/or survival in both cell types, but only in MCF-7 and MCF-7/IGF-IR cells, IGF-I was able to oppose this effect. Targeting p38 kinase with SB203580 reduced survival of MDA-MB-231 and MDA-MB-231/IGF-IR cells, and to a lesser extent the growth and survival of MCF-7 and MCF-7/IGF-IR cells.

IGF-I was not able to reverse the anti-mitogenic action of the p38 kinase inhibitor in either of the cell lines studied (Tab. 2).

Cumulatively, these results suggested that in ER-positive cells, IGF-I transmits mitogenic signals through PI-3K and ERK1/ERK2 pathways. By contrast, IGF-I does not induce growth or survival signal through these pathways in ER-negative cells.

IGF-I stimulates migration in MDA-MB-231 cells. Next, we investigated the non-mitogenic effects of IGF-I in ER-negative and ER-positive breast cancer cells. Unlike with the growth and survival responses, we found that IGF-IR was an effective transducer of non-mitogenic signals in MDA-MB-231 and MDA-MB-231/IGF-IR cells. Specifically, in the chemoattraction experiments, IGF-I placed in the lower chamber was stimulating migration of ER-negative cells in a manner reflecting IGF-IR content. In parallel experiments, the same doses of IGF-I induced migration in ER-positive cells (Tab. 3). The addition of IGF-I to the upper chamber always suppressed migration of all cell lines (data not shown).

IGF-I pathways regulating migration of MDA-MB-231 cells. Using the inhibitors of PI-3K, ERK1/ERK2, and p38 kinases, we determined which pathways of the IGF-IR are involved in migration of ER-positive and ER-negative cells. As demonstrated in Tab. 4, downregulation of PI-3K with LY294002 inhibited basal migration of both cell types, with a more pronounced effect in ER-negative cells. Similarly, blockade of p38 kinase reduced motility of all cell lines studied. Interestingly, inhibition of ERK1 and ERK2 with UO126 resulted in the stimulation of migration in both ER-positive and ER-negative cells. The addition of IGF-I as a chemoattractant significantly counteracted the effects of all

3 inhibitors, however, no clear association between the cellular levels of the IGF-IR and this competing action of IGF-I was noted (Tab. 4). These results suggested that IGF-I-dependent motility in both types of cells requires the PI-3K and p38 kinase pathways, and may be normally suppressed by the activation of ERK1/ERK2 kinases.

DISCUSSION

The experimental and clinical evidence supports the notion that hypercativation of the IGF-IR may be critical in early steps of tumor development, promoting cell growth, survival, and resistance to therapeutic treatments. However, the function of the IGF-IR in the later stages of the disease, including metastasis, is still obscure (1). For instance, whereas the IGF-IR has been found overexpressed in primary breast tumors, its levels, like ER levels, appear to undergo reduction during the course of the disease (1). According to Pezzino et al., who studied the IGF-IR status in two patient subgroups representing either a low risk (ER- and PgR-positive, low mitotic index, diploid) or a high risk (ER- and PgR-negative, high mitotic index, aneuploid) population, there is a highly significant correlation between IGF-IR expression and better prognosis (38). Similar conclusions were reached by Peyrat and Bonneterre (39). Therefore, it has been proposed that similar to the ER, the IGF-IR marks more differentiated tumors with better clinical outcome. However, it has also been argued that the IGF-IR may play a role in early steps of tumor spread since node-positive/IGF-IR-positive tumors appeared to have a worse prognosis than node-negative/ IGF-IR-positive tumors (1, 39). In addition, quite rare cases of ER-negative but IGF-IR-positive tumors are associated with shorter disease-free survival (40).

In breast cancer cell lines, a hormone-dependent and less aggressive phenotype correlates with a good IGF-IR expression (1, 18, 39). By contrast, highly metastatic ER-negative breast cancer cell lines express low levels of the IGF-IR and generally do not respond to IGF-I with growth (1, 18-21). However, many ER-negative cell lines appear to depend on the IGF-IR for tumorigenesis and metastasis. For instance, blockade of the IGF-IR in MDA-MB-231 cells by anti-IGF-IR antibody reduced migration *in vitro* and tumorigenesis *in vivo*, and expression of a soluble IGF-IR in MDA-MB-435 cells impaired growth, tumorigenesis and metastasis in animal models (1, 14, 16, 22). These observations suggest that some growth-unrelated pathways of the IGF-IR may be operative in the context of ER-negative cells.

Here we studied whether this particular IGF-I-dependence of metastatic breast cancer cells relates to the non-mitogenic function of the IGF-IR, such as cell migration. Our experiments indicated that the IGF-IR is an effective mediator of cell motility. Furthermore, IGF-I-induced migration was proportional to IGF-IR content. We demonstrated, for the first time, that in MDA-MB-231 ER-negative cells, IGF-IR signaling pathways responsible for cell movement include PI-3 and p38 kinases. Indeed, an acute IGF-I stimulation of MDA-MB-231 and MDA-MB-231/IGF-IR cells appears to induce both PI-3K and p38 kinases, suggesting that this short-time activation may be involved in migration. Both of these pathways have been previously shown to regulate cell motility in breast cancer cells and other cell types (34, 41). Interestingly, the migration of both ER-negative and ER-positive cells was stimulated in the presence of a specific MEK1/MEK2 inhibitor UO126. We observed this effect over a broad range of UO126 doses (1-10 uM)

and in several MDA-MB-231- and MCF-7-derived clones; the same doses always reduced IGF-I-dependent phosphorylation of ERK1/ERK1 (Fig. 7) and supressed cell proliferation in serum-containing medium and PRF-SFM (data not shown and Tab. 2). A slight stimulation of migration in MDA-MB-231, but not in MCF-7 cells, was also observed with another MEK (and ERK1/ERK2) inhibitor PD98059 at 5 uM (Surmacz, unpublished data). These peculiar effects suggest that normally the ERK1/ERK2 pathway positively regulates cell growth and survival, and negatively impacts on cell migration.

In contrast with the positive effects of IGF-I on cell motility in ER-negative and ER-positive breast cancer cells, this growth factor never stimulated the proliferation of MDA-MB-231 cells, while it induced the growth of MCF-7 cells and MCF-7-derived clones overexpressing the IGF-IR. It is has been established by Rubini et al. (29) and Reiss et al. (30) that mitogenic response to IGF-I requires a threshold level of IGF-IR expression (in fibroblasts, ~$1.5x10^4$). However, we demonstrated that increasing the levels of IGF-IR from ~$7x10^3$ up to ~$2.5x10^5$ and subsequent upregulation of IGF-IR tyrosine phosphorylation was not sufficient to induce the growth of MDA-MB-213 cells in IGF-I. Similar results were obtained by Jackson and Yee, who showed that overexpression of IRS-1 in ER-negative MDA-MB-435A and MDA-MB-468 breast cancer cells did not stimulate IGF-I-dependent mitogenicity (20). These authors suggested that the lack of IGF-I response, even in IRS-1 overexpressing ER-negative cells, was related to insufficient stimulation of ERK1/ERK2 and PI-3K pathways (20). Defective insulin response in ER-negative cell lines has also been described by Costantino et al. and linked with an increased tyrosine phosphatase activity (42).

Our experiments suggest that the lack of IGF-I mitogenicity in MDA-MB-231 and MDA-MB-231/IGF-IR cells is not related to the impaired IGF-IR or IRS-1 tyrosine phosphorylation. The cells are also able to respond to an acute IGF-I stimulation with a good activation of the PI-3K/Akt and ERK-1/ERK2 pathways. We hypothesize that this transient stimulation could be sufficient to induce some IGF-I response, such as cell motility. Mitogenic response, on the other hand, may rely on a more sustained activation of critical IGF-IR signals, as demonstrated by Swantek and Baserga in mouse embryo fibroblasts (35). Indeed, the most significant difference in IGF-I signal between ER-negative and ER-positive cells rested in the impaired long-term (2 days) stimulation of the PI-3K/Akt pathway: MDA-MB-231 and MDA-MB-231/IGF-IR cells were unable to sustain this IGF-I-induced signal for 2 days, while in MCF-7 and MCF-7/IGF-IR cells, the PI-3K/Akt pathway was still active at this time.

The subsequent experiments, however, demonstrated that increasing Akt activity is not sufficient to stimulate the survival or growth (with or without IGF-I) of ER-negative cells, which suggested that while Akt could be important in these processes, other pathways are also necessary. The identity of these pathways in presently unknown, but we obtained preliminary results indicating that the survival and growth of MDA-MB-231 cells can be significantly improved by the overexpression of an IGF-IR truncated at either aa 1229 or aa 1245 (Morelli and Surmacz, unpublished data). The anti-apoptotic effects induced by truncation of the IGF-IR have been described in other cell systems, but their molecular bases are not known (43).

In summary, our data suggest that IGF-IR signaling and function undergo evolution during breast cancer progression. In ER-positive cells, IGF-IR transmits various signals, such as growth, survival, migration, adhesion. In ER-negative cells, the growth-related functions of the IGF-IR become attenuated, but the receptor is still able to control non-mitogenic processes, such as migration. It is likely that this kind of evolution concerns also the response to other growth factors. Epidermal growth factor, for instance, is an effective mitogen for ER-positive breast cancer cells, but does not stimulate the proliferation or survival in MDA-MB-231 cells, despite high EGF-R expression (44). However, as recently demonstrated by Price et al., EGF is a potent chemoattractant for MDA-MB-231 cells. EGF-induced migration in MDA-MB-231 cells requires PI-3K and PLC gamma and is not inhibited by antagonists of ERK1/ERK2 (44).

In conclusion, mitogenic and non-mitogenic pathways induced by growth factors in breast cancer cells may be dissociated, and attenuation of one is not necessarily linked with the cessation of the other. Delineating the non-mitogenic responses will be critical for the development of drugs specifically targeting metastatic cells.

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TABLES

Tab. 1. Effects of IGF-I on apoptosis in ER-negative and ER-positive cells.

Cell Line	*Apoptosis (%)*	
	SFM	*SFM+IGF-I*
MDA-MB-231	**41.4**±3.0	**46.0**±1.9
MDA-MB-231/IGF-IR	**50.1**±4.1	**53.3**±4.2
MCF-7	**14.5**±0.2	**4.2**±0.1
MCF-7/IGF-IR	**10.1**±1.3	**2.8**±0.1

Apoptosis was studied in MDA-MB-231 cells, MDA-MB-231/IGF-IR clone 31, MCF-7 cells and MCF-7/IGF-IR clone 12. The cells were cultured for 48 h in PRF-SFM, and the apoptotic index (% apoptotic cells/total cell number in the field) was determined by TUNEL, as described under Materials and Methods. The results are average from at least 3 experiments; SD values are given.

Tab. 2. Effects of PI-3K and MAPK inhibitors on growth and survival of ER-negative and ER-positive breast cancer cells.

Cell Line	*Inhibition (%)*					
	LY294002 (PI-3K)		*UO126 (MEK)*		*SB203580 (p38)*	
	SFM	**+IGF**	**SFM**	**+IGF**	**SFM**	**+IGF**
MDA-MB-231	**9.4**±1.0	**7.8**±0.8	**35.0**±2.6	**39.0**±2.7	**47.8**±2.2	**42.5**±4.4
MDA-MB-231/IGF-IR	**11.1** ±1.2	**12.3**±0.9	**18.3**±0.9	**22.9**±1.3	**29.5**±2.0	**35.6**±3.6
MCF-7	**68.8**±3.3	**35.0**±1.2	**42.6**±3.8	**26.3**±2.5	**11.7**±1.2	**10.0**±0.4
MCF-7/IGF-IR	**73.2**±6.7	**34.6**±2.7	**49.4**±3.9	**20.2**±1.5	**24.7**±0.2	**25.9**±0.9

MDA-MB-231 cells, MDA-MB-231/IGF-IR clone 31, MCF-7 cells, and MCF-7/IGF-IR clone 12 were cultured in PRF-SFM with or without IGF-I in the presence or absence of the inhibitors for 2 days, as described under Materials and Methods. The difference (in percentages) between the number of live cells under treatment and the number of cells cultured under control conditions was defined as inhibition (%). The results are average from 3 experiments; SDs are given.

Tab. 3. IGF-I-induced migration in ER-negative and ER-positive breast cancer cells.

Cell Line	*IGF-I-Induced Migration (%)*
MDA-MB-231	29±3.0
MDA-MB-231/IGF-IR	74±4.5
MCF-7	11±0.2
MCF-7/IGF-IR	30±2.9

The migration of MDA-MB-231 and MCF-7 cells as well their IGF-IR overexpressing derivatives, MDA-MB-231/IGF-IR clone 31 and MCF-7/IGF-IR clone 12 was determined as described under Materials and Methods. The results are average (±SD) from at least 5 experiments.

Tab. 4. Effects of PI-3K and MAPK inhibitors on migration of ER-negative and positive breast cancer cells.

Cell Line	*Change (%)*					
	LY294002 (PI-3K)		*UO126 (MEK)*		*SB203580 (p38)*	
	SFM	**+IGF**	**SFM**	**+IGF**	**SFM**	**+IGF**
MDA-MB-231	**-47.2**±3.3	**-13.3**±1.0	**+53.4**±3.5	**+36.4**±2.2	**-30.2**±2.9	**-8.5**±0.7
MDA-MB-231/IGF-IR	**-41.0**±4.2	**-9.2**±0.4	**+29.0**±2.0	**+12.6**±0.7	**-40.1**±0.4	**-2.5**±0.0
MCF-7	**-15.4**±0.8	**-8.7**±0.2	**+94.9**±3.9	**+56.4**±1.7	**-18.9**±1.1	**-5.6**±0.2
MCF-7/IGF-IR	**-33.1**±2.7	**-12.8**±0.3	**+65.6**±5.4	**+23.8**±1.9	**-24.8**±0.8	**-1.7**±0.1

The migration of MDA-MB-231 cells, MDA-MB-231/IGF-IR clone 31, MCF-7 cells, and MCF-7/IGF-IR clone 12 was tested in modified Boyden chambers as described under Materials and Methods. The inhibitors were added to the cells in the upper chamber at the time of cell plating, and their effect on basal (SFM) or IGF-I-induced (+IGF) migration was assessed 24h later. The values represent % change relative to the migration at basal conditions in PRF-SFM (SFM) without inhibitors or chemoattractants. The experiments were repeated at least 3 times; the average results ±SD are given.

LEGENDS TO FIGURES

Fig. 1. MDA-MB-231/IGF-IR clones.

MDA-MB-231/IGF-IR cells were generated by stable transfection with an IGF-IR expression vector, as described under Materials and Methods. In several G418-resistant clones, the expression of the IGF-IR protein was tested by WB with anti-beta subunit IGF-IR pAb (Santa Cruz) in 50 ug of total protein lysate. For comparison, MCF-7 cells and MCF-7/IGF-IR clone 15 with known levels of IGF-IR ($6x10^4$ and $3x10^6$, respectively) (13) are shown. Low levels of IGF-IR in MDA-MD-231 cells (~$7x10^3$ receptors/cell) are not visible in this blot, but were detectable in its phosphorylated form by IP and WB in 500 ug of protein lysates (see Fig. 4A). The estimated expression of the IGF-IR in clones 2, 21, and 31 is $1.5x10^4$, $3x10^4$, and $2.5x10^5$ receptors/cell, respectively.

Fig. 2. Effect of IGF-IR overexpression on the growth of ER-negative and ER-positive cells in serum-containing medium.

MDA-MB-231 cells, MDA-MB-231/IGF-IR clones 2, 21, and 31 **(A),** and their ER-positive counterparts, MCF-7 cells and MCF-7/IGF-IR, clones 12 and 15 **(B),** were plated in DMEM:F12 plus 5% CS. The cells were counted at 50% confluence (day 0), and at subsequent days 1, 2, and 4. The results are average from 3 experiments.

Fig. 3. IGF-I-dependent growth and survival of ER-negative and ER-positive breast cancer cells.

MDA-MB-231 cells and MDA-MB-231/IGF-IR clone 31 **(A)** as well as MCF-7 cells and MCF-7/IGF-IR clone 12 **(B)** were synchronized in PRF-SFM and treated with IGF-I, as described in Materials and Methods. The cells were counted at days 0, 1, 2, and 4 of treatment. The results are average from at least 3 experiments.

Fig. 4. IGF-I signaling in ER-negative and ER-positive breast cancer cells.

The activation of IGF-IR/IRS-1/PI-3K signaling **(A** and **B)**, Akt/GSK-3 signaling **(C and D),** and ERK1/ERK2 and p38 kinase signaling **(E** and **F)** was tested in MDA-MB-231 cells, MDA-MB-231/IGF-IR clone 31, MCF-7 cells, and MCF-7/IGF-IR clone 12. The cells were synchronized in PRF-SFM and treated with IGF-I for 15 min or 2 days. The cellular levels of the IGF-IR and IRS-1 were detected by IP and WB in 500 ug of total protein lysate using specific antibodies (Materials and Methods). IGF-IR and IRS-1 tyrosine phosphorylation (PY) was assessed upon striping and reprobing the same filters with the anti-PY20 antibody. The levels of IRS-1-bound p85 subunit of PI-3K (p85/IRS-1) were analyzed in IRS-1 IPs by WB with anti-p85 Ab. The levels and activity of Akt, GSK-3, ERK1/ERK2, and p38 kinases were probed by WB in 50 ug of total cellular lysates using specific Abs. The Abs used are listed under Materials and Methods. Representative results are shown. Note decreased IRS-1 expression under 15 min IGF-I treatment in ER-positive cells, as described before (46).

Fig. 5. IGF-I-induced PI-3 kinase activity in ER-negative and ER-positive cells.

MDA-MB-231 cells, MDA-MB-231/IGF-IR clone 31, MCF-7 cells, and MCF-7/IGF-IR clone 12 were synchronized in PRF-SFM and treated with IGF-I for 15 min or 2 days. IRS-1-bound PI-3K activity was measured in vitro in IRS-1 IPs as described under Materials and Methods. The experiments were repeated at least 3 times; the bars indicate SDs.

Fig. 6. Effect of increased Akt activity on the survival of MDA-MB-231 cells.

MDA-MB-231 cells were transiently transfected with expression plasmids encoding 2 different constitutively active Akt kinase mutants (Myr-Akt and Akt/E40K). The Akt vectors contained HA-tag for easy detection. The cells treated with the transfection mixture lacking plasmid DNA (Mock) served as control. The expression of the plasmids as well as the activity and the levels of Akt kinase in the transfected cells were monitored at 2 and 4 days after transfection. 50 ug of total protein lysates were sequentially probed by WB with anti-HA, anti-active Akt, and then anti-total Akt specific Abs (described under Materials and Methods). Representative results are shown **(A)**.

In parallel, the number of cells was assessed at days 0 (post-transfection media change), 1, 2, and 4 after transfection. The results are average from at least 3 times. For the clarity of the graph, the SDs are not pictured **(B)**.

Fig. 7. PI-3K and ERK1/ERK2 kinase inhibitors.

Synchronized cultures of MDA-MB-231 and MCF-7 cells were treated with LY294002 or UO126 in the presence or absence of IGF-I for 15 min, as described under Materials and Methods. The activities of Akt kinase, a downstream substrate of PI-3K, and ERK1/ERK2 kinases were determined by WB in 50 ug of protein lysates using specific antibodies. Representative results are shown.

ABBREVIATIONS

CS, calf serum; EGF, epidermal growth factor; EGFR, EGF receptor; ER, estrogen receptor; FACS, fluorescence-assisted cell sorting; GSK-3, glycogen synthase kinase-3; IGF-I, insulin-like growth factor I; IGF-IR, IGF-I receptor; IP, immunoprecipitation; IRS-1, insulin receptor substrate 1; mAb, monoclonal antibody; MAPK, mitogen activated kinases; pAb, polyclonal antibody; PI-3K, phosphatidyl inositol 3-kinase; PLC-gamma, phospholipase C gamma; PgR, progesteron receptor; PRF-SFM, phenol red-free serum-free medium; PY, tyrosine phosphorylation; SFM, serum free medium; WB, Western blotting.

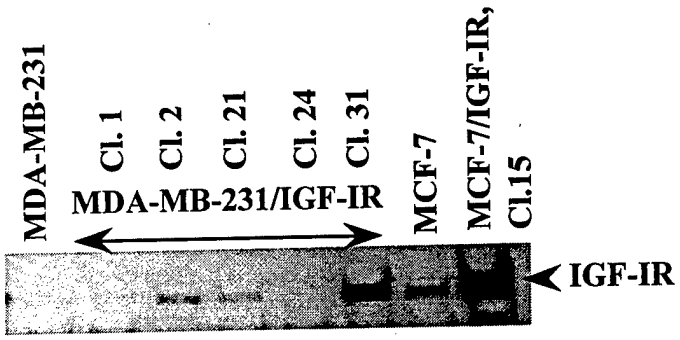

MDA-MB-231
Cl. 1
Cl. 2
Cl. 21
Cl. 24
Cl. 31
MDA-MB-231/IGF-IR
MCF-7
MCF-7/IGF-IR, Cl.15
IGF-IR

FIGURE 1

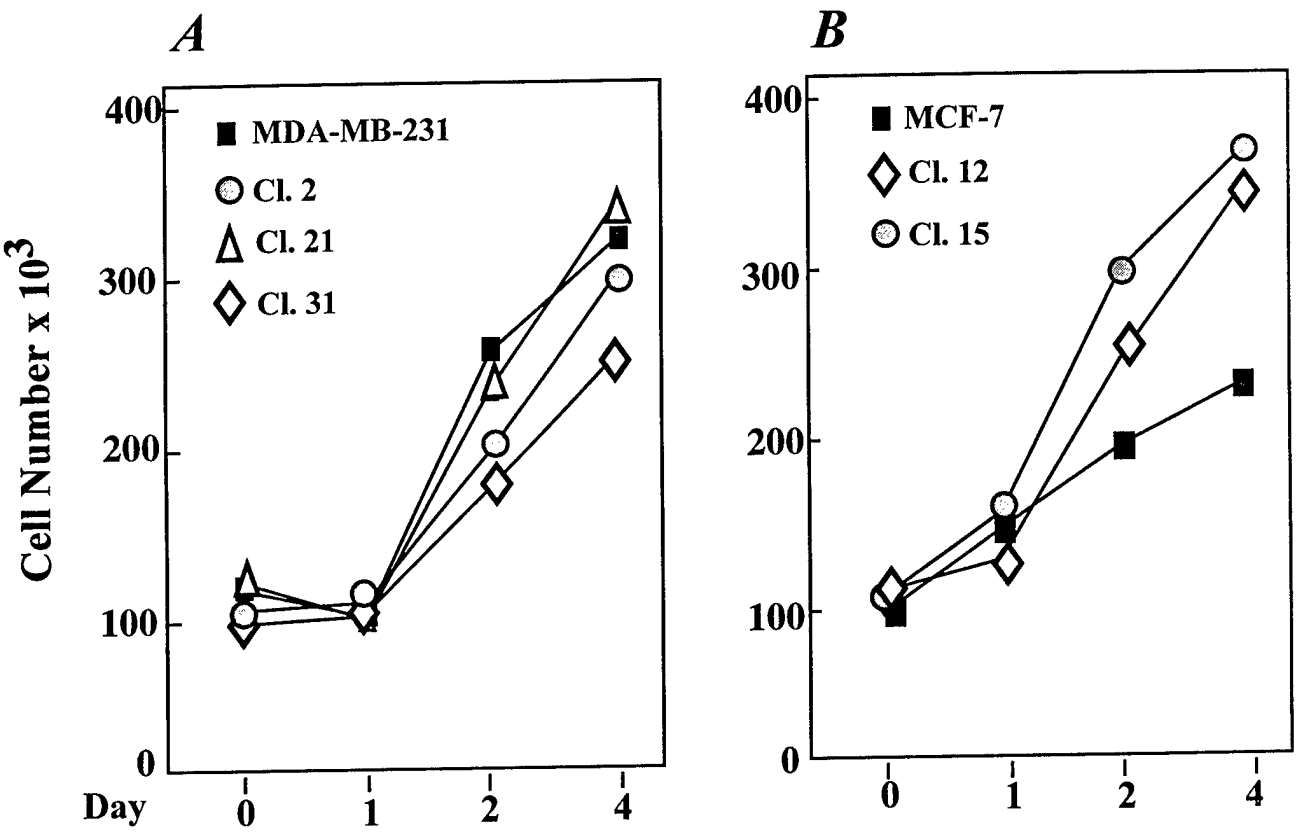

A
B
Cell Number x 10^3
400
300
200
100
0
Day
0
1
2
4
MDA-MB-231
Cl. 2
Cl. 21
Cl. 31
MCF-7
Cl. 12
Cl. 15

FIGURE 2

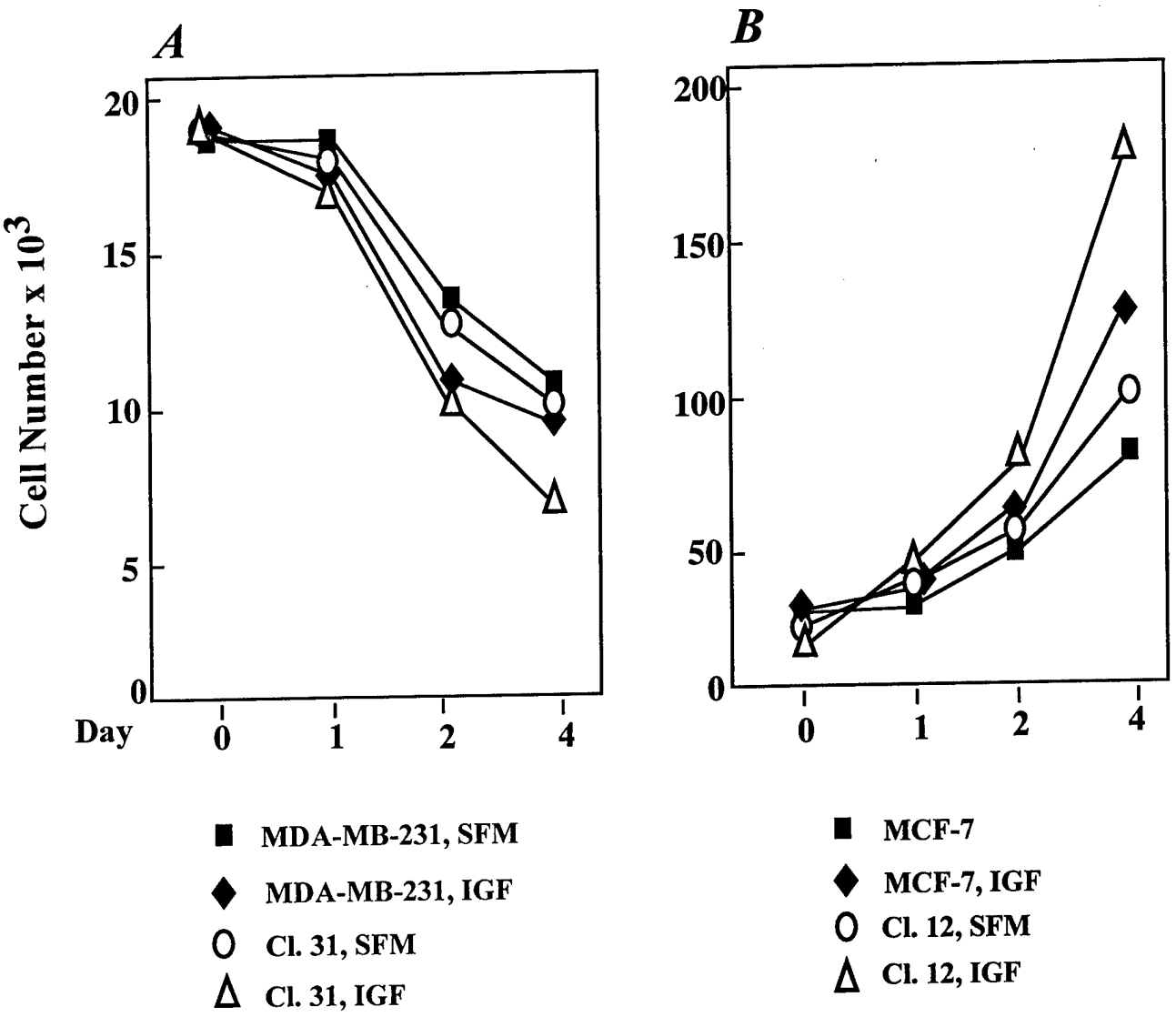

A
B
Cell Number x 10^3
20
15
10
5
0
Day
0
1
2
4
200
150
100
50
0
MDA-MB-231, SFM
MDA-MB-231, IGF
Cl. 31, SFM
Cl. 31, IGF
MCF-7
MCF-7, IGF
Cl. 12, SFM
Cl. 12, IGF

FIGURE 3

A

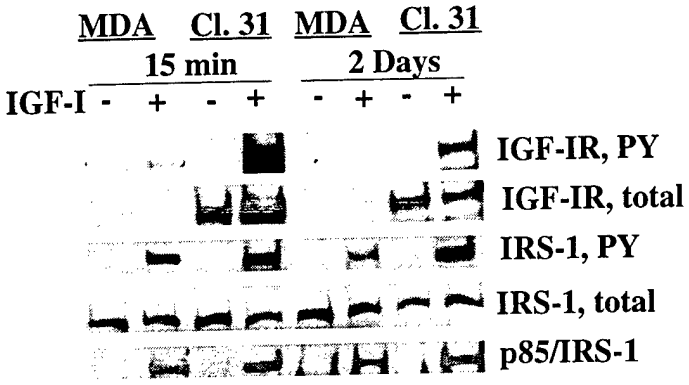

MDA
Cl. 31
MDA
Cl. 31
15 min
2 Days
IGF-I - + - + - + - +
IGF-IR, PY
IGF-IR, total
IRS-1, PY
IRS-1, total
p85/IRS-1

FIGURE 4

B

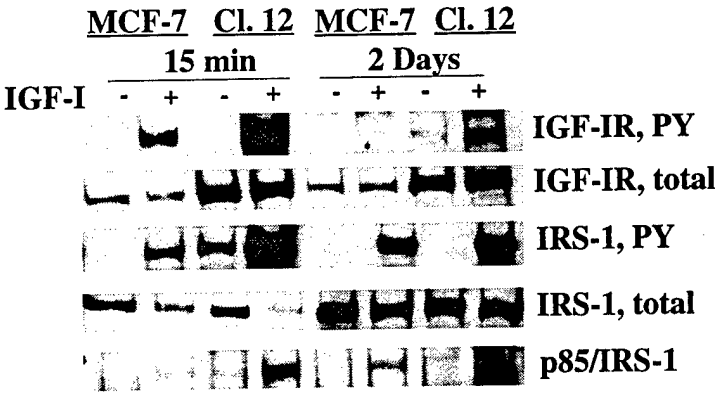

MCF-7
Cl. 12
MCF-7
Cl. 12
15 min
2 Days
IGF-I
- + - + - + - +
IGF-IR, PY
IGF-IR, total
IRS-1, PY
IRS-1, total
p85/IRS-1

FIGURE 4

C

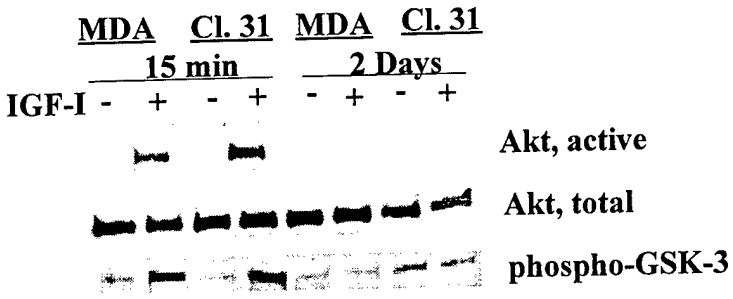

MDA
Cl. 31
MDA
Cl. 31
15 min
2 Days
IGF-I - + - + - + - +
Akt, active
Akt, total
phospho-GSK-3

FIGURE 4

D

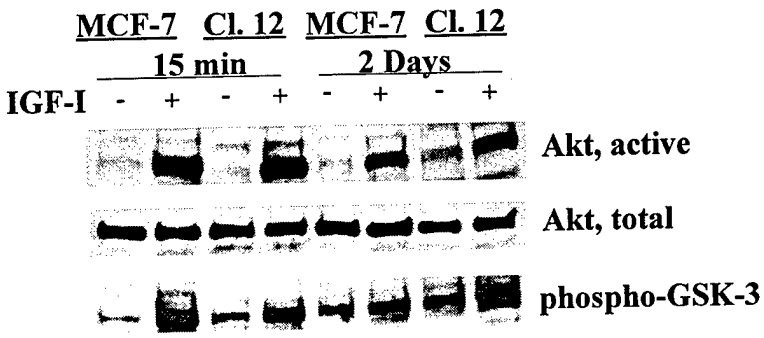

MCF-7
Cl. 12
MCF-7
Cl. 12
15 min
2 Days
IGF-I - + - + - + - +
Akt, active
Akt, total
phospho-GSK-3

FIGURE 4

E

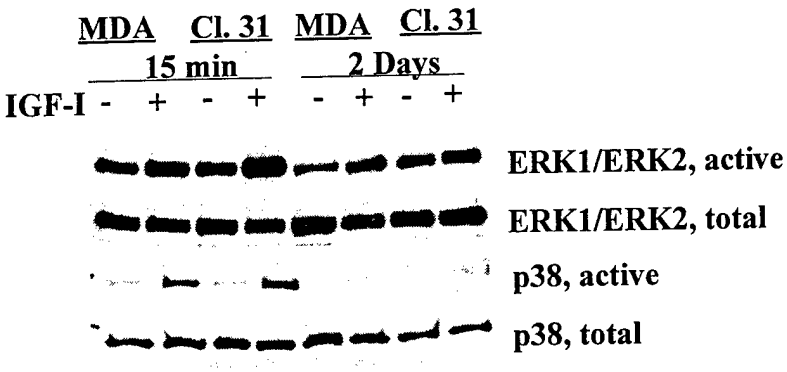

MDA
Cl. 31
MDA
Cl. 31
15 min
2 Days
IGF-I - + - + - + - +
ERK1/ERK2, active
ERK1/ERK2, total
p38, active
p38, total

FIGURE 4

F

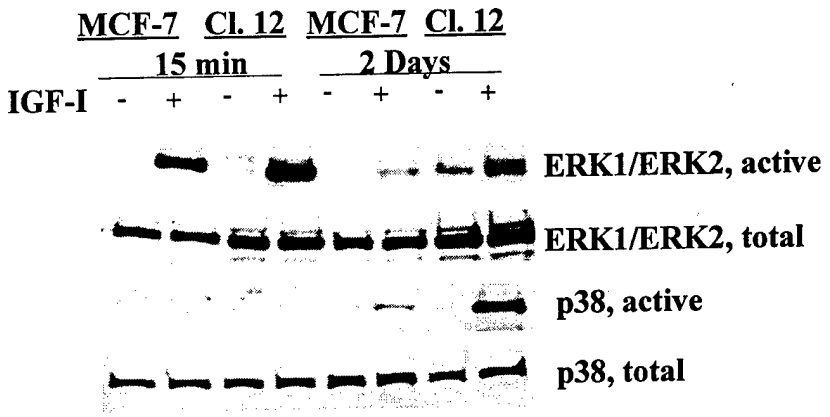
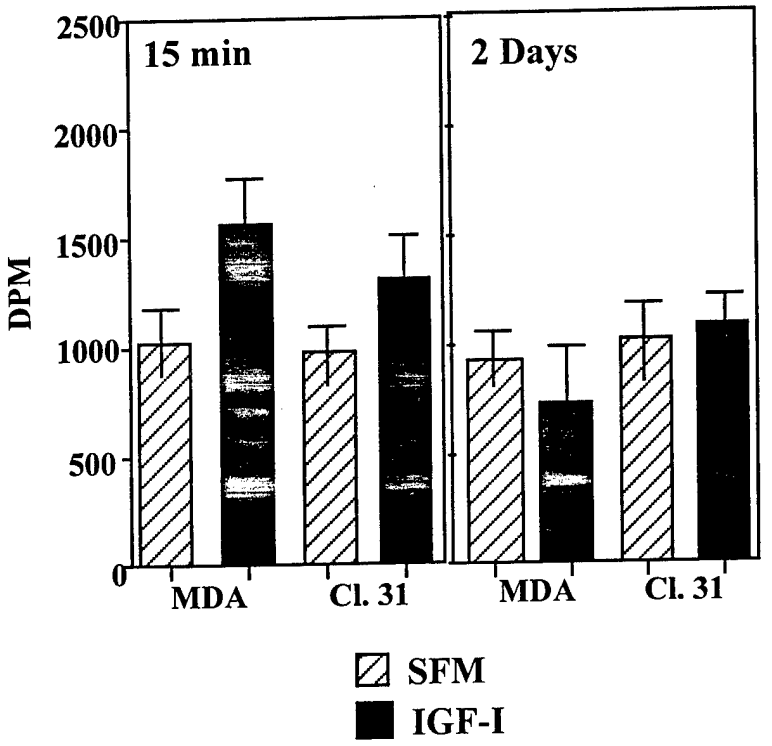

MCF-7 Cl. 12 MCF-7 Cl. 12
15 min 2 Days
IGF-I - + - + - + - +
ERK1/ERK2, active
ERK1/ERK2, total
p38, active
p38, total

FIGURE 4


15 min
2 Days
DPM
2500
2000
1500
1000
500
0
MDA
Cl. 31
MDA
Cl. 31
SFM
IGF-I

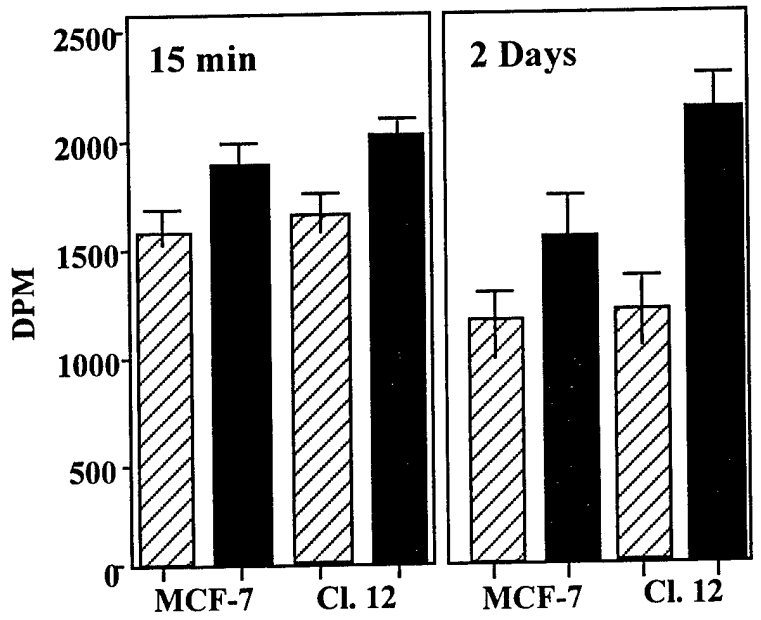

15 min
2 Days
DPM
2500
2000
1500
1000
500
0
MCF-7
Cl. 12
MCF-7
Cl. 12

FIGURE 5

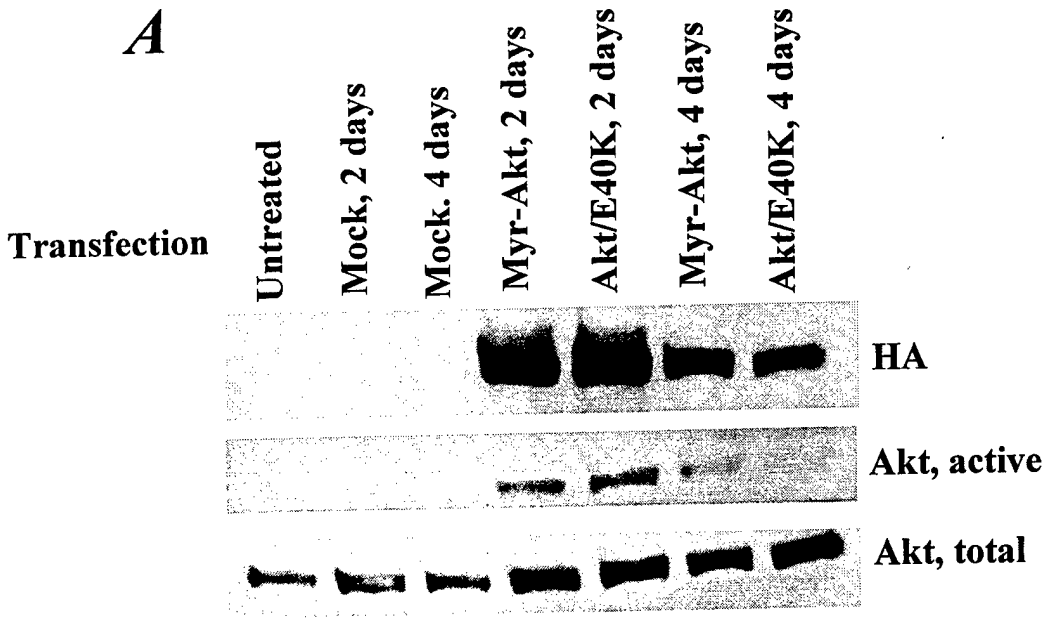

A
Transfection
Untreated
Mock, 2 days
Mock. 4 days
Myr-Akt, 2 days
Akt/E40K, 2 days
Myr-Akt, 4 days
Akt/E40K, 4 days
HA
Akt, active
Akt, total

FIGURE 6

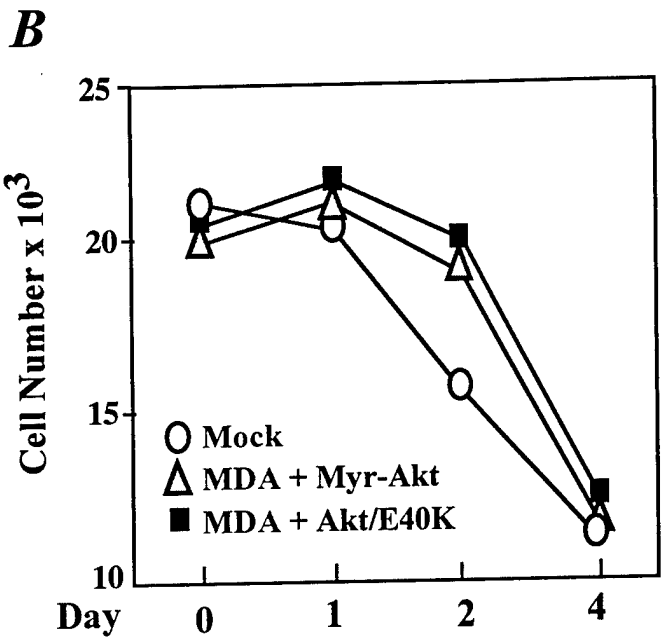

B
25
20
15
10
Cell Number x 10^3
Mock
MDA + Myr-Akt
MDA + Akt/E40K
Day
0
1
2
4

FIGURE 6

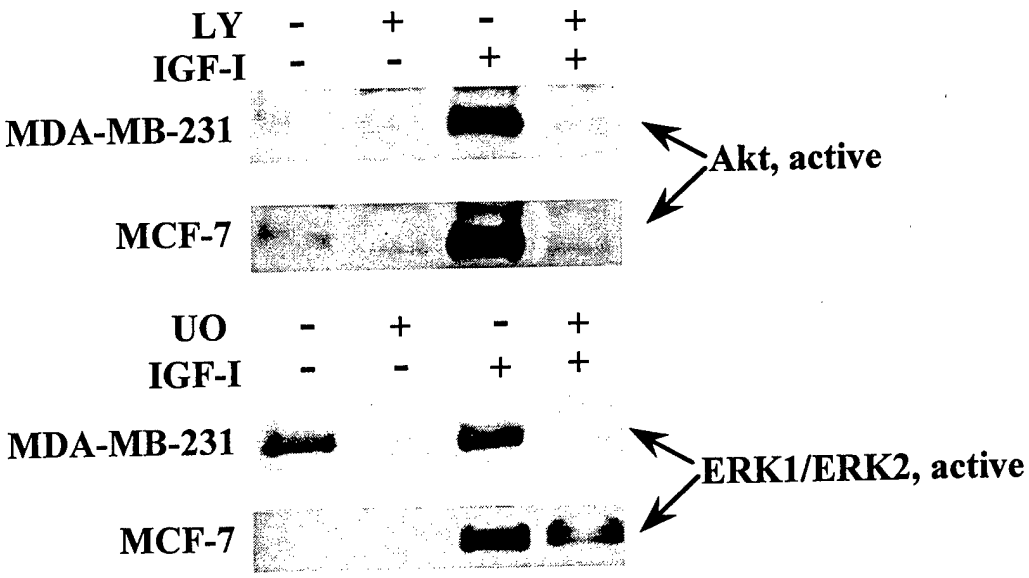

LY - + - +
IGF-I - - + +
MDA-MB-231
MCF-7
Akt, active
UO - + - +
IGF-I - - + +
MDA-MB-231
MCF-7
ERK1/ERK2, active

FIGURE 7