Oncogenic ras Causes Resistance to the Growth Inhibitor Insulin-like Growth Factor Binding Protein-3 (IGFBP-3) in Breast Cancer Cells*

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Insulin-like growth factor binding protein-3 (IGFBP-3) inhibits proliferation and promotes apoptosis in normal and malignant cells. In MCF-10A human mammary epithelial cells, 30 ng/ml human plasma-derived IGFBP-3 inhibited DNA synthesis to 70% of control. This inhibition appeared IGF-independent, since neither an IGF-receptor antibody nor IGFBP-6 inhibited DNA synthesis. Malignant transformation of MCF-10A cells by transfection with Ha-ras oncogene abolished the inhibitory effect of IGFBP-3, concomitant with an increase in IGFBP-3 secretion and cell association of approximately 60 and 300%, respectively. When mitogen-activated protein (MAP) kinase activation was partially inhibited using PD 98059, IGFBP-3 sensitivity in ras-transfected cells was restored, with a significant inhibitory effect at 10 ng/ml IGFBP-3. PD 98059 had no effect on IGFBP-3 secretion or cell association by ras-transfected or parent MCF-10A cells. Hs578T, a tumor-derived breast cancer cell line that expresses activated Ha-ras, similarly has a high level of secreted and cell-associated IGFBP-3. In the absence of PD 98059, DNA synthesis by Hs578T cells was reduced to 70% of control by 1000 ng/ml IGFBP-3. PD 98059 increased sensitivity to IGFBP-3, so that this level of inhibition was achieved with 100 ng/ml IGFBP-3. These results suggest that MAP kinase activation by oncogenic ras expression causes IGFBP-3 resistance, a possible factor in the dysregulation of breast cancer cell growth.

The potent growth promoting effects of the insulin-like growth factors (IGFs) are regulated in all tissues by the six members of a family of structurally and functionally related proteins, the IGF-binding proteins (IGFBPs), which bind the IGFs with affinities of between 10^5 and 10^11 liters/mol (1). While sharing considerable amino acid homology, the IGFBPs undergo considerable post-translational modification to yield differently glycosylated, phosphorylated, and proteolytically cleaved products (2). IGFBP-3, a 45-kDa glycoprotein, is the predominant carrier of the growth factors in the circulation where, as a component of a 150-kDa complex consisting of IGFBP-3, IGF-I or -II, and the acid-labile subunit, it maintains a circulating reservoir of the IGFs, limits their insulin-like hypoglycemic potential, and helps to control their egress from the circulation to the extravascular tissues (3). In addition to its presence in the circulation, IGFBP-3 is found in the pericellular environment of many tissues, where it functions as a paracrine or autocrine modulator of the mitogenic effects of the IGFs.

There is now good evidence that IGFBP-3, a p53-inducible protein (4), inhibits proliferation and promotes apoptosis in normal and malignant cells by mechanisms that may be independent of its effects on IGF bioactivity (5–9). An important role for IGFBP-3 in the modulation of breast cancer cell growth is suggested by the observation that DNA synthesis and cell proliferation in tumor-derived breast cell lines may be reduced by treatment with recombinant IGFBP-3 (7). In addition, the antiproliferative effects of agents as diverse as transforming growth factor-β (TGF-β), retinoic acid, vitamin D analogs, and anti-estrogens is accompanied by induction of IGFBP-3 (6, 10–12), and prevention of this induction with antisense oligonucleotides restores DNA synthesis. Very little is known of the mechanisms by which IGFBP-3 exerts these effects, however, and the factors involved in regulating cell sensitivity to IGFBP-3 are completely uncharacterized. We now show that breast epithelial cells expressing a constitutively active Ras protein are resistant to IGFBP-3 and that abrogation of the MAPK/ERK signaling pathway restores sensitivity to IGFBP-3. These findings implicate the Ras-MAPK/ERK pathway in the development of IGFBP-3 insensitivity in breast cancer.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture reagents and plasticware were from Trace Biosciences (North Ryde, New South Wales, Australia) and Nunc (Roskilde, Denmark). Bovine serum albumin (BSA), bovine insulin, hydrocortisone, epidermal growth factor (EGF), and protein A were purchased from Sigma. Cholera enterotoxin was purchased from ICN Biomedicals Australasia (Seven Hills, New South Wales, Australia). The MAP kinase inhibitor PD 98059, and monoclonal antibody against type 1 IGF receptor, a1R-3, were obtained from Calbiochem-Novabiochem (Alexandria, New South Wales, Australia). Polyclonal antibodies against Thr202/Tyr204 phosphorylated and total p44/42 MAP kinase were from New England Biolabs (Beverley, MA). Recombinant human TGF-β1 was purchased from Austral Biologicals (San Ramon, CA), and recombinant human IGF-1 was the gift of Pharmacia and Upjohn (Stockholm, Sweden). Human IGFBP-3 was purified from Cohn fraction IV of human plasma and IGFBP-6 from SV-40-transformed fibroblast-conditioned medium, as described previously (13, 14). Electrophoresis reagents were purchased from Bio-Rad and Amrad-Pharmacia (Ryde, New South Wales, Australia). Protein A and IGF-1 were radiolabeled with 125I (ICN) using chloramine T.
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**Cell Culture**—The MCF-10A cell line, and its control vector-transfected and Ha-ras-transfected derivatives, were the kind gift of Drs. Robert Pauley and Herbert Soule at the Karmanos Cancer Institute, Detroit, MI (15). The parental, vector-transfected and oncogenic ras-transfected cells were used between passages 158–162, 31–35 (post-transfection), and 26–30 (post-transfection), respectively. Cells were maintained in DMEM/F-12 containing 15 mm Hepes, 5% horse serum, 10 μg/ml bovine insulin, 20 ng/ml EGF, 100 ng/ml cholera enterotoxin, and 0.5 μg/ml hydrocortisone. The Ha578T cell line (used between passages 63 and 68) was purchased from ATCC and maintained in RPMI containing 15 mm Hepes, 5% fetal bovine serum, and 10 μg/ml bovine insulin.

**[3H]Thymidine Incorporation**—For analysis of DNA synthesis, confluent cultures of cells in 24-well plates were changed to Hepes-buffered DMEM/F-12 containing 0.1% BSA for 48 h prior to addition of treatments. Spent media were replaced by fresh serum-free medium containing additives as indicated for individual experiments, and incubations were continued for 20 h. One μCi/well [3H]thymidine (35 Ci/μmol, ICN) was added in 50 μM of medium for a further 4-h incubation at 37 °C. Monolayers were rinsed twice with ice-cold saline and fixed with 250 μl of each lysate mixed with scintillant (Optima-Gold, Amersham Pharmacia Biotech) before counting for 2 min in a Hewlett-Packard β counter.

**SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting**—Cell-conditioned media from control and IGFBP-3-treated cells were fractionated by 12% SDS-PAGE under nonreducing conditions. Media conditioned by MCF-10A and MCF-10A ras-transfected cells were harvested whether the absence of IGFBP-3 inhibitory activity in MCF-10A ras-transfected cells was due to increased degradation of the protein. Media conditioned by MCF-10A and MCF-10A ras-transfected cells treated with IGFBP-3 were analyzed by IGF ligand blot. By this method, increased proteolysis results in decreased signal attributable to intact 43-kDa IGFBP-3 (20), as the proteolyzed protein has reduced binding to ido-IGF-I (21). As shown in Fig. 1E, there was no loss of 43-kDa IGFBP-3 in the MCF-10A ras-transfected medium compared with the untransfected MCF-10A cells by ligand blot, confirming that the lack of an inhibitory effect of IGFBP-3 was not due to increased degradation of exogenous IGFBP-3 by the MCF-10A ras-transfected cells. MCF-10A ras-transfected cells also showed a similar sensitivity to the inhibitory effects of TGF-β as MCF-10A (Fig. 1F), indicating that resistance to IGFBP-3 was not due to a general change in sensitivity to inhibitory factors as a result of constitutive activation of the Ras signaling pathway. Taken together, these data indicate that the inhibition of DNA synthesis in breast epithelial cells by IGFBP-3 is not a simple consequence of IGF binding by IGFBP-3 and that expression of oncogenic ras by these cells results in resistance to inhibition by IGFBP-3.

**Signal transduction**—Using Statview software for Macintosh (SAS Institute, Inc), signal transduction was monitored whether sensitivity to IGFBP-3 in MCF-10A ras-transfected cells could be changed in the presence of specific inhibitors of these pathways. The inhibitor wortmannin (10 nM), which blocks the phosphatidylinositol 3-kinase pathway, had no effect on IGFBP-3 sensitivity in MCF-10A or MCF-10A ras-transfected cells (not shown). However, PD 98059, a synthetic flavone that acts as a specific inhibitor of activation of the MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase) kinase MEK (23) had a marked effect on IGFBP-3 sensitivity in ras-transfected cells. When exposed to 5 μM PD 98059, each cell line showed 30–40% inhibition of DNA synthesis over 24 h, an effect similar to that described in other cell types (23). Addition of 10–100 ng/ml IGFBP-3 resulted in further inhibition of DNA synthesis.
synthesis (Fig. 2A), implying that sensitivity to IGFBP-3 in these cells is restored when MEK activation is inhibited.

Increased signaling through Ras-dependent pathways, either as the result of mutations in ras itself as seen in a small proportion of breast tumors or via increased activity of factors that activate Ras, is believed to be an important contributing factor in the development and progression of breast malignancies (24). The tumor-derived Ha-ras cell line expresses an activated Ha-ras gene (25), and we found that, like ras-transfected MCF-10A cells, Ha-ras cells were relatively insensitive to growth inhibition by IGFBP-3, requiring 1000 ng/ml IGFBP-3 for significant inhibition of DNA synthesis (Fig. 2B). These results indicate that in either transfected or tumor-derived cell models, breast epithelial cells expressing constitutively active Ras are relatively resistant to the inhibitory effects of IGFBP-3. As was seen with the MCF-10Aras cells, however, co-incubation of Ha-ras cells with PD 98059 (5 μM) and IGFBP-3 at a concentration of 100 ng/ml resulted in significant inhibition of DNA synthesis (Fig. 2B), indicating a 10-fold increase in sensitivity compared with IGFBP-3 treatment in the absence of PD 98059. To confirm that this reagent was inhibiting MAP kinase phosphorylation in the MCF-10Aras and Ha-ras cells, lysates from cells treated for 24 h with PD 98059 were analyzed by immunoblot using antibodies against phosphorylated p44/42 MAPK (Fig. 2C). Incubation with 5 or 20 μM PD 98059 reduced phosphorylated MAPK by 50–60% (determined by densitometric scanning of the autoradiograms shown), in the absence of any change in total MAPK. Thus, partial blockade of Ras signaling by inhibiting MAPK/ERK activation restores sensitivity to IGFBP-3 in breast cells expressing oncogenic ras.

One possible mechanism by which PD 98059 might increase sensitivity to inhibition by IGFBP-3 would be through increasing the responsiveness to endogenous IGFs. However, in the presence of PD 98059, neither aIR-3 nor IGFBP-6 caused further inhibition of DNA synthesis in MCF-10Aras or Ha-ras cells (not shown), indicating that restoration of IGFBP-3 inhibitory activity was unrelated to its binding of endogenous IGFs. Furthermore, Ha-ras cells, which show no response to exogenous IGF-I (27), did not show increased IGF sensitivity in the presence of PD 98059 (not shown).

It has been proposed that the intracellular actions of IGFBP-3 are initiated through its interaction with a cell-surface receptor (26), although no signaling receptor has been identified. In common with other peptide receptors, the cell-surface expression of such a receptor might be down-regulated by a high concentration of its ligand. In contrast with many other breast cancer cells which secrete little or no IGFBP-3 (10), ras transfected MCF-10A cells similarly secreted higher levels than their nontransfected counterparts (Table I). To determine whether the restoration of sensitivity to IGFBP-3 in response to PD 98059 was the result of decreased production of IGFBP-3, and thereby release from down-regulation of a putative signaling receptor, IGFBP-3 concentrations in medium conditioned by cells treated with PD 98059 were measured. As shown in
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**DISCUSSION**

Although antiproliferative and pro-apoptotic effects of IGFBP-3 have been reported in a variety of breast cancer cell lines, its actions in nonmalignant breast cells have not been described previously. The data presented in this report indicate that normal breast epithelial cells are considerably more sensitive to IGFBP-3 than tumor-derived breast cancer cells, which require 500–1500 ng/ml IGFBP-3 to achieve a similar level of inhibition to that seen with 10–100 ng/ml in the nontransformed cells (7, 27). Our findings also indicate that resistance to IGFBP-3 is induced in breast cells transfected with oncogenic ras, thereby implicating increased activity of Ras-dependent signaling pathways in the development of IGFBP-3 insensitivity. In contrast, sensitivity toward the growth inhibitor TGF-β was unaffected by ras transfection, suggesting some specificity in the IGFBP-3 effect.

Activation of the MAPK/ERK pathway, whether by ras mutation or other mechanisms such as growth factor receptor activation, may be a common feature of the malignant growth of breast epithelial cells (24), and our results would indicate that in these situations resistance to the tumor-suppressive activity of endogenous IGFBP-3 would be one result of such activation. Several lines of evidence indicate that changes in sensitivity to IGFBP-3 resulting from oncogenic ras expression or MAPK blockade are unrelated to effects on IGF signaling or sensitivity. First, MCF-10A cells are not inhibited by IGFBP-6 or the type 1 IGF receptor antagonist, aRI-3, despite their sensitivity to IGFBP-3. Second, transfection of MCF-10A cells with oncogenic ras actually increased their sensitivity to IGF-1, so that loss of IGF-I responsiveness could not explain their loss of inhibition by IGFBP-3. Third, the insensitivity of HS578T cells to IGF-I is not altered in the presence of PD 98059.

Increased proteolysis of exogenous IGFBP-3 in the MCF-10Aras cells (A) and HS578T cells (B) were incubated with the indicated concentrations of IGFBP-3 in the absence (closed symbols) or presence (open symbols) of 5 μM PD 98059 for 24 h at 37 °C. Cell-associated IGFBP-3 was determined immunologically as described under “Experimental Procedures.” Results are expressed as the percent of 125I-protein A bound to cells in the absence of added IGFBP-3 or PD 98059 and are the mean ± S.E. of triplicate wells from one of two experiments with identical results. *, p < 0.05 and **, p < 0.0001 compared with untreated control; †, p < 0.05 and ††, p < 0.001 compared with 5 μM PD 98059 in the absence of IGFBP-3, by analysis of variance and Fisher’s PLSD. There was no significant effect of PD 98059 at any concentration of IGFBP-3 in either cell line.

**TABLE I**

Effects of PD 98059 on secreted IGFBP-3 levels in nontransformed and transformed breast cells

| Secreted IGFBP-3  | No addition | +5 μM PD 98059 |
|-------------------|-------------|---------------|
|                   | ng/well     |               |
| MCF-10A           | 32.9 ± 0.5  | 36.3 ± 1.3    |
| MCF-10Aras        | 22.8 ± 0.9* | 20.9 ± 0.9*   |
| MCF-10Avector     | 51.5 ± 1.8* | 52.6 ± 1.0*   |
| HS578T            | 40.7 ± 2.5  | 37.1 ± 0.9    |

a p < 0.001 compared with MCF-10A.

b p < 0.001 compared with MCF-10Avector for either no addition or PD 98059-treated cells (by analysis of variance and Fisher’s PLSD).

Table I, PD 98059 treatment, while restoring sensitivity to exogenous IGFBP-3, had no significant effect on IGFBP-3 secretion in any of the cell lines tested.

Cell association of IGFBP-3 has been shown in some breast cancer cell lines to correlate with its growth inhibitory effect (26). We therefore investigated whether the amount of cell-associated IGFBP-3 changed under conditions where sensitivity to its inhibitory effects was increased. In the absence of IGFBP-3 or PD 98059, cell-associated IGFBP-3 levels were similar in the MCF-10Aras and HS578T cells and 2–3-fold higher than in either the parent or control vector-transfected MCF-10A cell lines (data not shown). As shown in Fig. 3A for MCF-10Aras cells, addition of IGFBP-3 significantly increased the amount of IGFBP-3 associated with the cell at all doses tested. However, co-addition of 5 μM PD 98059 had no effect on cell-associated protein in the absence or presence of concentrations of IGFBP-3 shown previously (in Fig. 2) to inhibit DNA synthesis. In the HS578T cells, a significant increase in the amount of cell-associated IGFBP-3 was seen with 100 ng/ml dose of exogenous IGFBP-3 only, and this did not change in the presence of PD 98059 (Fig. 3B). These observations argue against a direct link between the degree of cell association of IGFBP-3 and its inhibition of DNA synthesis.

![Fig. 3. Cell association of IGFBP-3 in PD 98059-treated cells.](image)
10Aras cell line was also investigated as a possible cause of the lack of response to its inhibitory effects. In other cell systems, limited degradation of IGFBP-3 has been demonstrated to affect its IGF-independent (28) and -dependent (29) actions; however, there was no significant loss of intact IGFBP-3 over the 24-h treatment period for the MCF-10Aras cells. We also investigated the possibility that a high level of endogenous IGFBP-3, which is characteristic of both the Hs578T and MCF-10Aras cell lines, was leading to down-regulation of a putative signaling receptor, but found that sensitivity to IGFBP-3 could be reinstated in the absence of any change in the level of secreted IGFBP-3.

It has been reported that the antiproliferative effects of IGFBP-3 correlate with its presence on the cell surface of the cell (27). This observation has been interpreted as evidence for the existence of a signaling receptor (26), which has been suggested recently to be the type V TGF-β receptor (30) despite a lack of specific evidence that this receptor mediates either TGF-β or IGFBP-3-dependent events. Although we identified IGFBP-3 in association with the surface of MCF-10A and MCF-10Aras cells, its concentration did not correlate with sensitivity to its effects. In IGFBP-3-insensitive MCF-10Aras cells, addition of IGFBP-3 resulted in an increase in the amount of cell-associated IGFBP-3 without inhibiting DNA synthesis, while treatment with PD 98059, which restored sensitivity to the growth inhibitory effects of IGFBP-3 in both Hs578T and MCF-10Aras cells, did not alter the amount of cell-associated IGFBP-3. This suggests that cell binding of IGFBP-3 per se does not lead to inhibition of DNA synthesis and implies the existence of other factors, either intracellular or extracellular, involved in the regulation of IGFBP-3 inhibitory signaling. Our findings would also indicate that the expression or activity of such factors is Ras-dependent.

We showed recently that in T47D breast cancer cells transfected to overexpress IGFBP-3, the cell population changed over several passages from a state of relative cell cycle arrest by IGFBP-3 to a state of insensitivity to its inhibitory effect, where cell growth was uninhibited by high levels of secreted and cell-bound IGFBP-3 (31). Although the mechanism for this transition is unknown, we have now demonstrated that the expression of oncogenic ras, either in a transfected cell line or in breast tumor-derived cells, is associated with resistance to IGFBP-3 inhibitory signaling. IGFBP-3 is abundant in the human circulation (32), where a high concentration is a negative risk factor for the development of breast and prostate cancer (33, 34). We propose that the development of resistance to IGFBP-3 may be a key step in the dysregulation of breast cancer cell growth, raising the possibility that future therapies could be directed toward maintaining or restoring sensitivity to the growth inhibitory effects of this protein.

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