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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 recently implicated in breast cancer. The IGF-IR is often overexpressed in estrogen receptor (ER)-positive breast tumors and this feature predicts enhanced tumor drug- and radio-resistance and cancer recurrence at the primary site. Our previous work demonstrated that the IGF-IR induces cell survival, growth and estrogen-independence in hormone-sensitive cells. Our next goal was to study IGF-IR function in metastatic breast cancer cells that have lost the expression of important markers of good prognosis such as E-cadherin and the ER. Using MDA-MB-231 metastatic breast cancer cells and their IGF-IR-overexpressing derivatives, we demonstrated that, unlike in ER-positive cells, IGF-I has no growth or survival function in these cells 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, 2).

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. 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).

Using in vitro model systems developed in our laboratory, we asked how the IGF-IR and its different signaling pathways contribute to breast tumor development and progression. We focused on abnormal proliferation and survival, enhanced resistance to anti-tumor treatments, and augmented migration and invasion. Our previous data strongly suggested that hyperactivation of the IGF-IR in ER-positive breast cancer cells stimulates cell proliferation and survival, and contributes to the development of antiestrogen resistance. Overexpression of the IGF-IR also improved cell-cell adhesion through an E-cadherin-dependent mechanism.

During this reporting period, we addressed the function of the IGF-IR in ER-negative, E-cadherin-negative breast cancer cells characterized by a less differentiated phenotype and enhanced metastatic properties.

TECHNICAL REPORT

Administrative Note: This postdoctoral fellowship was originally awarded to Dr. M. Guvakova. After the completion of the second year, Dr. Guvakova transferred to another institution and since then the program has been carried out by Dr. M. Bartucci under the guidance of the same mentor (Dr. E. Surmacz) whithin the recipient institution. Due to administrative delays caused by this transfer, there was an interruption in funding and research from September 1999 to January 2000. Consequently, a 1 year no-cost extension has been requested and granted till August 2001, and the research plan has been extended into Year 4.

Science Report. During the reporting performance period, the experiments proceeded according to the SOW Year 3. Using MDA-MB-231 cells, a well-described metastatic cell line lacking E-cadherin 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).*

Since IGF-I is an important mitogen 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 IGF-IR signaling is different in ER-positive and ER-negative cells. In ER-positive non-metastatic cells, the IGF-IR regulates growth, survival, and migration, whereas in ER-negative metastatic cells, only motogenic (migratory) signaling is operative;

- 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. Mauro, L., Sisci, D., Bartucci, M., Salerno, M., Kim, J., Tam, T., Guvakova, M., Ando, S., Surmacz, E. SHC-alpha5 beta1 integrin interactions regulate breast cancer cell adhesion and motility. Exp. Cell Res., 252: 439-448, 1999.

Presentations/Abstracts:

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

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: None

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Conclusions

The IGF-IR has different functions in ER-positive and ER-negative cells. Targeting the IGF-IR in ER-positive cells will result in the inhibition of cell growth and survival. Blocking the IGF-IR in ER-negative cells will not affect growth properties (at least in vitro), but will inhibit cell migration. Thus, anti-IGF strategies may prove beneficial at different stages of tumor progression.

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3. 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.

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.

FIGURE 1

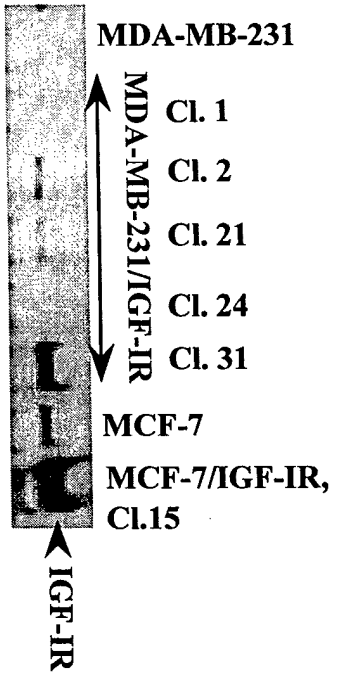
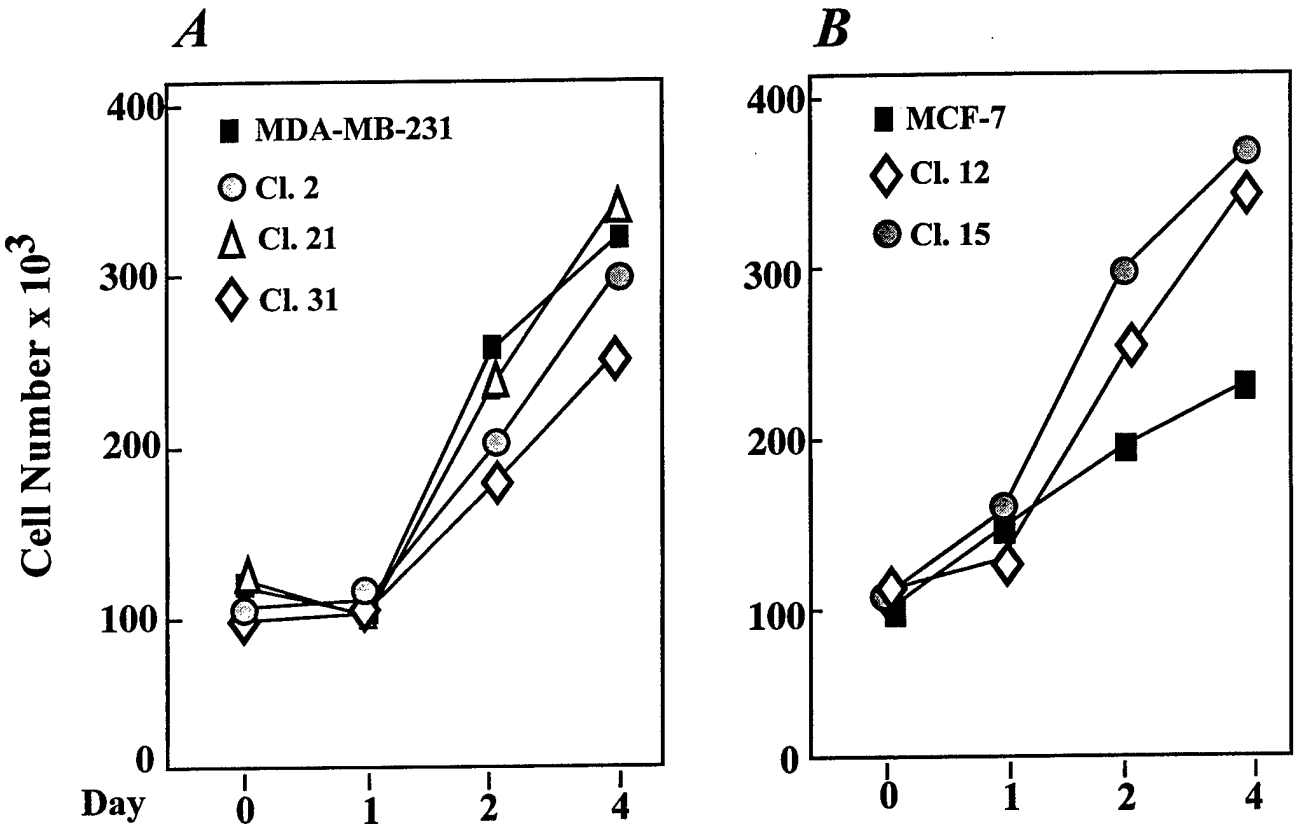

MDA-MB-231
MDA-MB-231/IGF-IR
Cl. 1
Cl. 2
Cl. 21
Cl. 24
Cl. 31
MCF-7
MCF-7/IGF-IR, Cl.15
IGF-IR

FIGURE 2


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

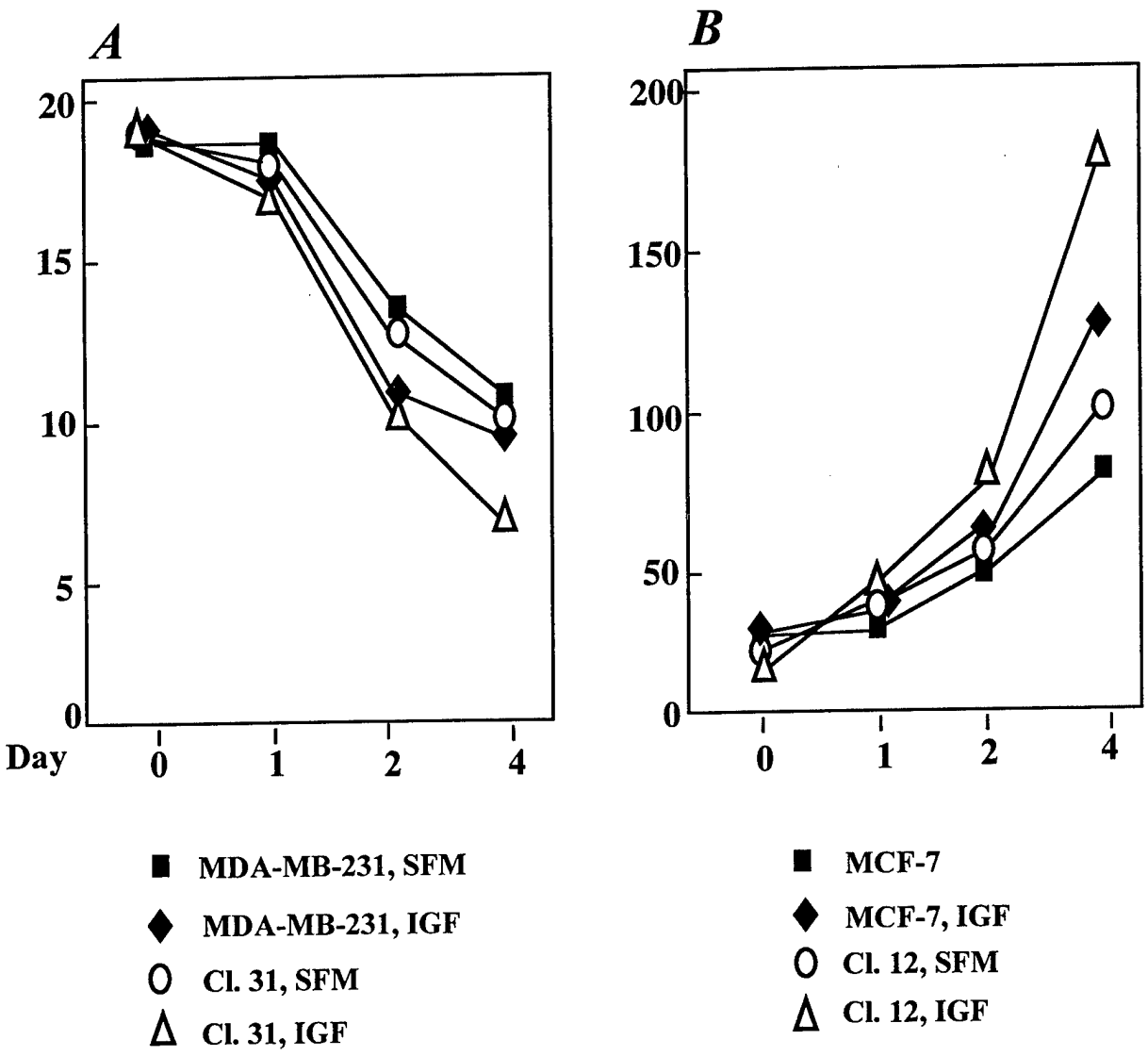

A
B
Cell Number x 10^3
20
15
10
5
0
200
150
100
50
0
Day
0
1
2
4
MDA-MB-231, SFM
MDA-MB-231, IGF
Cl. 31, SFM
Cl. 31, IGF
MCF-7
MCF-7, IGF
Cl. 12, SFM
Cl. 12, IGF

FIGURE 3

A

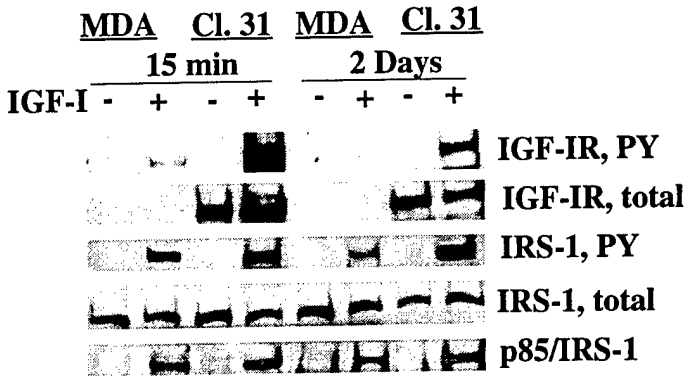

MDA
Cl. 31
MDA
Cl. 31
15 min
2 Days
IGF-I - + - + - + - +
IGF-IR, PY
IGF-IR, total
IRS-1, PY
IRS-1, total
p85/IRS-1

FIGURE 4

B

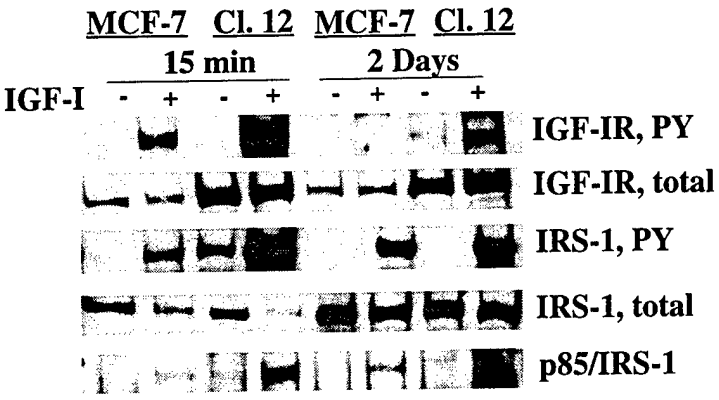


FIGURE 4 (CONTINUED)

C

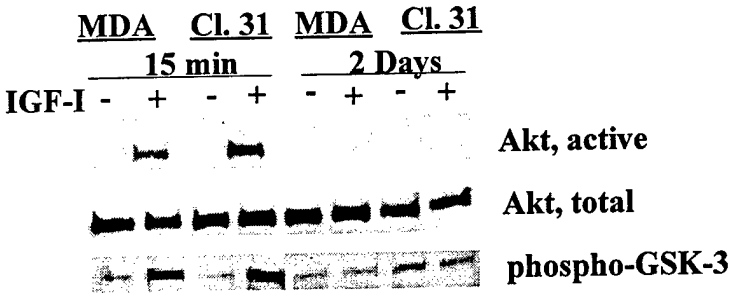


FIGURE 4 (CONTINUED)

D

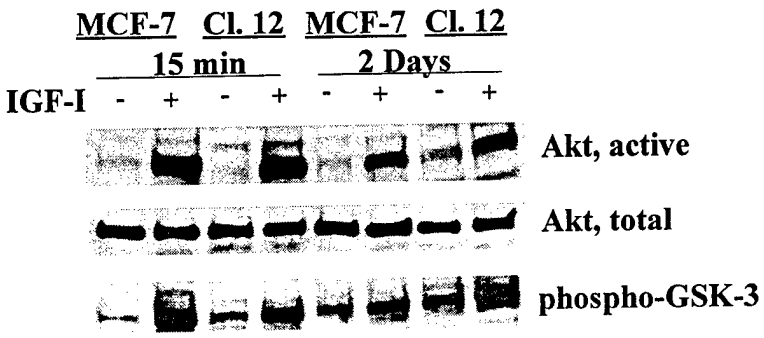


FIGURE 4 (CONTINUED)

E

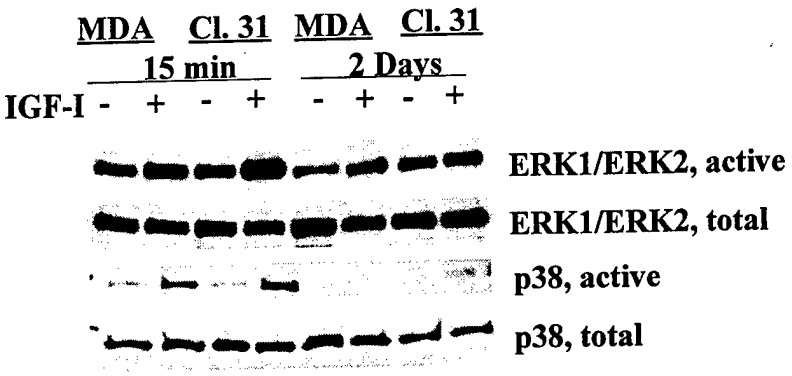


FIGURE 4 (CONTINUED)

F

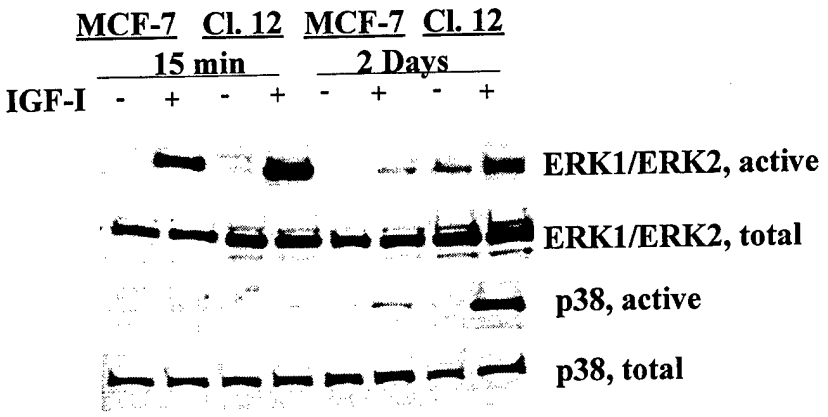


FIGURE 4 (CONTINUED)

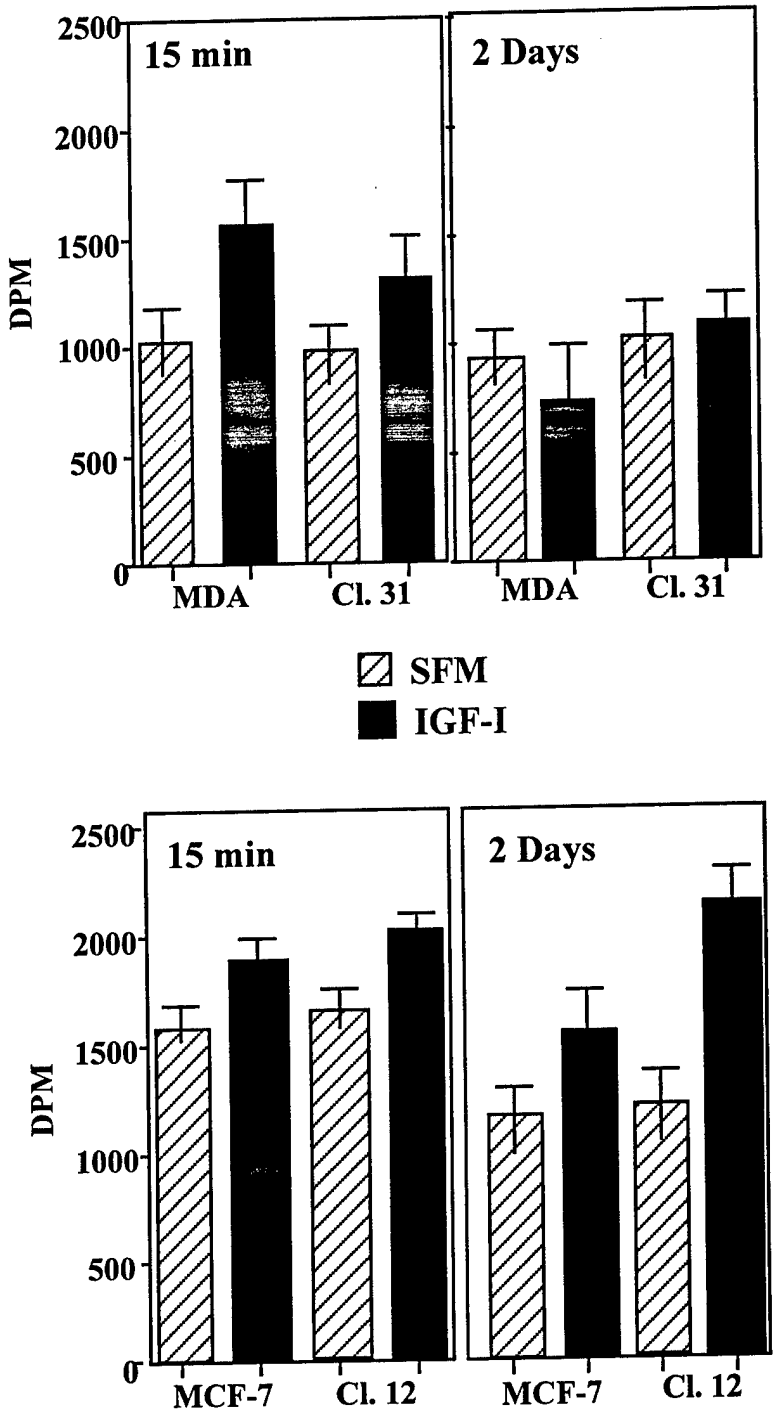

15 min
2 Days
DPM
2500
2000
1500
1000
500
0
MDA
Cl. 31
MDA
Cl. 31
SFM
IGF-I
15 min
2 Days
DPM
2500
2000
1500
1000
500
0
MCF-7
Cl. 12
MCF-7
Cl. 12

FIGURE 5

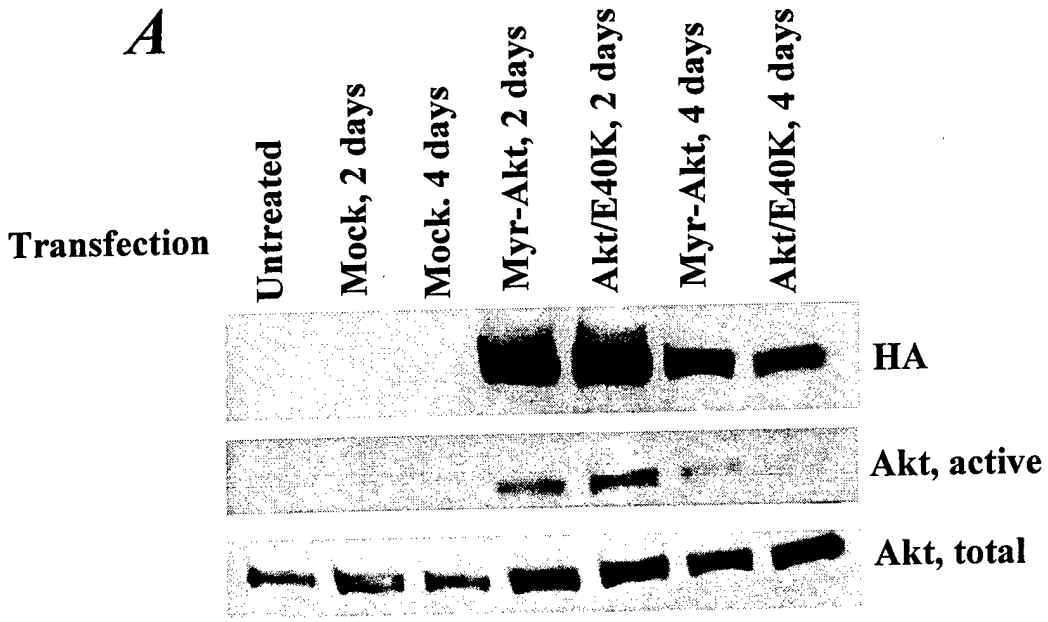
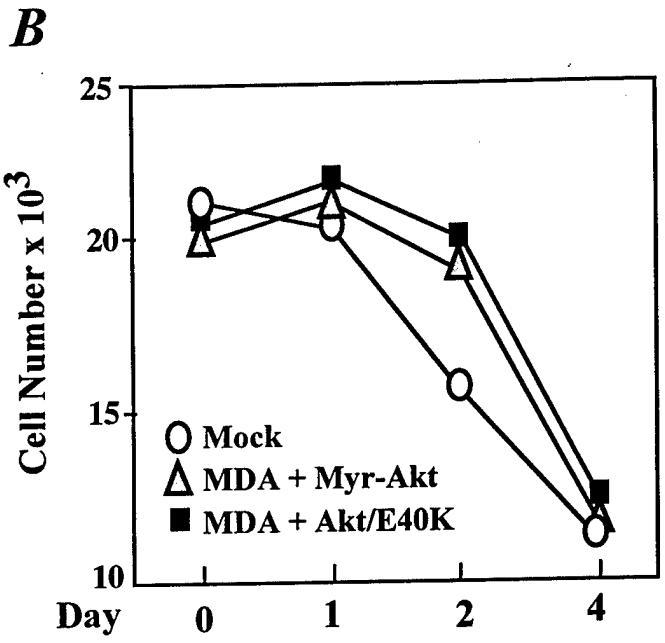

A
Transfection
Untreated
Mock, 2 days
Mock. 4 days
Myr-Akt, 2 days
Akt/E40K, 2 days
Myr-Akt, 4 days
Akt/E40K, 4 days
HA
Akt, active
Akt, total

FIGURE 6

FIGURE 6 (CONTINUED)



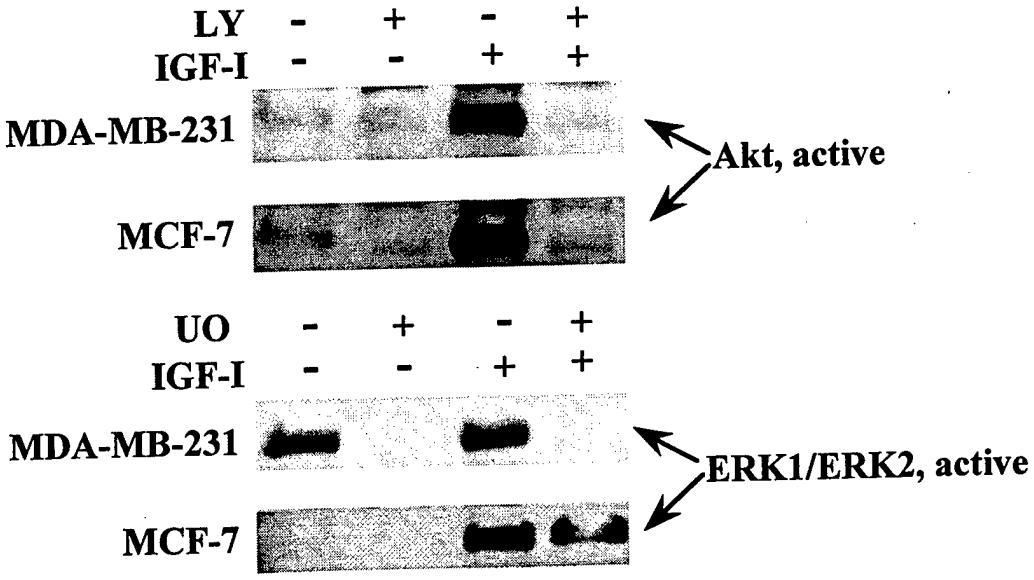

LY - + - +
IGF-I - - + +
MDA-MB-231
MCF-7
Akt, active
UO - + - +
IGF-I - - + +
MDA-MB-231
MCF-7
ERK1/ERK2, active

FIGURE 7

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SHC–$\alpha5\beta1$ Integrin Interactions Regulate Breast Cancer Cell Adhesion and Motility

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The oncogenic SHC proteins are signaling substrates for most receptor and cytoplasmic tyrosine kinases (TKs) and have been implicated in cellular growth, transformation, and differentiation. In tumor cells overexpressing TKs, the levels of tyrosine phosphorylated SHC are chronically elevated. The significance of amplified SHC signaling in breast tumorigenesis and metastasis remains unknown. Here we demonstrate that seven- to ninefold overexpression of SHC significantly altered interactions of cells with fibronectin (FN). Specifically, in human breast cancer cells overexpressing SHC (MCF-7/SHC) the association of SHC with $\alpha5\beta1$ integrin (FN receptor) was increased, spreading on FN was accelerated, and basal growth on FN was reduced. These effects coincided with an early decline of adhesion-dependent MAP kinase activity. Basal motility of MCF-7/SHC cells on FN was inhibited relative to that in several cell lines with normal SHC levels. However, when EGF or IGF-I was used as the chemoattractant, the locomotion of MCF-7/SHC cells was greatly (approx fivefold) stimulated, while it was only minimally altered in the control cells. These data suggest that SHC is a mediator of the dynamic regulation of cell adhesion and motility on FN in breast cancer cells. © 1999 Academic Press

Key Words: **SHC; $\alpha5\beta1$ integrin; fibronectin; motility; breast cancer.**

INTRODUCTION

The ubiquitous SH2 homology/collagen homology (SHC) proteins (p46, p52, and p66) are overlapping SH2-PTB adapter proteins that are targets and downstream effectors of most transmembrane and cytoplasmic tyrosine kinases (TKs) [1, 2]. Consequently, overexpression of $p52^{SHC}$ and $p46^{SHC}$ (referred to as SHC hereinafter) amplifies various cellular responses; for instance, it induces mitogenic effects of growth factors in NIH 3T3 mouse fibroblasts and myeloid cells [1, 3], stimulates differentiation in PC12 rat pheochromocytoma [4], and augments hepatocyte growth factor (HGF)-induced proliferation and migration in A549 human lung adenocarcinoma [5]. Overexpressed SHC is oncogenic in NIH 3T3 mouse fibroblasts, but amplification of $p66^{SHC}$ isoform does not induce transformation [1, 6, 7] and may even inhibit growth pathways [8]. Importantly, increased tyrosine phosphorylation of SHC, which has been noticed in different tumor cell lines, is a marker of receptor or cytoplasmic TKs overexpression [2]. In breast cancer, for instance, SHC is hyperphosphorylated in cells overexpressing ERB-2 and c-Src [9, 10]. Whether such amplification of SHC signaling contributes to the development of a more aggressive phenotype of breast tumor cells remains unknown.

The effector pathways downstream of SHC are partially known. Upon tyrosine phosphorylation by TKs, SHC associates with the GRB2/SOS complex and subsequently stimulates the canonical Ras–MAPK (p42 and p44 mitogen-activated protein kinases) signal transduction cascade [1, 6, 7]. SHC/GRB2 binding and the activation of Ras are prerequisites for SHC-induced mitogenesis and transformation in NIH mouse fibroblasts [6]. In addition, SHC has been described as associating with adapters Crk II [11] and GRB7 [12] as well as with a signaling protein p145 [13, 14] and PEST tyrosine phosphatase [15] in various experimental systems. However, SHC pathways incorporating these signaling intermediates and their biological significance are not well understood.

There is substantial evidence suggesting that in addition to its role in mitogenesis and transformation, SHC regulates nongrowth processes, such as cell adhesion and motility. For instance, overexpressed SHC improved motility in HGF-stimulated melanoma cells [5], and downregulation of SHC reduced epidermal growth factor (EGF)-dependent migration in MCF-7 breast cancer cells [16]. SHC was also essential for

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kidney epithelial cell scattering mediated by the receptors c-met, c-ros, and c-neu [17]. The mechanisms by which SHC regulates cell adhesion and motility are still obscure.

In several cell types (Jurkat, HUVEC, MG-63, and A431 cells), SHC couples with certain ECM receptors, specifically with the integrins $\alpha1\beta1$, $\alpha5\beta1$, $\alpha v\beta3$, and $\alpha6\beta4$, but not with $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha6\beta1$ [18]. Association of SHC with integrins may result in phosphorylation of SHC by integrin-associated TKs (Fyn, other Src-like kinases, FAK, or FAK-associated kinases) and subsequent activation of MAPK [18–21]. The biological significance of integrin-stimulated MAPK activity is not well understood. However, recent data indicated that it positively regulates cell growth and survival [18, 22], but is not essential for cell migration [23].

This work addresses the consequences of amplified SHC signaling on proliferation, transformation, adhesion, and motility in breast cancer MCF-7 cells. In these cells, SHC is an important intermediate of different signaling pathways. Growth factors present in serum, such as IGF-I and EGF, can induce SHC through their cognate receptors [1, 16, 24]. Estrogens (also contained in serum) may elevate tyrosine phosphorylation of SHC via cytoplasmic TKs of the Src family [25]. In addition, SHC can be stimulated by cytoplasmic TKs as a result of cell spreading on ECM [18]. MCF-7 cells express several integrin receptors: $\alpha2\beta1$, $\alpha3\beta1$, $\alpha5\beta1$, and $\alpha v\beta5$ [26]. Of those, $\alpha5\beta1$, a FN receptor, is known to associate with SHC, while $\alpha2\beta1$ and $\alpha3\beta1$ are not SHC binding proteins [18].

The interactions of cells with FN have been reported to influence or control different processes regulating the behavior of cancer cells, namely cell migration, invasion, and metastasis as well as survival and proliferation [27]. The exact role of $\alpha5\beta1$ FN receptors in tumor progression is not clear. It has been shown that extracellular matrix recognition by $\alpha5\beta1$ integrin is a negative regulator of cell growth and may be lost in some tumor cells [28]. In agreement with this, overexpression of $\alpha5\beta1$ integrin and improved cell spreading on FN can reduce cell growth and transformation *in vivo* and reverse tumorigenicity *in vitro* [29, 30]. On the other hand, FN receptors may play a role in later stages of tumor progression since blocking $\alpha5\beta1$ integrin abrogated cell spread in experimental breast metastasis [31]. The importance of SHC signaling in the interactions of breast cancer cells with FN has not been studied and is a subject of this work.

MATERIALS AND METHODS

Cell lines and cell culture conditions. MCF-7 cells are estrogen receptor positive cells of a low tumorigenic and metastatic potential. The growth of MCF-7 cells is controlled by estrogens, such as estradiol (E2), and growth factors, such as IGF-I and EGF [32, 33]. MCF-7 cells express several integrins, including $\alpha5\beta1$ (FN receptor), $\alpha2\beta1$ (collagen, COL receptor), $\alpha3\beta1$ (COL/FN/laminin 5 receptor), and $\alpha v\beta5$ (vitronectin receptor) [26].

MCF-7/SHC clones 1 and 9 are MCF-derived cells stably transfected with the expression plasmid pcDNA3/SHC containing a human SHC cDNA encoding $p55^{SHC}$ and $p47^{SHC}$. The clones expressing the transgene were selected in 2 mg/ml G418, and the levels of SHC expression in 20 G418-resistant clones were determined by Western blotting (WB) in whole cell protein lysates, as described below.

As control cells, we used several MCF-7-derived clones with modified growth factor signaling pathways: specifically, MCF-7/IRS-1, clones 3 and 18, which are MCF-7 cells overexpressing insulin receptor substrate 1 (IRS-1) [32]; MCF-7/IGF-IR, clone 17, which is an MCF-7-derived clone overexpressing the insulin-like growth factor 1 receptor (IGF-IR) [33]; and MCF-7/anti-SHC, clone 2, with SHC levels decreased by 50% due to the stable expression of an anti-SHC RNA [16].

MCF-7 cells were grown in DMEM:F12 (1:1) containing 5% calf serum (CS). MCF-7-derived clones were maintained in DMEM:F12 plus 5% CS plus 200 μg/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 μM FeSO4, and 2 mM L-glutamine (referred to as PRF–SFM).

Monolayer growth. Cells were plated at a concentration 1.5–2.0 $\times$ 10^5 in six-well plates in the growth medium; the following day (day 0), the cells at approximately 50% confluence were shifted to PRF–SFM containing 1 or 20 ng/ml IGF-I or 1 or 10 ng/ml EGF. After 4 days, the number of cells was determined by direct counting.

Anchorage-independent growth. Transforming potential of the cells (anchorage independence) was measured by their ability to form colonies in soft agar, as previously described [32]. The cells, 1 $\times$ 10^5/35-mm plate, were grown in a medium solidified with 0.2% agarose. The solidified medium contained either (i) DMEM:F12 supplemented with 10% FBS or 5% CS or (ii) PRF–SFM with 200 ng/ml IGF-I, 50 ng/ml EGF, or 200 ng/ml IGF-I plus 50 ng/ml EGF. After 21 days of culture, the colonies greater then 100 μm in diameter were counted using an inverted phase–contrast microscope.

Adhesion on FN or COL. Cells synchronized for 24 h in PRF–SFM were seeded in 60-mm plates coated with FN (50 μg/ml) or COL (20 μg/ml). Before the experiment, the plates were blocked with 3% BSA for 3 h at 37°C and then washed once with PBS. To inactivate $\alpha5\beta1$ integrin, the cells were incubated with a blocking $\alpha5\beta1$ Ab 10 μg/ml (Chemicon) for 30 min before plating. Cell morphology was recorded using an inverted phase–contrast microscope with a camera. Percentage of nonadherent cells was determined by counting the number of floating cells vs. the number of cells originally inoculated in the plate.

Growth on FN. Cells (0.5 $\times$ 10^5/ml) were seeded in 12-well plates coated with FN (50 μg/ml) in normal growth medium with or without EGF (10, 50, or 100 ng/ml) or IGF-I (20 or 100 ng/ml). The cells were counted after 4 days of culture.

Motility assay. Motility was tested in modified Boyden chambers containing porous (8-mm) polycarbonate membranes. The undersides of membranes were coated with either 20 μg/ml COL IV or 50 μg/ml FN, as described by Mainiero *et al.* [34]. According to this protocol, collagen (COL) or FN covered not only the underside of the membrane, but also diffused into the pores where cell contact with ECM was initiated. Synchronized cells (2 $\times$ 10^4) suspended in 200 μl of PRF–SFM were plated into upper chambers. Lower chambers contained 500 μl of PRF–SFM with EGF (1 and 10 ng/ml) or IGF-I (20 ng/ml). After 12 h, 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 (0.25 g Coomassie blue/45 ml water/45 ml methanol/10 ml glacial acetic acid) for 5 min. After that, the chambers were washed three times with H_2O. The cells that migrated to the lower chamber were counted under the microscope as described before [16].

Immunoprecipitation and Western blotting. Proteins were obtained by lysis of cells with a buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 100 mM NaF, 0.2 mM Na_3VO_4, 1% PMSF, 1% aprotinin. The expression of SHC in transfectants and the parental cells was assessed in 50 μg of total cell lysate using an anti-SHC monoclonal antibody (mAb) (Transduction Laboratories). Alternatively, SHC was detected by immunoprecipitation (IP) from 250–1000 μg (specific amounts are given under the figures) of protein lysate with an anti-SHC polyclonal antibody (pAb) (Transduction Laboratories), followed by WB with an anti-SHC mAb (Transduction Laboratories). Tyrosine phosphorylation of SHC was measured by WB using an anti-phosphotyrosine mAb PY20 (Transduction Laboratories). The levels of $\alpha5\beta1$ integrin were assessed in 1 mg of protein lysate by IP with an anti-$\alpha5\beta1$ pAb (Chemicon) and WB using an anti-$\beta1$ mAb (Chemicon). The amounts of integrin-associated SHC were measured in $\alpha5\beta1$ integrin immunoprecipitates with an anti-SHC pAb (Chemicon). The intensity of bands representing relevant proteins was measured by laser densitometry scanning.

MAPK activity. The phosphorylated forms of p42 and p44 MAPK were identified by WB in 50 μg of whole cell lysates with an anti-phospho-MAPK (Thr202/Tyr204) mAb (New England Biolabs). The total levels of MAPK were determined with an anti-MAPK pAb (New England Biolabs). Adhesion-induced MAPK activity was assessed in cells plated either on COL IV or FN and then lysed at different times after plating (0–24 h). To determine EGF-induced MAPK activity, the cells were plated on different substrates, allowed to attach for 1 h, and then treated with 10 ng/ml EGF. The cells were lysed at different times (0–24 h) of the treatment and MAPK activity was measured as described above.

Statistical analysis. The results of cell growth experiments were analyzed by Student t test.

RESULTS

Basal and growth factor-induced SHC tyrosine phosphorylation is increased in MCF-7/SHC cells. To investigate the implications of increased SHC signaling in breast cancer cells, we developed several MCF-7-derived clones stably overexpressing $p46^{SHC}$ and $p52^{SHC}$. Of 20 G418 resistant clones, 7 exhibited SHC overexpression, as determined by WB (data not shown). Two representative clones, MCF-7/SHC, 1 and MCF-7/SHC, 9, with a seven- and ninefold SHC amplification, respectively, were selected for subsequent experiments (Fig. 1). The greater amount of SHC in these clones was reflected by increased levels of SHC tyrosine phosphorylation, which was evident in both continuously proliferating (Fig. 1A) and EGF-stimulated cultures (Fig. 1B). The extent of SHC tyrosine phosphorylation roughly corresponded to the cellular levels of the protein (Fig. 1A and B).

Overexpression of SHC has minimal effects on cell growth on plastic and does not enhance transformation in soft agar. The significant hyperactivation of SHC in MCF-7/SHC cells suggested that growth properties of these cells might have been altered. First we determined that in serum-containing medium or PRF–SFM, the growth rate of MCF-7/SHC clones was comparable to that of the parental cells or other cell lines with normal SHC levels (data not shown). Next, we studied mitogenic response to EGF and IGF-I, growth factors which stimulate tyrosine phosphorylation of SHC [1, 24] and require SHC for their growth action [16]. We found that relative to MCF-7 cells, MCF-7/SHC clones exhibited only moderately (20–40%) enhanced growth response to IGF-I or EGF (Fig. 1C). Under the same conditions, the proliferation of a control clone MCF-7/anti-SHC, 2 was substantially (at least by 50%) reduced (Fig. 1C), as we demonstrated previously [16].

Since SHC is oncogenic when overexpressed in NIH mouse fibroblasts [1], we assessed transforming potential of MCF-7/SHC clones in soft agar assay. Despite significant SHC overexpression in these cells, in several repeat experiments and under different growth conditions used, anchorage-independent growth of MCF-7/SHC cells was never enhanced relative to that seen in MCF-7 cells (Table 1). In the same assay, MCF-7 cells overexpressing IRS-1, i.e., MCF-7/IRS-1, clone 3, exhibited increased transformation in the presence of serum or IGF-I [32], typical for these cells.

SHC associates with $\alpha5\beta1$ integrin (FN receptor) in MCF-7 cells, and the abundance of SHC/$\alpha5\beta1$ integrin complexes is increased in MCF-7/SHC cells. The limited or absent effects of SHC overexpression on mitogenic and transforming potential of MCF-7 cells prompted us to assess the role of SHC in nongrowth processes, specifically, adhesion and motility. Because interactions of cells with FN have been implicated in the growth and metastatic behavior of breast cancer cells [30, 31] and since SHC is a potential mediator of FN receptor signaling [18, 21], we investigated how overexpressed SHC affects the function of $\alpha5\beta1$ integrin in MCF-7 cells. The first observation was that the levels of SHC, especially $p46^{SHC}$, associated with $\alpha5\beta1$ were markedly increased (~sevenfold) in MCF-7/SHC clones compared with that in MCF-7 cells or in several cell lines with normal levels of SHC but overexpressing other signaling proteins (IRS-1 or the IGF-IR) (Fig. 2). Interestingly, the amount of $p46^{SHC}$ associated with $\alpha5\beta1$ integrin was similar in both MCF-7/SHC clones, regardless of the level of SHC overexpression (Fig. 2). This suggests that the extent of SHC/$\alpha5\beta1$ binding is not directly proportional to total cellular SHC levels and that $\alpha5\beta1$/SHC complex formation is a saturable process, possibly determined by the limited expression of $\alpha5\beta1$ integrin in MCF-7 cells [30].

MCF-7/SHC cells exhibit increased adhesion to FN. Because of the enhanced association of SHC with $\alpha5\beta1$ integrin in MCF-7/SHC cells, we examined the role of SHC in cellular interactions with FN using cell lines with normal, amplified, or reduced SHC levels. We found that the overexpression of SHC was associated with an accelerated cell adhesion to FN, while the reduction of SHC levels blocked cell spreading on the substrate (Fig. 3 and Table 2). The differences in the

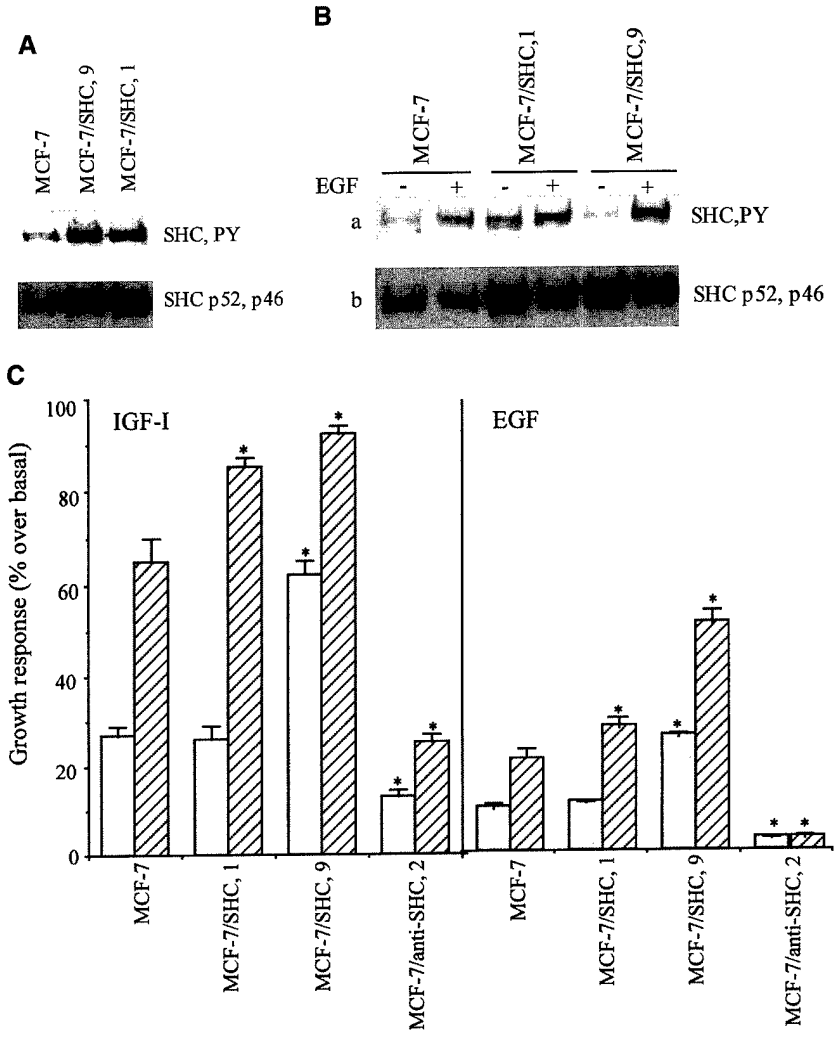

FIG. 1. MCF-7/SHC cells. (A) SHC expression and tyrosine phosphorylation in proliferating cells. The protein levels and tyrosine phosphorylation (PY) of $p52^{SHC}$ and $p46^{SHC}$ in two selected MCF-7/SHC clones 1 and 9 were determined in 750 μg of protein lysate by IP and WB with specific antibodies, as detailed under Materials and Methods. Cell lysates were isolated from logarithmic cultures maintained in normal growth medium. (B) SHC expression and tyrosine phosphorylation in growth-factor-stimulated cells. 70% confluent cultures were synchronized in PRF-SFM for 24 h and then stimulated with 10 ng EGF for 15 min. SHC levels and tyrosine phosphorylation (PY) were studied by IP and WB in 250 μg of protein lysates. Note that lane MCF-7/SHC, 1, EGF (−) is overloaded. (C) Growth response of MCF-7/SHC cells to IGF-I and EGF. The cells at 50% confluence were synchronized in PRF–SFM and stimulated with mitogens for 4 days as described under Materials and Methods. Abscissa, cell lines; ordinate, the percentage of growth increase over that in PRF-SFM. Solid bars, low doses: IGF-I 1 ng/ml or EGF 1 ng/ml; striped bars, high doses, IGF-I 20 ng/ml or EGF 10 ng/ml. High doses of IGF-I or EGF are the EC_{50} concentrations in these cells. SD is marked by solid bars; asterisks indicate statistically significant differences ($P \leq 0.05$) between the growth responses of MCF-7/SHC or MCF-7/anti-SHC cells and identically treated MCF-7 cells. The results are average of four experiments.

dynamics of cell interactions with FN were most evident at 1 h after plating (Fig. 3B and Table 2). Specifically, while at this time both MCF-7/SHC clones were well spread on FN, and almost no floating cells were observed, only ~50% of MCF-7 cells exhibited initial attachment to the substrate (cells were still rounded but with distinct membrane protrusions), and MCF-7/anti-SHC cells remained completely suspended. At 2 and 6 h after plating, MCF-7 and MCF-7/SHC clones were attached to FN and the differences in adhesion among these cell lines were unremarkable. At the same time, MCF-7/anti-SHC cells were in minimal contact with FN (Table 2). After 24 h, MCF-7/anti-SHC cells formed small aggregates demonstrating limited contact with the substrate, but all other tested cell lines (represented here by MCF-7 cells) were fully attached (Fig. 3D and Table 2). At 48 h, MCF-7/anti-SHC cells were completely detached, while MCF-7 and MCF-7/SHC cells begun proliferation on FN (data not shown).

Our experiments also indicated that the adhesion of MCF-7/SHC cells to FN was mediated by $\alpha 5\beta 1$ integrin, since this process was totally blocked with a spe-

TABLE 1

Anchorage-Independent Growth of MCF-7/SHC Cells

Cell line	Number of colonies				
	10% FBS	5% CS	SFM + IGF-I	SFM + EGF	SFM + IGF-I + EGF
MCF-7	172	105	2	1	12
MCF-7/SHC, 1	164	90	0	0	9
MCF-7/SHC, 9	155	88	0	0	10
MCF-7/IRS-1, 3	213	131	25	10	22

Note. The cells were tested in soft agar as described under Materials and Methods. The agar-solidified medium was either DMEM:F12 with 10% FBS or 5% CS or PRF–SFM with EGF (200 ng/ml), IGF-I (50 ng/ml), or EGF plus IGF-I (50 plus 200 ng/ml, respectively). MCF-7/IRS-1, clone 3, characterized by an increased transforming potential [30], was used as a positive control. The experiment was repeated seven times. Average number of colonies of the size at least 100 μm in diameter is given.

cific anti-$\alpha5\beta1$ blocking antibody (Fig. 3C), but not with a control goat IgG (not shown).

In contrast with the results obtained on FN, the dynamics of cell adhesion on COL, which is mediated by an integrin not associating with SHC ($\alpha5\beta1$) [18], were similar in all tested cell lines, regardless of the levels of SHC expression. Specifically, all cells tested initiated contacts with COL at 15 min and completed attachment at 1 h after plating (data not shown).

Overexpression of SHC modulates adhesion-dependent, but not growth factor-induced, MAP kinase activity on FN. Cell adhesion to ECM and the activation of different integrin-associated cytoplasmic TKs result in the stimulation of MAPK activity [35]. This process can be mediated through SHC, which, as a substrate of TKs (e.g., Fyn, other c-Src-like kinases, or FAK), is tyrosine phosphorylated, binds the GRB2/SOS complex, and stimulates Ras [18, 19, 21]. The integrin–MAPK pathway can also be induced in a SHC-independent way, through FAK–GRB2-SOS–Ras signaling [20, 21]. Here we studied the effect of SHC amplification on adhesion-dependent MAPK response in MCF-7 and MCF-7/SHC cells. Figure 4 demonstrates representative results obtained with MCF-7/SHC, 1 cells; the results with the clone MCF-7/SHC, 9 were similar.

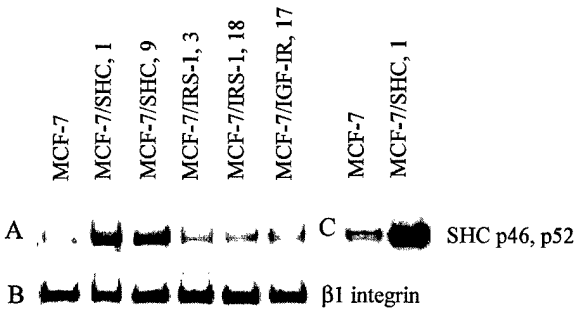

FIG. 2. SHC associates with $\alpha5\beta1$ integrin. The amounts of SHC associated with $\alpha5\beta1$ integrin in MCF-7/SHC cells, MCF-7 cells, and several control clones with normal SHC levels but increased levels of IRS-1 (MCF-7/IRS-1, clones 3 and 18) or the IGF-IR (MCF-7/IGF-IR, clone 17) were determined in 750 μg of protein lysate by IP with an anti-$\alpha5\beta1$ pAb and WB with an anti-SHC pAb (A). The expression of $\alpha5\beta1$ integrin in the cells was determined after stripping the above blot and reprobing with the anti-$\beta1$ pAb (only the β subunit is shown) (B). To locate the position of SHC isoforms on the gel, SHC proteins were precipitated from 250 μg of MCF-7 and MCF-7/SHC, 1 cell lysates with an anti-SHC pAb, run in parallel with $\alpha5\beta1$ integrin IP samples and probed with an anti-SHC mAb (C). Note: The $\alpha5\beta1$ integrin IP samples could not be reprobed with the SHC mAb because of strong antibody cross-reaction.

First, we found that overexpression of SHC significantly modulated MAPK activation in cells spread on FN, but on COL (Fig. 4A). Specifically, on COL, MCF-7 and MCF-7/SHC cells responded similarly—the activation of MAPK was biphasic, with a peak between 30 min and 4 h after plating, followed by a decline of activity at 8 h, and then an increased activity between 12 and 24 h. In contrast, on FN, the dynamics of MAPK response was different—in MCF-7 cells, the stimulation of MAPK was the highest at 1 h after plating and the kinases remained highly stimulated for up to 8 h. In MCF-7/SHC cells, MAPK was activated at 30 min after plating, reached the maximum at 1 h, and rapidly declined at 4 h to reach basal levels at 24 h (Fig. 4A). The activation of MAPK in suspended cells was undetectable (not shown).

Next, we investigated whether SHC overexpression affects growth-factor-induced MAPK response in cells plated on FN. We used EGF in this experiment since this mitogen induces SHC phosphorylation more strongly than IGF-I in MCF-7 cells (Surmacz *et al.*, unpublished observations). The patterns of EGF-stimulated MAPK activity were remarkably similar on COL and FN (a peak between 15 min and 1 h after treatment followed by a decline to basal levels) in both MCF-7 and MCF-7/SHC cells (Fig. 4B).

Since MAPK pathway contributes to cell growth, we studied whether the reduced duration of adhesion-dependent MAP activity reflects mitogenicity of MCF-7/

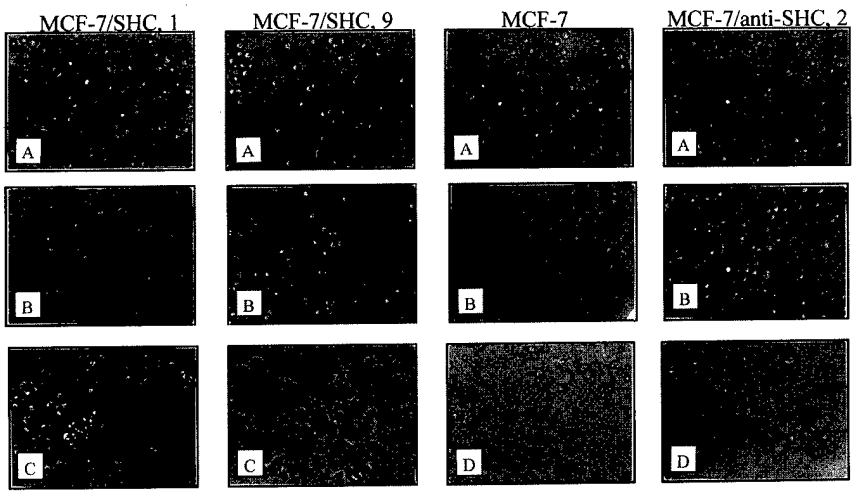

FIG. 3. Adhesion of MCF-7/SHC clones on FN. MCF-7/SHC clones 1 and 9 (amplified SHC), MCF-7 cells (normal SHC levels) and MCF-7/anti-SHC, clone 2 (reduced SHC levels) were synchronized for 24 h in PRF–SFM and plated on FN (50 μg/ml) in PRF–SFM. The cells were photographed at times 0 (A) and 1 h (B). The role of $\alpha5\beta1$ integrin in the adhesion of MCF-7/SHC clones 1 and 9 was assessed by blocking the FN receptor with a specific antibody 30 min before cell plating (C), as described under Materials and Methods. The long-term (24 h) adhesion of MCF-7 and MCF-7/anti-SHC, clone 2, is shown in panels D.

SHC cells cultured on FN (Table 3). Indeed, we found that overexpression of SHC coincided with a significant (~twofold) growth inhibition. Interestingly, the addition of EGF (different doses, up to 100 ng/ml) to growth medium did not improve proliferation of MCF-7/SHC or MCF-7 cells on FN. The addition of IGF-I (doses up to 100 ng/ml) only minimally (9–22%) stimulated growth under the same conditions (Table 3).

Overexpression of SHC inhibits basal motility on FN, and IGF-I or EGF mobilizes MCF-7/SHC cells. We investigated whether increased binding of SHC to $\alpha5\beta1$ integrin affects cell motility in FN-coated inserts. We found that basal migration of MCF-7/SHC cells was significantly (~fourfold) reduced compared with that of MCF-7 cells and several MCF-7-derived cell lines containing normal amounts of SHC (Fig. 5). In contrast, the motilities of MCF-7/SHC clones in COL-coated inserts were similar ($P \geq 0.05$) to those seen with other tested cell lines (Fig. 5).

The use of EGF or IGF-I as chemoattractants significantly (~five- to sevenfold, $P \leq 0.01$) improved the migration of MCF-7/SHC cells toward FN, but not to COL. The mitogens did not affect motility of other cells to COL, except some inhibition of MCF-7/IRS-1, clone 18 with 10 ng/ml EGF. Interestingly, in FN-coated inserts, 10 ng/ml EGF stimulated the migration of MCF-7/IRS-1 cells; however, the extent of this stimulation was much lower than that of SHC overexpressing clones (Fig. 5). The increased EGF sensitivity of MCF-7/IRS-1 clones has been noticed before [16].

TABLE 2

Dynamics of Cell Attachment to FN

Cell line	% Nonadherent cells					
	0 h	0.5 h	1 h	2 h	6 h	24 h
MCF-7	100	74	55	8	5	2
MCF-7/SHC, 1	100	33	5	5	4	4
MCF-7/SHC, 9	100	25	6	5	2	3
MCF-7/anti-SHC, 2	100	99	100	78	80	76

Note. 0.5×10^5 cells suspended in PRF–SFM were plated into 60-mm plates coated with 50 μg/ml FN as described under Materials and Methods. At the time of plating (0 h) and at 0.5, 1, 2, 6, and 24 h after plating, the floating cells were collected and counted. The values represent the percentages of cells floating vs cells originally plated and are averages from three experiments.

DISCUSSION

SHC is a signaling substrate of most receptor-type and cytoplasmic TKs and therefore may amplify various cellular responses [2]. In consequence, the significance of SHC amplification must depend on the intracellular and extracellular cell context. Breast cancer cells, unlike normal breast epithelium, frequently overexpress TKs, such as c-Src (80%), or ERB2 (~20–30%), which may result in constitutive activation of SHC [9, 10]. The contribution of amplified SHC signaling to development and progression of breast cancer is not known. We addressed this question by examining the effects of SHC overexpression in MCF-7 cells (representing an early stage of breast cancer and characterized by moderate c-Src amplification). The major findings of this work are that unlike in fibroblasts, hyperactivation of SHC is not sufficient to provide sig-

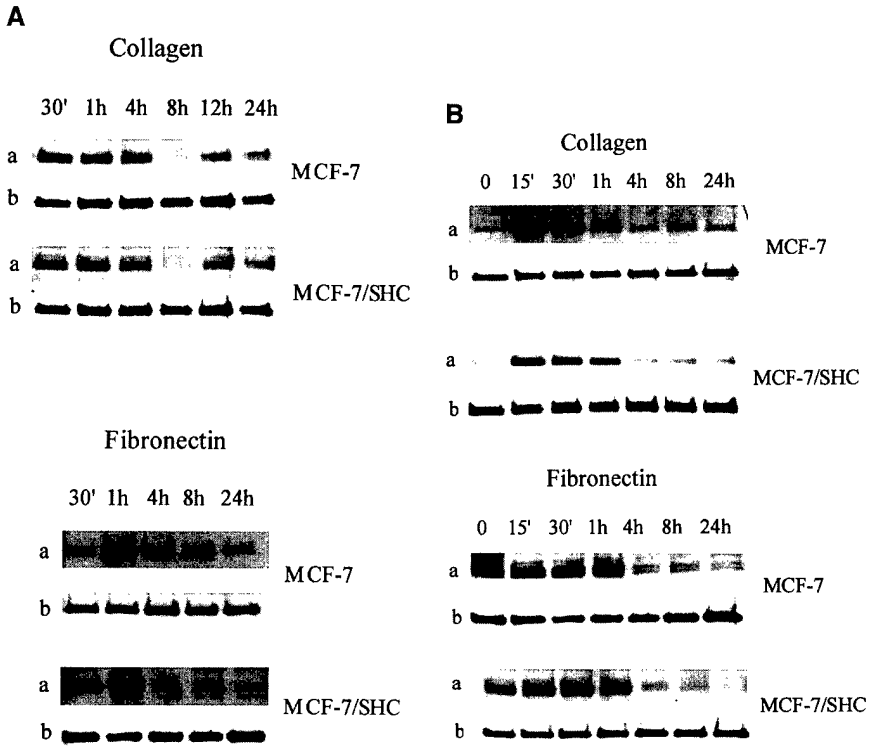

FIG. 4. Adhesion-induced (A) and EGF-dependent (B) MAPK activity in MCF-7/SHC cells. To measure adhesion-induced MAP kinase activity (A), MCF-7 and MCF-7/SHC cells were plated on COL IV or FN. The cells were lysed at the indicated times after plating. The phosphorylated forms of p42 and p44 MAPK were determined as described under Materials and Methods. EGF-induced MAP kinase activity (B) was determined in MCF-7 and MCF-7/SHC cells. The cells were plated on COL IV or FN, allowed to attach for 1 h, and then treated with 10 ng/ml EGF. The cells were lysed at different times (0–24 h) of the treatment. In (A) and (B), panels (a) represent phosphorylated MAPK, panels (b) total cellular levels of MAPK. The representative results demonstrating MAPK response in MCF-7 cells and MCF-7/SHC, clone 1, are shown; results with MCF-7/SHC, clone 9, were analogous to that obtained in clone 1.

nificant growth or transforming advantage in breast cancer cells. High levels of SHC, however, increase cell connections with FN and modulate cell growth and migration on this substrate, which may have consequences in cell spread and metastasis.

TABLE 3

Growth of MCF-7/SHC Cells on FN

	Cell number		
Cell line	5% CS	5% CS + EGF	5% CS + IGF-I
MCF-7	2.2×10^5	2.2×10^5	2.4×10^5
MCF-7/SHC, 1	1.1×10^5	1.0×10^5	1.3×10
MCF-7/SHC, 9	0.9×10^5	0.8×10^5	1.1×10^5

Note. The growth of cells on FN in normal growth medium (DMEM:F12 + 5% CS) or growth medium containing EGF (100 ng/ml) or IGF-I (100 ng/ml) was tested as described under Materials and Methods. The cells were plated at the concentration 0.5×10^5 cells/ml and counted after 4 days of culture. The values represent cell numbers/ml and are average results from three independent experiments.

SHC in epithelial cell growth and transformation. In mouse fibroblasts, overexpression of SHC resulted in increased SHC tyrosine phosphorylation, augmented EGF-, or IGF-I-dependent MAPK response, accelerated cell cycle progression through G1 phase in the absence of growth factors, and enhanced anchorage-independent growth in soft agar and tumorigenicity in nude mice [1, 6, 24]. Increased levels of SHC also potentiated growth factor response in myeloid and A549 adenocarcinama cells [3, 5]. Consistent with these findings are our previous data demonstrating that downregulation of SHC results in reduced sensitivity to mitogenic action of EGF and IGF-I and growth inhibition in breast cancer cells [16]. The present studies indicated that in SHC overexpressing epithelial cells, like in fibroblasts, basal and growth-factor-induced SHC tyrosine phosphorylation was increased, and cell responsiveness to IGF-I and EGF was moderately augmented in monolayer culture on plastic. However, amplification of SHC did not potentiate MAPK activity or proliferation of cells in complete serum-containing medium. This, again, was consistent with

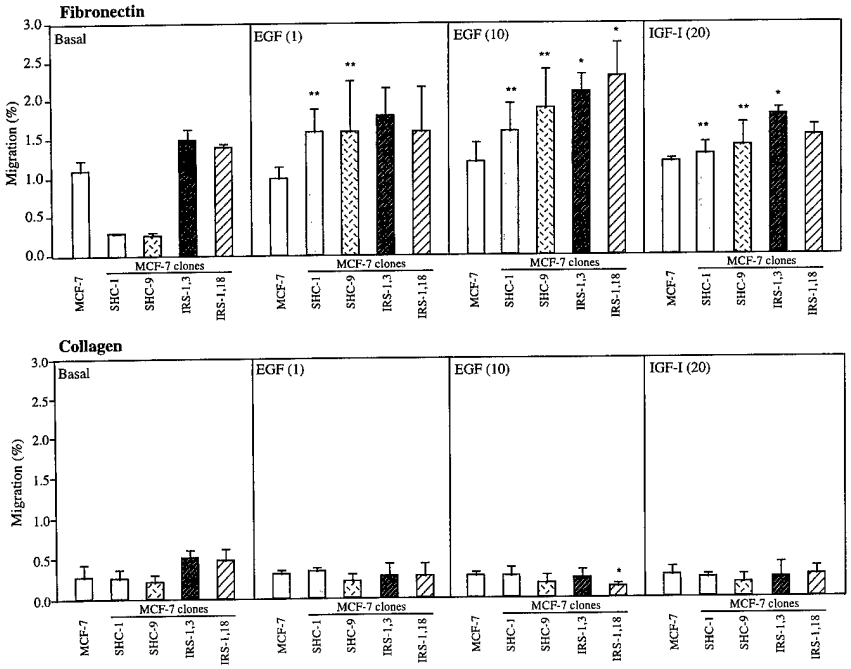

FIG. 5. Motility of MCF-7/SHC cells in FN or COL inserts. The motility of MCF-7/SHC cells and several control cell lines with normal SHC levels was tested as described under Materials and Methods. The upper and lower chambers contained PRF–SFM. Growth-factor-induced motility was assessed by supplementing PRF–SFM in lower chambers with either EGF (1 or 10 ng/ml) or IGF-I (20 ng/ml). The percentage of cells that migrated to the underside of inserts (relative to the number of cells plated) is shown. The experiments were repeated four times. Average values are given. Asterisks indicate statistically significant ($*P \leq 0.05$, $**P \leq 0.01$) differences between the basal and growth factor induced migration of a given cell line.

the effects observed in SHC overexpressing NIH 3T3 fibroblasts [6].

Note that high levels of SHC did not promote transformation of MCF-7 cells, whereas overexpression (at a similar level) of another signaling substrate IRS-1 markedly augmented anchorage-independent growth [32]. Since anchorage-independent growth reflects tumorigenic potential of breast cancer cells [36] and other cell types [1], our results indicate that, unlike in NIH 3T3 cells, SHC is not oncogenic in MCF-7 cells. This may reflect differences between pathways controlling transformation in fibroblasts and epithelial cells.

SHC in cell adhesion and motility. In contrast with the minimal impact of SHC overexpression on growth and transforming processes, high levels of SHC significantly modulated cell interactions with ECM in breast epithelial cells. SHC was found associated with $\alpha5\beta1$ integrin, the FN receptor, and $\alpha5\beta1$/SHC complexes were more abundant in SHC overexpressing cells than in other cell lines with SHC normal levels. The increased SHC/$\alpha5\beta1$ binding in MCF-7/SHC cells was paralleled by accelerated cell attachment to FN, reduced basal motility, abbreviated adhesion-mediated MAPK activity, and inhibited proliferation on the substrate. These effects were absent on COL (whose receptor does not bind SHC in MCF-7 cells), which suggests a specific role of SHC-$\alpha5\beta1$ interactions in the above processes.

The association of SHC with certain classes of integrins has been noted in several other cell systems. In A431 cells and other cell lines, binding and tyrosine phosphorylation of SHC to $\beta1$ integrin was induced by cell contact with ECM or by integrin cross-linking with a specific antibody [18]. Similarly, an association of SHC with $\alpha6\beta4$ integrin was observed in attached, but not suspended, A431 cells [34]. In several cell types, ligation of SHC-binding integrins, but not other integrins, has been reported to enhance cell cycle progression [18]. In our cell system, however, the increased association of SHC with $\alpha5\beta1$ integrin and the enhanced attachment to FN coincided with growth inhibition. Consistent with these findings are the observations of Wang *et al.,* who reported that in MCF-7 cells, $\alpha5\beta1$ integrin overexpression and improved interactions of cells with FN resulted in reduced proliferation on the substrate and impaired tumorigenicity *in vivo* [30].

Cell growth and survival on ECM are reflected by enhanced MAPK activity [18, 19, 35]. This pathway is induced by various integrin-associate TKs (e.g., c-Src-like TKs or FAK) and often involves activation of the SHC–GRB2–Ras pathway [19–21]. We found that the

amplification of SHC corresponded to the reduced duration of adhesion-mediated MAPK response on FN but not on COL. Note that, in mouse fibroblasts, an early decline of MAPK activity coincided with growth inhibition, whereas a prolonged activity marked cell cycle progression [37]. Thus, the abbreviated MAPK response in MCF-7/SHC cells may reflect their significantly slower proliferation on FN. In our experiments, treatment of cells spread on FN with EGF induced MAPK but did not stimulate cell growth, which confirms that MAPK signaling is required but not sufficient for the proliferation of MCF-7 cells [38].

Reduced growth and better attachment to FN in MCF-7/SHC cells were also associated with significantly reduced basal migration. However, EGF or IGF-I induced motility of MCF-7/SHC clones more strongly than other control cell lines when tested in FN-coated inserts. Such an enhancement of growth-factor-induced migration in SHC overexpressing cell lines has been reported before [5]. The increased chemotaxis was probably mediated by pathways other than MAPK, since MAPK activities were similar in MCF-7 and MCF-7/SHC cells treated with EGF.

In summary, in MCF-7 cells, the impact of the amplified SHC on cell growth and transformation is not significant; however, SHC plays an important role in the regulation of cell adhesion and motility on FN through its interaction with $\alpha 5\beta 1$ integrin. The significance of SHC-mediated interactions with FN in breast cancer metastasis is not known and will be pursued in an animal model.

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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.

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908 *IGF Signaling and Binding Proteins Oral Session, Thursday, 6/22, 1:00 PM, Room 701*

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.