Growth Inhibition by Insulin-like Growth Factor-binding Protein-3 in T47D Breast Cancer Cells Requires Transforming Growth Factor-β (TGF-β) and the Type II TGF-β Receptor*‡

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Susan Fanayan, Sue M. Firth, Alison J. Butt, and Robert C. Baxter‡
From the Kolling Institute of Medical Research, University of Sydney, Royal North Shore Hospital, St. Leonards, New South Wales 2065, Australia

This study explores the relationship between anti-proliferative signaling by transforming growth factor-β (TGF-β) and insulin-like growth factor-binding protein-3 (IGFBP-3) in human breast cancer cells. In MCF-7 cells, the expression of recombinant IGFBP-3 inhibited proliferation and sensitized the cells to further inhibition by TGF-β1. To investigate the mechanism, we used T47D cells that lack type II TGF-β receptor (TGF-βRII) and are insensitive to TGF-β1. After introducing the TGF-βRII by transfection, the basal proliferation rate was significantly decreased. Exogenous TGF-β1 caused no further growth inhibition, but immunoneutralization of endogenous TGF-β1 restored the proliferation rate almost to the control level. The addition of IGFBP-3 did not inhibit the proliferation of control cells but caused dose-dependent inhibition in TGF-βRII-expressing cells when exogenous TGF-β1 was also present. Similarly, receptor-expressing cells showed dose-dependent sensitivity to exogenous TGF-β1 only in the presence of exogenous IGFBP-3. This indicates that in these cells, anti-proliferative signaling by exogenous IGFBP-3 requires both the TGF-βRI and exogenous TGF-β1. To investigate this synergism, the phosphorylation of TGF-β signaling intermediates, Smad2 and Smad3, was measured. Phosphorylation of each Smad was stimulated by TGF-β1 and, independently, by IGFBP-3 with the two agents together showing a cumulative effect. These data suggest that IGFBP-3 inhibitory signaling requires an active TGF-β signaling pathway and implicate Smad2 and Smad3 in IGFBP-3 signal transduction.

Transforming growth factor-β (TGF-β)1 is a member of a family of structurally homologous dimeric proteins, which are multifunctional growth factors (1). TGF-β has been shown to display a variety of biological activities including the negative and positive regulations of cell growth, stimulation of extracellular matrix formation, stimulation of angiogenesis, and induction of differentiation of several cell lineages (2, 3). All human breast tumor cell lines secrete all three isoforms of TGF-β, namely TGF-β1, TGF-β2, and TGF-β3 (4, 5), and levels are elevated with increased malignancy (6).

TGF-β is synthesized and secreted as a high molecular weight latent complex that restricts its in vivo availability (7, 8). TGF-β must be released from this complex before it can exert its actions, which is an important regulatory step in the action of this growth factor. Biological activities of TGF-β are believed to be mediated through specific cell surface receptors (9). A number of different size receptors have been identified in cultured cells and tissues, which include types I, II, III, IV, V, and VI receptors (10, 11). Of them, only type I receptor (TGF-βRI) and type II receptor (TGF-βRII) have been shown to be directly involved in signal transduction (12). This is supported by the observation that the lack of response to TGF-β in some types of cancer cell lines correlates with the loss or low expression levels of TGF-βRI and/or TGF-βRII (13, 14).

Smads are molecules of relative molecular mass 40–60 kDa with two regions of homology at the NH2- and COOH-terminals termed Mad homology domains (MH1 and MH2, respectively), connected with a proline-rich linker sequence (15). They fall into three classes based on sequence similarity and function. Class I Smads or pathway-restricted Smads couple to different receptors. Of these, Smad2 and Smad3 are phosphorylated after stimulation by TGF-β (16) or activin (17), whereas Smad1 and Smad5 are involved in bone morphogenetic protein signaling (18). In the COOH-terminal region, pathway-restricted Smads have a characteristic Ser-Ser-X-Ser motif, the two most COOH-terminal serine residues of which are phosphorylated by activated TGF-βRI (16, 18). Class II Smads that are represented by Smad4 (20) appear to be a general partner for the pathway-restricted Smads by bringing the cytoplasmic Smads into the nucleus where they can activate transcriptional responses (16, 21). Class III Smads, which include Smad6 and Smad7, are known as the inhibitory Smads because they bind to TGF-βRII and interfere with the phosphorylation of the pathway-restricted Smads (22, 23).

After the binding of TGF-β to TGF-βRII, a constitutively active serine-threonine kinase, TGF-βRI is recruited into the complex where it is phosphorylated by the type II receptor. The activated TGF-βRI then interacts with and phosphorylates Smad2 and Smad3, thus inducing their association with Smad4 followed by the translocation of the heteromeric complex to the nucleus where they can potentiate the transcription of target genes (21, 24).

Insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3) is a member of a family of six well characterized IGF-binding proteins, IGFBP-1 to IGFBP-6 (25). IGFBP-3 is the most abundant IGFBP in the circulation, serves as a storage depot for IGFs (26), and has been shown to have both IGF-dependent and IGF-independent effects on cell proliferation. In its IGF-
dependent actions, IGFBP-3 modulates the interaction between IGFs and their cell surface receptors, resulting in either inhibition or stimulation of cellular growth (27, 28). IGFBP-3 has also been shown to act as a growth inhibitor in the absence of IGFs (29), an effect that may be mediated by putative IGFBP-3 receptor(s) on the cell surface. No signaling receptor for IGFBP-3 has yet been unequivocally identified.

MCF-7 human breast cancer cells express intact TGF-β signaling machinery and have been shown to be responsive to TGF-β growth inhibition. On the other hand, T47D human breast cancer cells lack TGF-βRII and are unresponsive to TGF-β treatment (30). We have found that T47D cells also fail to respond to exogenous IGFBP-3, although we previously showed that transfection with IGFBP-3 is growth inhibitory to these cells (31). In this study, we demonstrate that restoration of active TGF-β signaling is required for T47D cells to respond to exogenous IGFBP-3 and that IGFBP-3 stimulates the phosphorylation of the signaling intermediates Smad2 and Smad3, suggesting a previously unrecognized pathway of IGFBP-3 signaling.

EXPERIMENTAL PROCEDURES

Materials—T47D and MCF-7 cells were purchased from the American Type Culture Collection (Rockville, MD). Anti-human TGF-βRII polyclonal antibody (C-16) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human TGF-β1 monoclonal antibody (MAB240) was purchased from R&D Systems (Minneapolis, MN). Anti-human Smad2 (LPB2), anti-human Smad3 (LPC3), and anti-phospho-Smad2 (Poly-Z-PS1) polyclonal antibodies were purchased from Zymed Laboratories, Inc. (San Francisco, CA). Recombinant human TGF-β1 was purchased from Austral Biologicals (San Ramon, CA). IGFBP-3 was isolated from Cohn fraction IV of human plasma (32).

Cell Culture—Stock cultures of T47D and MCF-7 cells were routinely maintained in RPMI 1640 medium (Cytosystems, North Ryde, Australia) supplemented with 10 μg/ml insulin, 2.92 mg/ml glucose, and 10% (v/v) fetal bovine serum. Transfection—A 1.7-kilobase pair TGF-βRII cDNA fragment (a gift from J. P. Pujol, Caen, France) was cloned into the expression vector pcDNA3 (Invitrogen, Leek, The Netherlands) to generate pcDNA3/TGF-βRII. A 1.1-kilobase pair EcoRI-PvuII fragment isolated from pIIF6 (33), which contains the full coding sequence of hIGFBP-3 (provided by W. I. Wood, Gentech, San Francisco, CA), was inserted into the vector pOP13 (Stratagene, San Francisco, CA) to generate pOP13/IGFBP-3. Transfections were performed by the LipofectAMINE-mediated procedure as recommended by the manufacturer (Life Technologies, Inc.). Transfected cells were maintained in media containing Genetin (500 μg/ml for T47D, 800 μg/ml for MCF-7) for 21 days post-transfection to select for stable transfectants. Experiments were performed on mixed population cultures of transfectants.

Northern Blot—Total cellular RNA was extracted from cells by the guanidine isothiocyanate/acid-phenol technique (34). Total RNA samples (20 μg) were electrophoresed in 1% agarose gels containing 2.2 M formaldehyde. The RNA was transferred by capillary blotting to Zetablot membranes (Bio-Rad) and cross-linked by baking at 80 °C.

Immunoprecipitation of Cell Lysates—Cells in 60-mm dishes (20 g) were electrophoresed in 1% agarose gels containing 2.2 M formaldehyde. The RNA was transferred by capillary blotting to Zetablot membranes (Bio-Rad) and cross-linked by baking at 80 °C. The signals were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

 FIG. 1. A, immunoblot analysis of media conditioned by control vector-transfected MCF-7 cells and IGFBP-3 transfected MCF-7 cells. For 72 h, conditioned media from subconfluent cultures of vector-transfected cells (lanes 1, 3, and 5) or IGFBP-3 transfected MCF-7 cells (lanes 2, 4, and 6) were either concentrated 20-fold (lanes 1 and 2) or immunoprecipitated with hIGFBP-3 specific antibody (lanes 3–6). Samples were electrophoresed and transferred to nitrocellulose as described under "Experimental Procedures" and then probed with either 125I-IGF-I, 1 × 106 cpm/ml (lanes 1–4) or antisera R-30 to hIGFBP-3 (1.7500 final dilution) (lanes 5 and 6). The mobilities of molecular mass markers (a-pIPI/IGF-BP-3, TGF-β1, IGFBP-3, and IGFBP-5) are indicated on the left. B, responsiveness of MCF-7/IGFBP-3 cells to TGF-β1 growth inhibition. Vector-transfected MCF-7 cells and IGFBP-3 transfected MCF-7 cells were seeded at 5 × 104 cells/well in media containing 10% (v/v) fetal bovine serum. Cells were treated with various concentrations of TGF-β1 for 4 days, the monolayers were trypsinized, and total cell numbers were determined in a hemocytometer. Values shown are the means ± S.E. of triplicate wells from three independent experiments. * p < 0.0001 for MCF-7/IGFBP-3 versus MCF-7/vector cells.

IGF-I, 1 Enzyme-linked Immunosorbent Assay of Conditioned Media—Cells were plated in six-well plates in the presence of 10% (v/v) fetal bovine serum. After reaching 80–90% confluence, cultures were changed to serum-free media for 96 h. Conditioned media were then collected and assayed for both active and total TGF-β1, using TGF-β1 Immunoassay System (Promega, Madison, WI) that measures both naturally processed (endogenous active) and acid-treated (total) TGF-β1. To measure total TGF-β1, 100 μl of conditioned medium was first acid-treated with 5 μl of 1 M HCl for 15 min at 22 °C and then neutralized with 5 μl of 1 M NaOH.

Measurement of Cell Growth—Cells were seeded at 5 × 104 cells/well in 12-well plates in the presence of 10% (v/v) fetal bovine serum. After 3 days, cells were washed with serum-free media and treated with various concentrations of TGF-β1 in the absence or presence of exogenous IGFBP-3 for 4 days. Cell monolayers were then trypsinized, and the viable cell numbers were counted in a hemocytometer.

Statistical Analysis—Statistical analysis was carried out using StatView 4.02 (Abacus Concepts, Inc., Berkeley, CA). Differences between the groups were evaluated by Fisher PLSD (Protected Least Significant Difference) test after analysis of variance using repeated measures or factorial analysis where appropriate.

IGF-β Action in MCF-7 Cells Transfected with hIGFBP-3 cDNA—To determine whether IGFBP-3 has a physiological relevance in cellular responses to TGF-β, we introduced the IGFBP-3 cDNA into MCF-7 cells, which have intact TGF-β signaling machinery. Analysis of culture medium conditioned by IGFBP-3 transfected MCF-7 (MCF-7/IGFBP-3) cells for 3 days revealed up to 50 ng/ml IGFBP-3, which was determined by radiommunoassay. In contrast, IGFBP-3 was not detected in medium-conditioned control vector-transfected cells (MCF-7/vector). 125I-IGF-I ligand blot analysis (Fig. 1A) of concentrated culture medium, conditioned by MCF-7/vector cells, indicated the presence of three IGFBPs (IGFBP-2, IGFBP-4, and IGFBP-5) as described previously (36). In addition to these IGFBPs, a 43-kDa IGF-I ligand was also present in MCF-7/IGFBP-3 conditioned medium, which was detected by 125I-IGF-I ligand

TBS (150 mM NaCl, 10 mM Tris) containing 1% (w/v) bovine serum albumin and 0.05% (v/v) NaN3 at 4 °C. After washing in cold TBS, the membrane was incubated at 22 °C with radiiodinated protein A followed by several washes with TBS buffer. The dried membrane was exposed to Hyperfilm-MP for 48–72 h before developing.
In the absence of exogenous TGF-β1, IGFBP-3-expressing MCF-7 cells demonstrated a basal proliferation rate, which was almost half that of the control vector-transfected cells ($p < 0.0001$) (Fig. 1B). The lack of growth inhibitory effect of TGF-β1 on vector-transfected MCF-7 cells was hypothesized to be due to the presence of endogenous TGF-β1 produced by the cells, which might render the cells less responsive to additional TGF-β1 treatment. Because most cell lines have been shown to secrete TGF-β1 in a latent form requiring acidification to become active (37), we measured TGF-β1 activity in neutral-treated and acid-treated media from MCF-7 cells. TGF-β1 enzyme-linked immunosorbent assay of medium conditioned by MCF-7/vector cells revealed the presence of 3.2 ng/ml total TGF-β1, of which approximately 1.5 ng/ml was present in the active form. The level of TGF-β1 expressed was similar in the presence of endogenous IGFBP-3 in MCF-7/IGFBP-3 cells (Table I).

Expression of TGF-βRII by T47D Cells Transfected with TGF-βRII cDNA—Because the sensitivity of MCF-7 cells to TGF-β1 appeared to be enhanced when the cells were expressing IGFBP-3, we used the TGF-βRII-negative T47D cell line to investigate possible synergism between TGF-β and IGFBP-3 signaling. To demonstrate that TGF-β1 responsiveness could be restored in T47D cells after TGF-βRII expression, cells were stably transfected with either the plasmid vector pcDNA3 or the recombinant plasmid pcDNA3/TGF-βRII. Northern blot analysis of total RNA isolated from the transfected cells revealed the presence of a 1.7-kilobase band, corresponding to the TGF-βRII mRNA transcript in T47D/TGF-βRII cells but not in control T47D/vector cells (Fig. 2A). In addition, immunoprecipitated cell lysates of T47D/vector and T47D/TGF-βRII cells were examined using SDS-polyacrylamide gel electrophoresis and Western immunoblotting with an anti-TGF-βRII antibody. Fig. 2B shows that TGF-βRII-transfected cells but not vector-transfected T47D cells expressed the ~75-kDa TGF-βRII.

Effect of TGF-βRII Expression on T47D Cell Proliferation—As shown in Fig. 3A, the basal proliferation rate of T47D/TGF-βRII cells, measured over 4 days serum-free after the initial plating period, was significantly lower in the absence of exogenous TGF-β1 than in vector-transfected cells ($p < 0.0001$). The addition of exogenous TGF-β1 (1–10 ng/ml) slightly stimulated T47D/TGF-βRII cell growth ($p = 0.005$) but had no effect on the proliferation of the control T47D/vector cells.
The absence of open symbols alone did not affect control T47D/vector cell growth, although T47D/TGF-β1 cells treated with increasing concentrations of IGFBP-3 (A and B) in the absence (open symbols) and presence (closed symbols) of 500 ng/ml IGFBP-3 for 4 days. C and D, cells were treated with increasing concentrations of IGFBP-3 in the absence (open symbols) and presence (closed symbols) of 2.5 ng/ml TGF-β1 for 4 days. The monolayers were then trypsinized, and total cell numbers were determined as described under "Experimental Procedures." Values shown are the means ± S.E. of triplicate wells from four independent experiments for A and B and from two independent experiments for C and D. Only error bars larger than the symbols used are shown. *p < 0.0001 (repeated measures of analysis of variance) for TGF-β1 (0 ng) versus TGF-β1 (2.5 ng) in T47D/TGF-βRII cells treated with increasing concentrations of IGFBP-3 (D); and †, p < 0.0001 for IGFBP-3 (0 ng) versus IGFBP-3 (500 ng) in T47D/TGF-βRII cells treated with increasing concentrations of TGF-β1 (B).

The presence of endogenous active TGF-β1, which only inhibits proliferation in the presence of functional TGF-βRII. Treatment of both cell lines with 15 μg of non-immune IgG had no effect on the proliferation of either cell line (data not shown).

**IGFBP-3 and TGF-β1 Act Synergistically to Inhibit Cell Growth**—To investigate the functional interaction between TGF-β1 and IGFBP-3, T47D/TGF-βRII and T47D/vector cells were treated with various concentrations of exogenous TGF-β1 (0–10 ng) in the absence or presence of 500 ng of human plasma-derived IGFBP-3 for 4 days. The vector-transfected T47D cells, which showed no response to treatment by exogenous TGF-β1 alone, were slightly growth-stimulated in the presence of 500 ng of IGFBP-3 at the higher concentrations of TGF-β1 (p = 0.001) (Fig. 4A). However, in T47D/TGF-βRII cells, which were slightly growth-stimulated after treatment with TGF-β1 alone (Figs. 3A and 4B), the basal cell number was further reduced in the presence of TGF-β1 together with IGFBP-3 (p < 0.0001) in a TGF-β1 dose-dependent manner (Fig. 4B).

Similar observations were made when cells were treated with fixed concentration (2.5 ng/ml) of exogenous TGF-β1 together with increasing concentrations of IGFBP-3. IGFBP-3 alone did not affect control T47D/vector cell growth, although when TGF-β1 was added together with IGFBP-3, cells were slightly growth-stimulated (p < 0.0001) (Fig. 4C). Similarly, T47D/TGF-βRII cells did not respond to treatment with IGFBP-3 alone (Fig. 4D); however, when co-treated with IGFBP-3 and TGF-β1, they were growth-inhibited in a IGFBP-3 dose-dependent manner (p < 0.0001). These data indicate a synergism between IGFBP-3 and TGF-β1, which requires the presence of the TGF-βRII.

**Intracellular Signaling Mechanism Involved in TGF-β1 and IGFBP-3 Regulation of Cell Growth**—To investigate whether the TGF-β signaling intermediates, Smads, were involved in the synergism between TGF-β1 and IGFBP-3, Smad2 and Smad3 serine phosphorylation was analyzed. Both T47D/vector and T47D/TGF-βRII cells were treated with exogenous TGF-β1 at 2.5 ng/ml for 0–30 min before analyzing anti-phosphoserine antibody immunoprecipitated cell lysates by SDS-polyacrylamide gel electrophoresis. There was an undetectable level of phosphorylated Smad2 or Smad3 in T47D/vector cells. However, in T47D/TGF-βRII cells, Smad2 and Smad3 showed maximal phosphorylation when exposed to TGF-β1 at different time points after treatment with the optimal time for Smad2 phosphorylation occurring at 5–10 min followed by a decrease to the basal level by about 30 min. On the other hand, Smad3 phosphorylation occurred slightly later with maximum phosphorylation detected at 15 min post-TGF-β1 treatment, which then decreased to the basal level by 30 min (Fig. 5A). IGFBP-3 also induced Smad2 and Smad3 phosphorylation in T47D/vector cells, although with a different time course to TGF-β-induced phosphorylation. IGFBP-3-induced phosphorylation of Smad2 occurred later than that induced by TGF-β1, increasing at 15 min post-treatment and peaking after 30 min followed by a decrease after 3 h of treatment (Fig. 5B). IGFBP-3 phosphorylation of Smad3 peaked later than that of Smad2, with a maximal phosphorylation observed 90 min after treatment with exogenous IGFBP-3, declining by 3 h (Fig. 5B). Like TGF-β1, IGFBP-3 was unable to induce Smad2 or Smad3 phosphorylation in control vector-transfected T47D cells (data not shown), suggesting that both TGF-β1 and IGFBP-3 require functional TGF-β signaling machinery to induce phosphorylation of Smad proteins.

Because the co-treatment of T47D/TGF-βRII cells with TGF-β1 and IGFBP-3 revealed their synergistic effect on cell growth and based on the individual ability of TGF-β1 and
IGFBP-3 to induce Smad2 and Smad3 phosphorylation (Fig. 5, A and B), we examined the effect of their co-treatment on both Smad2 and Smad3 phosphorylations. This study was done by performing a single time point of TGF-β1 treatment, which induced maximal Smad2 or Smad3 phosphorylation, while IGFBP-3 treatment was carried out for 15, 30, 90, 180, or 240 min. Based on the results from TGF-β1-induced phosphorylation of Smad2 and Smad3 (Fig. 5A), each IGFBP-3 time point incorporated a final 10 min of TGF-β1 treatment for the Smad2 experiment, whereas to test Smad3 phosphorylation, a final 15-min TGF-β1 treatment was combined with each IGFBP-3 treatment. Fig. 5C shows that T47D/TGF-βRII cells treated with exogenous IGFBP-3 for 15 min, in the last 10 min of which TGF-β1 was also added, exhibited maximum Smad2 phosphorylation. Smad3 phosphorylation, on the other hand, was more significant after a 30-min IGFBP-3 treatment, which included TGF-β1 treatment in the last 15 min (Fig. 5C). These levels of Smad2 and Smad3 phosphorylation were greater than the levels induced by either TGF-β1 or IGFBP-3 alone, suggesting a synergism between TGF-β1 and IGFBP-3 in Smad phosphorylation.

**DISCUSSION**

Intracellular signals from TGF-β are transduced by a mechanism that involves the transmembrane serine-threonine kinase receptors, TGF-βRI and TGF-βRII (38). TGF-β binds primarily to the TGF-βRII followed by the recruitment of TGF-βRI to form a ternary complex that allows TGF-βRII to phosphorylate TGF-βRII. This activation of the type I receptor kinase is the first necessary step in transducing the TGF-β signal downstream.

There is now considerable evidence that the loss of TGF-βRII expression occurs in a variety of human neoplasms, resulting in a lack of response to TGF-β growth inhibition in these cells. Loss of TGF-βRII expression is caused by various mechanisms including homozygous gene losses, gross gene rearrangements, and truncated transcripts of this receptor (13, 30, 39, 40). Inactivation of the TGF-β pathway has also been shown to be caused by the mutation or loss of TGF-βRII (41, 42). Furthermore, the deletion or mutation of Smad2, Smad3, or Smad4 has also been demonstrated in several cancer cell lines (43). Any of these mechanisms provides a selective advantage by allowing the cells to escape from TGF-β-mediated growth control.

In this study, we have investigated possible interactions between TGF-β and IGFBP-3 signaling pathways. We first showed that the overexpression of IGFBP-3 in MCF-7 human breast cancer cells, which normally express low levels of the protein (44), led to a decreased proliferation rate. Exogenous IGFBP-3 is known to inhibit MCF-7 cell proliferation (45), and the growth inhibitory effect of the anti-estrogen ICI 182780 (46) and vitamin D (47) on these cells is believed to be mediated in part by the induction of IGFBP-3 gene expression. In contrast, transfection of MCF-7 cells with IGFBP-3 cDNA was reported by Chen et al. (48) to enhance IGF-I-stimulated proliferation. Although the different responses to IGFBP-3 in different studies are not easily explained, they may be related to the presence of IGFBP-3 proteolytic activity secreted by MCF-7 cells (49), which has recently been shown to be stimulated by estrogen and inhibited by TGF-β (50).

We found that in IGFBP-3-transfected MCF-7 cells, sensitivity to growth inhibition by TGF-β was unexpectedly increased, raising the possibility of an interaction between TGF-β and IGFBP-3 inhibitory signaling. Late passage MCF-7 cells are reported to be less sensitive to TGF-β than early passage MCF-7 cells, a difference associated with a 3-fold reduction in TGF-βRII expression (51). To investigate further how TGF-β signaling might be influenced by IGFBP-3, we turned to the T47D breast cancer cell line, which is reported in some studies to totally lack the TGF-βRII expression. In fact, there are conflicting reports regarding the level of TGF-βRII in T47D cells and the sensitivity of these cells to TGF-β treatment. TGF-β inhibition of T47D cell proliferation has been demonstrated by several groups (52–54), whereas others (55, 56) have demonstrated a resistance to TGF-β effects in these cells. Kalkhoven et al. (30) have shown that resistance to TGF-β growth inhibition was due to the lack of sufficient TGF-βRII expression. Pouliot and Labrie (43) have also shown that T47D cells lack TGF-βRII but express mRNAs for Smad2, Smad3, and Smad4. The differences in the TGF-β responsiveness of T47D cell lines reported by various groups may reflect the variation between clonal lines used in different studies.

In the present study, we used a T47D cell line with demonstrable resistance to TGF-β1 due to lack of TGF-βRII. Transfection of these cells with a TGF-βRII expression plasmid resulted in a significantly lower basal proliferation rate compared with control vector-transfected cells. This observation suggested that other components in the TGF-β signal transduction pathway are intact and that the loss of TGF-βRII expression is the mechanism by which these T47D cells escape TGF-β growth inhibition. Treatment with exogenous TGF-β1 alone did not have any further growth inhibitory effect on the T47D/TGF-βRII cells over 4 days, although it increased the basal phosphorylation levels of both Smad2 and Smad3 rapidly (within 10–15 min) and transiently. The lack of effect of exogenous TGF-β1 on 4-day growth inhibition may be due to the presence of a sustained level of endogenous TGF-β1, neutralization of which was able to stimulate cell growth almost to the level of TGF-βRII-negative control cells.

IGFBP-3 has been shown to mediate the growth inhibitory effects of a number of anti-proliferative agents on breast cancer cells. Growth inhibition by TGF-β (57, 58), retinoic acid (59), anti-estrogens (46), and the tumor suppressor p53 (60) has been shown to correlate with the induction of IGFBP-3 at both the transcriptional and translational levels. However, the mechanism by which IGFBP-3 can mediate these growth inhibitory effects is not well understood. In a previous study, we showed that T47D cells transfected with an IGFBP-3 expression plasmid were growth-inhibited at low passage numbers post-transfection but became refractory to the IGFBP-3 growth inhibitory effect at higher passage numbers (31). The mechanism by which the early passage cells producing endogenous IGFBP-3 were sensitive to the IGFBP-3 growth inhibitory effect is not clear. In the light of the present study, this requires further investigation because we have shown here that exogenous IGFBP-3 is not inhibitory to either T47D/vector or T47D/TGF-βRII cells in the absence of exogenous TGF-β. This finding suggests that IGFBP-3 expressed within the cell may be able to bypass the step that requires TGF-β interaction with TGF-βRII at the cell surface to sensitize the cells to exogenous IGFBP-3.

In the presence of an active TGF-β signaling pathway, T47D/TGF-βRII cells are responsive to exogenous IGFBP-3 and TGF-β1, whereas the same combined treatment is inactive in T47D/vector cells. This finding suggests that both active TGF-β signaling pathway and exogenous IGFBP-3 are required to cause growth inhibition in T47D/TGF-βRII cells and that there is synergism between IGFBP-3 and TGF-β1 in their growth inhibitory actions.

In investigating the mechanism of this synergism, we have shown the ability of exogenous IGFBP-3 as well as TGF-β1 to potentiate Smad2 and Smad3 phosphorylation. This is the first report of phosphorylation of intracellular signaling intermediates known to be involved in growth inhibitory signals in response to IGFBP-3. The synergistic effect of IGFBP-3 and
TGF-β1 was also evident in their potentiation of Smad2 and Smad3 phosphorylation, which was greater in the presence of both effectors than either agent alone. This provides evidence for some commonality in the inhibitory signaling pathways used by TGF-β1 and IGFBP-3. We recently reported that the sensitivity to IGFBP-3 in MCF-10A mammary epithelial cells was abrogated by the expression of oncogenic ras and restored when mitogen-activated protein kinase phosphorylation was blocked by the inhibitor PD98059 (61). Whether the mitogen-activated protein kinase pathway provides a link between the inhibitory effects of TGF-β1 and IGFBP-3 in T47D cells remains to be determined.

The identity of the proteins on the T47D cell surface or elsewhere that interact with IGFBP-3 to facilitate the intracellular TGF-β signaling cascade remains elusive. Several cell-associated proteins that bind IGFBP-3 have been described (58, 62, 63), although the specificity of the binding and whether these IGFBP-3-binding proteins play a functional role in the growth inhibition by IGFBP-3 have yet to be confirmed. The type V TGF-β receptor (TGF-βRIV) has recently been described as having a role in mediating the IGF-independent growth inhibitory effect of IGFBP-3 in mink lung cells (64). Interestingly, phosphorylation of Smad2 and Smad3 was not stimulated by IGFBP-3 in this cell line, despite an effect of TGF-β on Smad phosphorylation (65). Thus, the functional link of the reported signaling of IGFBP-3 through TGF-βRIV and the synergy between IGFBP-3 and TGF-β, which is dependent on the presence of TGF-βRII that we have described in T47D cells, is not clear.

In conclusion, we have demonstrated that the well recognized TGF-β signaling pathway requiring the presence of TGF-βRII and involving the phosphorylation of receptor-associated Smads can be activated by IGFBP-3 in a way that apparently increases the sensitivity toward TGF-β, thus leading to an enhancement of growth inhibition in the presence of both agents. The level at which IGFBP-3 interacts and the mechanism by which T47D cells transfected with IGFBP-3 cDNA may bypass the early steps of the TGF-β signaling cascade remain important areas for investigation.

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