Signaling through the Smad Pathway by Insulin-like Growth Factor-binding Protein-3 in Breast Cancer Cells

RELATIONSHIP TO TRANSFORMING GROWTH FACTOR-β1 SIGNALING*

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We previously demonstrated in T47D cells transfected to express the transforming growth factor-β receptor type II (TGF-βRII) that insulin-like growth factor binding protein-3 (IGFBP-3) could stimulate Smad2 and Smad3 phosphorylation, potentiate TGF-β1-stimulated Smad phosphorylation, and cooperate with exogenous TGF-β1 in cell growth inhibition (Fanayan, S., Firth, S. M., Butt, A. J., and Baxter, R. C. (2000) J. Biol. Chem. 275, 39146–39151). This study further explores IGFBP-3 signaling through the Smad pathway. Like TGF-β1, natural and recombinant IGFBP-3 stimulated the time- and dose-dependent phosphorylation of TGF-βRI as well as Smad2 and Smad3. This effect required the presence of TGF-βRII. IGFBP-3 mutated in carboxyl-terminal nuclear localization signal residues retained activity in TGF-βRI and Smad phosphorylation, whereas IGFBP-3 was inactive. Immune neutralization of endogenous TGF-β1 suggested that TGF-β1 was not essential for IGFBP-3 stimulation of this pathway, but it increased the effect of IGFBP-3. IGFBP-3, like TGF-β1, elicited a rapid decline in immunodetectable Smad4 and Smad4/Smad2 complexes. IGFBP-3 and nuclear localization signal mutant IGFBP-3 stimulated the activation of the plasminogen activator inhibitor-1 promoter but was not additive with TGF-β1, suggesting that this end point is not a direct marker of the IGFBP-3 effect on cell proliferation. This study defines a signaling pathway for IGFBP-3 from a cell surface receptor to nuclear transcriptional activity, requiring TGF-βRII but not dependent on the nuclear translocation of IGFBP-3. The precise mechanism by which IGFBP-3 interacts with the TGF-β receptor system remains to be established.

Transforming growth factor-β (TGF-β)† is a multifunctional growth factor secreted by many cell types. The nature of its action on target cells depends not only on the cell type but also on its state of differentiation and other growth factors present (1, 2). One of the biological effects of TGF-β is the inhibition of the proliferation of epithelial cells including some but not all types of malignant cells.

TGF-β signaling from the cell surface to the nucleus requires a series of interdependent events. It is initiated by the association between TGF-β and the type II TGF-β receptor (TGF-βRII), resulting in the recruitment of the type I TGF-β receptor (TGF-βRI) into a heteromeric complex, which allows TGF-βRII to phosphorylate and activate TGF-βRI (3). Signaling intermediates Smad2 and Smad3 are phosphorylated by active TGF-βRII followed by their association with Smad4 and the translocation of heteromeric Smad complexes to the nucleus (4, 5) where they can potentially regulate the transcription of target genes either through binding to elements in the DNA or indirectly by binding to other transcription factors.

The disruption of any of these steps can lead to the loss of TGF-β signaling. In many cases, this resistance to TGF-β-induced growth inhibition is the result of a loss or mutational inactivation of one or several of the genes that encode the signaling intermediates of the TGF-β signaling pathway. For example, inactivating mutations in TGF-βRII occur in most human colorectal and gastric carcinomas with microsatellite instability (6). We and others (7, 8) have previously shown that a lack of responsiveness to TGF-β1 in T47D cells is attributable to the absence of TGF-βRII expression, because restoring receptor expression rendered these cells sensitive to autocrine inhibition by endogenous TGF-β1 (7).

Insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3), the major serum transport protein for the IGFs (9), also inhibits proliferation and stimulates apoptosis in a variety of cell types (10–12). In some epithelial cancer cells, the growth inhibitory effect of TGF-β on cell growth has been shown to be mediated by the up-regulation of IGFBP-3 mRNA and protein levels such that the blockade of IGFBP-3 up-regulation by antisense oligonucleotides ablates the inhibitory effect of TGF-β (13, 14). However, these studies did not suggest a functional interaction between IGFBP-3 and TGF-β signaling in their regulation of cell growth. We demonstrated this interaction by showing that in T47D cells transfected to express the TGF-βRII (T47D-RII cells), TGF-β and IGFBP-3 act together to inhibit cell proliferation, an effect not seen in control cells transfected with empty vector (T47D-vec cells). A mechanism for this concerted action was suggested by the observation that IGFBP-3 stimulates the phosphorylation of Smad2 and Smad3 in these cells and potentiates the stimulation of Smad phosphorylation by TGF-β (7).

The aim of this study was to further examine the role of IGFBP-3 signaling through the Smad pathway by determining its initiation through TGF-βRII phosphorylation and its downstream consequences. We now report that IGFBP-3 can stimulate phosphorylation of the cell surface receptor TGF-βRII and activate the promoter of the TGF-β-responsive gene, PAI-1, which encodes plasminogen activator inhibitor-1 (PAI-1). PAI-1 blocks the activation of plasminogen to plasmin, a protease
that causes partial fragmentation of IGFBP-3, thus limiting its ability to inhibit IGF action (15, 16). Furthermore, we show that basic residues in the carboxyl-terminal region of IGFBP-3 previously shown to be involved in its cell surface association (17) and nuclear translocation (18) are not involved in IGFBP-3 stimulation of the Smad pathway.

EXPERIMENTAL PROCEDURES

Materials—Plasma-derived IGFBP-3 was purified from Cohn fraction IV of human plasma (19). Recombinant human IGFBP-3, recombinant human IGFBP-3 (K225M/G226D/K227E/R228A/K229D) (basic domain mutant), and human IGFBP-5 were prepared from an adenoviral expression system (17, 20, 21). Recombinant human TGF-β1 was purchased from Austral Biologicals (San Ramon, CA). The rabbit anti-human TGF-βRI antibody, C-16, and rabbit anti-human TGF-βRI antibody, H-100, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-human TGF-β1 monoclonal antibody (MAB240) was purchased from R&D Systems (Minneapolis, MN). Anti-phosphoserine (Poly-Z-PS1), anti-human Smad2 (LPB2), and anti-human Smad3 (LP3C) antibodies were from Zymed Laboratories Inc. (San Francisco, CA), and anti-Smad4 antibody was from Upstate Biotechnology, NY.

Cell Models—T47D and MCF-7 cells were purchased from the American Type Culture Collection (Manassas, VA). 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 glutamine, and 10% fetal bovine serum (Cytosystems, North Ryde, Australia) supplemented with 10% fetal bovine serum. T47D cells stably transfected with either pcDNA3 (T47D-vec cells) or pcDNA3/TGF-RII (T47D-RII cells) have been described previously (7). MCF-7 cells were transfected with pcDNA3/TGF-βRII by the LipofectAMINE-mediated procedure as recommended by the manufacturer (Invitrogen), and stable transfectants (MCF-7-RII cells) were selected by main-
observed 15 min post-IGFBP-3 treatment (Fig. 1B). Little or no TGF-βRI phosphorylation was seen in T47D-vec cells, which lack TGF-βRII, despite their abundant level of TGF-βRI, consistent with the essential role of TGF-βRII in this process (data not shown). The effect of TGF-β1 and IGFBP-3 co-treatment was examined by treating the cells with IGFBP-3 for 10, 15, or 30 min, of which the final 10 min included TGF-β1 treatment. Maximal phosphorylation of TGF-βRI induced by TGF-β1 and IGFBP-3 co-treatment occurred at 10–15 min post-IGFBP-3 addition to the cells, but when quantitated by densitometry there was no evidence of synergism between the two agents (data not shown). Measurement of total cell TGF-βRI by immunoblot (Fig. 1B) and quantitation by densitometry confirmed that the observed changes in phospho-TGF-βRI were not because of changes in total levels of receptor protein despite some variability in immunoprecipitated protein among lanes.

The carboxyl-terminal region of IGFBP-3 has been shown to be involved in its cell surface binding (17). However, IGFBP-3 mutated in key cell-binding residues (K228M/G229D/R230G/ R231E/R232A) surprisingly retained the ability to stimulate TGF-βRI phosphorylation. Fig. 1C compares wild-type and mutant recombinant IGFBP-3 with the plasma-derived IGFBP-3 used in previous experiments. All three preparations were active in stimulating TGF-βRI phosphorylation, indicating that the ability of IGFBP-3 to induce TGF-βRI phosphorylation does not require the presence of the carboxyl-terminal basic residues. IGFBP-5 is structurally related to IGFBP-3 with similar basic residues (G225R226K227K) in its carboxy-terminal region implicated in cell and matrix binding (25, 26). Despite this similarity to IGFBP-3, IGFBP-5 was not capable of stimulating TGF-βRI phosphorylation when compared with IGFBP-3-induced TGF-βRI phosphorylation (Fig. 1D).

MCF-7 breast cancer cells were also transfected with a TGF-βRII cDNA, resulting in their increased responsiveness to TGF-β treatment (data not shown). As seen in T47D cells, MCF-7 cells express a high level of endogenous TGF-βRII, which decreased upon TGF-βRII overexpression (Fig. 2A). Fig. 2B illustrates that, like T47D-RII cells, both TGF-β1 and IGFBP-3 induction of TGF-βRII phosphorylation in MCF-7 cells occurred rapidly. TGF-βRII phosphorylation by TGF-β1 was induced within 5 min of TGF-β1 addition, peaking at 10 min post-TGF-β1 treatment. IGFBP-3 phosphorylation of TGF-βRII was also a rapid process with maximum phosphorylation observed after 10 min. Co-treatment with TGF-β1 and IGFBP-3 also shown in Fig. 2B did not provide evidence of synergism between exogenous TGF-β1 and IGFBP-3. As with the T47D-RII cells, the measurement of total cell TGF-βRII by immunoblot (Fig. 2B) and quantitation by densitometry (data not shown) confirmed that the observed changes in phospho-TGF-βRII were not because of changes in total levels of receptor protein.

Smads as Intracellular Mediators of TGF-β1 and IGFBP-3 Signaling—As shown in Fig. 3A, Smad2 was undetectable in lysates of T47D-vec cells either without (lane 1) or with (lane 3) immunoprecipitation with anti-Smad2 antibody. In T47D-RII cells, Smad2 was undetectable without immunoprecipitation (lane 2) but was evident on immunoprecipitation with 5 μg of anti-Smad2 antibody (lane 4), indicating substantial up-regulation in the T47D-RII cells. Similarly, little Smad3 was detectable in T47D-vec cell lysate even when immunoprecipitated with 5 μg of anti-Smad3 antibody (lane 3), whereas abundant Smad3 was detectable when immunoprecipitated in T47D-RII cells (lane 4). Similar up-regulation of Smad2 and Smad3 was seen in MCF-7 cells transfected to increase TGF-βRII expression (Fig. 3B). As in the T47D cells, no Smad2 or Smad 3 was detectable without prior immunoprecipitation. When measured after immunoprecipitation, it was evident that although both Smad2 and Smad3 are present in MCF-7 cells, consistent with active TGF-β signaling in these cells, their levels are increased upon TGF-βRII overexpression.

We previously reported the stimulation of Smad2 and Smad3 phosphorylation by 2.5 ng/ml of TGF-β1 and 500 ng/ml IGFBP-3, concentrations that were optimal in the inhibition of cell proliferation (7). Fig. 4, A and D, shows dose response data for Smad2 and Smad3 phosphorylation, respectively, in which TGF-β1 treatment was carried out for 10 min (Smad2) or 15 min (Smad3), and IGFBP-3 treatment was carried out for 30 min, optimal times from previous experiments (7). Although lower concentrations of IGFBP-3 were capable of inducing both Smad2 and Smad3 phosphorylation, 500 ng/ml IGFBP-3 gave the maximal effect, whereas 1000 ng/ml was less effective. Similarly for TGF-β1, 2.5 ng/ml was optimal in stimulating the phosphorylation of Smad2 and Smad3 with 5 μg/ml being slightly less effective (Fig. 4, A and D). In view of these results, all subsequent TGF-β1 and IGFBP-3 treatment experiments used TGF-β1 at 2.5 ng/ml and IGFBP-3 at 500 ng/ml.

Compared with plasma-derived IGFBP-3, recombinant IGFBP-3 was equally effective in stimulating both Smad2 (Fig. 4B) and Smad3 (Fig. 4E) phosphorylation. However, as seen for
TGF-βRI phosphorylation, recombinant IGFBP-5 was unable to stimulate Smad2 phosphorylation (test was not conducted on Smad3). The basic domain mutant form of IGFBP-3, which was able to increase TGF-βRI phosphorylation, also increased the phosphorylation of both Smad2 (Fig. 4C) and Smad3 (Fig. 4F).

Is Endogenous TGF-β1 Required for IGFBP-3 Action?—Endogenous TGF-β1 produced by T47D cells was previously shown to reduce the proliferation rate of these cells when TGF-βRII was expressed, an effect that was partially reversed by immunoneutralization of the endogenous TGF-β1 (7). This finding indicates the potential for autocrine signaling by TGF-β1 in these cells, raising the question of whether the ability of IGFBP-3 to activate the Smad pathway might require endogenous TGF-β.

To test this hypothesis, cells were treated with IGFBP-3 in the absence or presence of 20 μg/ml anti-TGF-β1 antibody MAb240, a concentration expected from previous experiments to fully neutralize endogenous TGF-β1 in these cells (7). As shown in Fig. 5, no phosphorylated TGF-βRI or Smad2 was observed in vector-transfected T47D cells either in the absence or presence of IGFBP-3 or MAb240. In both T47D-RII and MCF-7 cells, immunoneutralization of the endogenous TGF-β1 reduced basal TGF-βRII and Smad2 phosphorylation to almost undetectable levels, an average of 98% decrease when quantitated by densitometry. IGFBP-3 when added alone to the cells increased TGF-βRII and Smad2 phosphorylation compared with the basal level. When co-treated with IGFBP-3 plus MAb240, the phosphorylation level of both TGF-βRII and Smad2, although reduced at an average of 54% compared with that induced by IGFBP-3 alone as quantitated by densitometry, remained much higher than the basal level of phosphorylation with TGF-β1 immunoneutralized. Similar results were seen with a higher concentration of MAb240 (30 μg/ml, data not shown). These results suggest that IGFBP-3 induction of signaling through the Smad pathway can occur independently of endogenous TGF-β1 but can be enhanced by it.

Role of Smad4 in TGF-β1 and IGFBP-3 Signaling—Phosphorylated Smad2 and Smad3 associate with Smad4 before translocation into the nucleus (5, 27). Therefore, we examined the level of total Smad4 protein in T47D and MCF-7 cells following treatment with exogenous TGF-β1 and IGFBP-3 (Fig. 6A). There was little apparent difference in the basal level of total Smad4 between T47D-vec and T47D-RII cells. In T47D-RII cells, IGFBP-3 treatment caused a rapid decline in detectable Smad4 from 15 to 30 min after treatment. TGF-β1 treatment resulted in an even greater reduction in Smad4 protein with the level almost abolished 10 min post-TGF-β1 treatment. This time course is consistent with TGF-β1 and IGFBP-3 induction of Smad2 and Smad3 phosphorylation. In T47D-vec cells, the level of Smad4 protein remained constant following IGFBP-3 treatment, whereas a small reduction was visible following TGF-β1 treatment (Fig. 6A). In contrast to the rapid decline in total Smad4 detectable after exposure of T47D-RII cells to TGF-β or IGFBP-3, no change in total Smad2 protein was detectable (Fig. 6B).

To further elucidate the role of Smad4 in TGF-β1 and IGFBP-3 signaling, we examined Smad4 interaction with Smad2. The lysates of cells treated with exogenous TGF-β1 or IGFBP-3 were immunoprecipitated with anti-Smad4 antibody followed by immunoblot analysis using anti-Smad2 antibody. Fig. 6C shows that no Smad2 is detected in T47D-vec cells.
indicting the absence of any detectable interaction between Smad2 and Smad4 under basal or treatment conditions in these cells and consistent with their very low level of Smad2 expression. In T47D-RII cells, Smad2 protein was co-precipitated with Smad4 under basal conditions (Fig. 6C, lane 1), but its detectable level decreased at 30 min after treatment with either IGFBP-3 (lane 2) or TGF-β1 (lane 3), consistent with the decreasing levels of total Smad4 as shown in Fig. 6A.

Comparable results were obtained with MCF-7 cells in which both exogenous TGF-β1 and IGFBP-3 treatment reduced the level of Smad4 protein as detected by immunoblot analysis. As seen in T47D cells, total Smad4 was reduced more substantially in MCF-7 cells treated with TGF-β1 than with IGFBP-3 (Fig. 6D). The level of Smad2 was only slightly decreased in MCF-7 cells following IGFBP-3 or TGF-β1 treatment (Fig. 6E). A similar experiment with Smad3 detection was not performed in this study.

**Regulation of PAI-1 Expression by TGF-β1 and IGFBP-3** — To examine the downstream consequence of activating signaling through the Smad pathway, we measured the activation of the promoter for PAI-1, a gene previously characterized as being TGF-β-responsive. Dose-response data for PAI-1 activation by TGF-β1 or IGFBP-3 are shown in Fig. 7, A and B. The TGF-β1 effect was maximal at 2.5–5 ng/ml (p < 0.0001 compared with base line), whereas at 10 ng/ml, TGF-β1 was unable to induce luciferase activity (p = 0.8 compared with base line), consistent with a biphasic effect also observed in TGF-β1-induced Smad phosphorylation. For IGFBP-3, maximal PAI-1 induction was seen at a concentration of 600 ng/ml (p < 0.0001 compared with base line) with significant stimulation seen by 200 ng/ml (p = 0.0002 compared with base line). Higher concentrations (800–1000 ng/ml) were less potent (Fig. 7D), again correlating with the dose-response effect of IGFBP-3 treatment on Smad phosphorylation.

Fig. 7C shows the time course of activation of the PAI-1/L construct in T47D-RII cells following treatment with TGF-β1, IGFBP-3, or both. At 30 min after treatment with either TGF-β1 or IGFBP-3, PAI-1 promoter activity was still similar to the basal level (data not shown). However, significant induction of luciferase expression by both TGF-β1 and IGFBP-3 was clearly seen at 60 and 120 min after TGF-β1 addition (p < 0.0001 by repeated measures of ANOVA). Despite their interaction in inhibiting cell growth (7), the effects of TGF-β1 and IGFBP-3 co-treatment on PAI-1 transcriptional activation were not greater than that of either agent alone (p > 0.05).

Fig. 7D compares the induction of the PAI-1 transcription by plasma-derived IGFBP-3 as well as wild-type and basic region mutant forms of recombinant IGFBP-3 in T47D-RII cells. Cells were treated with various concentrations of these proteins (0, 200, 400, or 600 ng/ml) for 120 min, after which luciferase activity of the cell lysates was measured. Comparing the potency of the three preparations over this dose range by repeated measures of ANOVA, there was no significant difference between the degree of PAI-1 transcriptional activation by natural or recombinant wild-type IGFBP-3 or the basic region mutant protein.

**DISCUSSION**

IGFBP-3 has been reported to mediate the growth inhibitory effects of a number of anti-proliferative agents in breast cancer cell models including TGF-β (13, 14), retinoic acid (28), and antiestrogens (29). In all of those studies, the anti-proliferative
action of these agents correlated with the induction of IGFBP-3 at both transcriptional and translational levels. Co-treatment with an IGFBP-3-neutralizing antibody or an IGFBP-3 antisense oligonucleotide abolished the growth inhibitory effect of these agents, leading to the conclusion that they acted through the induction of IGFBP-3. However, these studies did not address the possibility that IGFBP-3 inhibitory signaling might interact with intracellular pathways activated by the inducing agents. We recently demonstrated that this interaction might exist, at least for TGF-β, by showing that IGFBP-3 could act together with TGF-βRI in inducing Smad phosphorylation (7).

The aim of this study was to determine whether IGFBP-3 signaling through the Smad pathway could be traced both upstream and downstream of the Smads themselves. Our data show clearly that IGFBP-3 signaling in T47D and MCF-7 breast cancer cells can be initiated at the level of TGF-βRI activation and that this requires the presence of TGF-βRII. IGFBP-3 and TGF-β appear to act in a similar manner at this step but without evidence of synergism. By immunoneutralizing endogenous TGF-βRI, we showed that IGFBP-3 can stimulate this pathway, both TGF-βRI and Smad2 phosphorylation, independently of endogenous or exogenous TGF-β, although endogenous TGF-βRI appears to enhance the IGFBP-3 effect. Because all experiments were conducted under serum- and IGF-free conditions and T47D and MCF-7 cells do not produce detectable IGFs, it is improbable that IGFBP-3 acts in this system by modulating IGF signaling through the IGF type I receptor. Rather, these effects appear to be IGF receptor-independent.

The screening of various components of the TGF-β signaling pathway revealed that TGF-βRI was expressed in both T47D and MCF-7 cells. Interestingly, total TGF-βRI levels were down-regulated in both cell lines when TGF-βRII expression was increased, even though phospho-TGF-βRII could be readily stimulated in these cells by TGF-β or IGFBP-3. Although the mechanism for the decrease in total TGF-βRI was not established, it is consistent with the observation that after ligand binding, TGF-βRI/IGFBP-3 heterodimers internalize and may enter a degradative pathway (30). Increased TGF-βRII expression might thus accelerate TGF-βRII turnover as an integral step in TGF-β action.

Pouliot and Labrie (31) have demonstrated mRNA for both Smad2 and Smad3 in T47D cells in the absence of TGF-βRII expression. In our T47D-vec cells, Smad2 and Smad3 proteins were almost undetectable but were strongly up-regulated in T47D-RII cells. Similarly, both Smad2 and Smad3 proteins, although clearly expressed in MCF-7 cells, were up-regulated in MCF-7-RII cells. The mechanism for this marked induction or stabilization of these proteins by TGF-βRII is not clear. In contrast to the marked up-regulation of Smad2 and Smad3, basal Smad4 levels were hardly changed between T47D-vec and T47D-RII cells. However, the level of immunoprecipitable Smad4 appeared to decline within minutes of exposure to either TGF-β or IGFBP-3 as did Smad4-Smad2 complexes despite no short term change in total Smad2 levels.

Smad4 is believed to shuttle continuously between the nucleus and cytoplasm with nuclear retention promoted by the formation of a heterodimer with phosphorylated Smad2. Eventually, Smad2 becomes dephosphorylated, and Smad4 may return to the cytoplasm (32), whereas Smad2 can be ubiquitinated and lost through proteasome-mediated degradation (33). In view of the recycling of Smad4, it is not clear why TGF-β or IGFBP-3 would cause a rapid loss in total detectable Smad4. One possibility is that the epitope recognized by the Smad4 precipitating antibody is blocked by the dimerization of Smad4 with Smad2 or Smad3, an interaction that occurs through the carboxyl-terminal domain of Smad4 (4). In this case the apparent rapid disappearance of Smad4 following TGF-β or IGFBP-3 stimulation of Smad phosphorylation would reflect the rapid formation of heterodimers.

The basic residues KGRKR in the carboxyl-terminal region of IGFBP-3 have been shown to be essential for cell surface association of IGFBP-3 (17), although other central domain residues may also play a role (34). These carboxyl-terminal residues also have a role in the docking of IGFBP-3 with importin-β, the nuclear transport protein that mediates the entry of IGFBP-3 into the nucleus (18, 35). Although it might be predicted that residues involved in cell surface binding would also be required for IGFBP-3 activation of a signaling pathway involving the cell surface TGF-βRI, a mutant form of IGFBP-3 in which the key basic residues were substituted by corresponding acidic residues of IGFBP-1 was active in TGF-βRI and Smad activation, whereas IGFBP-5 containing similar basic residues involved in both cell binding and nuclear import (26, 35, 36) was inactive.

These observations raise the question of the nature of the interaction between IGFBP-3 and the Smad signaling pathway. It is possible that the basic region mutant form of IGFBP-3, although apparently unable to associate with the cell surface as determined by a relatively insensitive detection method, is in fact capable of interacting with low abundance binding sites. Conceivably, central domain sites (34) could be involved in this interaction, perhaps exposed after proteolytic removal of the carboxyl-terminal domain as suggested for IGFBP-5 (37). Specific cell surface receptors for IGFBP-3 have been suggested in a number of studies. Oh et al. (38) reported the identification of specific receptors for IGFBP-3 on the Hs578T cell surface, whereas Leal et al. (39, 40) suggested the TGF-β type V receptor to be the specific receptor for IGFBP-3 in mink lung cells. TGF-β type V receptor is unlikely to be involved in IGFBP-3 signaling through Smads in T47D or MCF-7 cells, because IGFBP-3 binding in mink lung cells was reported to have no effect on Smad phosphorylation (40). It remains to be shown that these putative receptors initiate a signaling cascade when IGFBP-3 is bound. Although we have now demonstrated that IGFBP-3 can initiate signaling through TGF-βRI and Smads in human breast cancer cells, the primary binding interaction that initiates this pathway is still unclear. It must also be considered that IGFBP-3 could interact with TGF-βRI intracellularly rather than from the cell surface, although there is currently no experimental evidence in support of this speculation.

A PAI-1 reporter construct containing the TGF-β-responsive element of the PAI-1 promoter was used as a marker to determine whether signaling initiated by either TGF-β or IGFBP-3 was transduced into the nucleus. PAI-1, which inhibits the activation of plasminogen to plasmin, has been extensively documented as a TGF-β-responsive gene, its promoter activated through Sp1 binding sites as a result of direct Smad-Sp1 interaction (41). Although we used the PAI-1 reporter system as a marker of Smad-mediated signaling, the plasmin system in fact appears to be involved in IGFBP-3 regulation in a number of cell types. For example, plasminogen binds to IGFBP-3 in vivo, an interaction proposed to lead to IGFBP-3 proteolysis (15). Plasmin-derived IGFBP-3 fragments can exert both inhibitory and stimulatory effects on cell proliferation and show greatly decreased IGF binding. Thus, plasmin proteolysis of IGFBP-3 can regulate IGF bioavailability (42). IGF-I in turn decreases plasminogen activity in cell-conditioned medium (16), suggesting a complex regulatory loop.

TGF-β1 and IGFBP-3 appeared equally capable of inducing luciferase reporter activity within 60 min of their addition to T47D-RII cells, suggesting that transcriptional activation by
either IGFBP-3 or TGF-β1 can occur rapidly. This is in contrast to previous reports of TGF-β1 induction of the PAI-1 promoter activity, which involved a longer treatment period (22, 43). It is possible that the relatively high level of TGF-βRII and strongly up-regulated Smad2 and Smad3 levels in T47D-RII cells allowed a transcriptional response to be observed quite rapidly. Despite their individual effects on PAI-1 promoter activity, TGF-β1 and IGFBP-3 added together were no more potent than either agent alone. This finding is in contrast with their inhibitory effect on cell proliferation where a combined effect of either agent alone. This finding is in contrast with their inhibitory effect on cell proliferation where a combined effect of either IGFBP-3 or TGF-β1 is observed quite rapidly.

In conclusion, we have identified for the first time a signaling pathway through which IGFBP-3 action is mediated from the activation of a cell surface receptor to the induction of gene transcription in the nucleus. This pathway requires the presence of TGF-βRII and is not dependent on the nuclear translocation of IGFBP-3. Exactly how IGFBP-3 initiates this pathway is an important unanswered question. The elucidation of target genes that lead to TGF-β- and IGFBP-3-induced growth inhibition also remains to be determined, and with the expression of ~4000 genes (~10% all genes) reported to undergo rapid change following TGF-β treatment (44), the identification of key target genes will be a complex task.

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