Interaction Between IGF Binding Protein-3 and TGFβ in the Regulation of Adipocyte Differentiation

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The development of white adipose tissue involves both the hypertrophy of existing adipocytes and the proliferation and differentiation of preadipocytes. Adipogenic differentiation is inhibited by TGFβ signaling through Smad2/3, and IGF binding protein-3 (IGFBP-3) is also known to activate Smad2/3 signaling in some cell types. We previously reported that exogenous or overexpressed IGFBP-3 inhibits adipogenesis in 3T3-L1 cells, but the role of endogenous IGFBP-3 in this process, and its possible interaction with TGFβ, is not known. During 10-d adipogenic differentiation initiated by insulin, dexamethasone, and 3-isobutyl-1-methylxanthine, 3T3-L1 cells expressed increasing levels of IGFBP-3 and TGFβ1, secreting over 1000 pg/ml of both proteins. Exogenous recombinant human IGFBP-3 paralleled TGFβ1 in stimulating Smad2 phosphorylation in 3T3-L1 preadipocytes, but no additive effect was observed for the two agents. In contrast, knockdown of endogenous IGFBP-3 by small interfering RNA (siRNA) significantly impaired Smad2 activation by 0.25 ng/ml TGFβ1. Transient expression of human IGFBP-3 significantly inhibited the induction of adipogenic markers adiponectin and resistin, and the appearance of lipid droplets, but down-regulation of endogenous IGFBP-3 by siRNA had little effect on the expression of either marker during the 10-d differentiation, compared with nonsilencing control siRNA. However, down-regulation of endogenous IGFBP-3 using two different siRNA significantly reversed the inhibitory effect of TGFβ1 on both adiponectin and resistin induction. We conclude that IGFBP-3 activates inhibitory Smad signaling in 3T3-L1 cells and that endogenous IGFBP-3 modulates their adipogenic differentiation by regulating cell sensitivity towards the inhibitory effect of TGFβ. (***Endocrinology* 153: 4799–4807, 2012)**

The development and expansion of white adipose tissue involves both the hypertrophy of existing adipocytes and the proliferation and differentiation of precursor cells (preadipocytes), the process of adipogenesis (1–5). Adipogenesis occurs in two steps: the differentiation of multipotent precursors, mesenchymal stem cells, to committed preadipocytes, and the terminal differentiation of preadipocytes to mature adipocytes (6, 7). Understanding the biochemical mechanisms that regulate adipogenesis would be an important step in controlling fat cell numbers and, thus, modulating obesity.

Among the significant drivers of the tightly controlled adipogenic program are members of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors and peroxisome proliferator-activated receptor-γ (PPARγ), described as a master regulator (7, 8). Conversely, among the most potent inhibitors of adipogenesis are members of the Wnt family of secreted proteins and cytokines of the TGFβ family (7, 9, 10). There are numerous points of cross talk between the stimulatory and inhibitory regulators: for example, TGFβ and its signaling intermediate Smad3 down-regulate C/EBP-mediated PPARγ expression (but not activity) (11) and Wnt signaling inhibits PPARγ expression (12), whereas PPARγ activation down-regulates many genes involved in both TGFβ and Wnt signaling (13).

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Abbreviations: C/EBP, CCAAT/enhancer-binding protein; FBS, fetal bovine serum; hIGFBP-3, human IGFBP-3; HMBS, hydroxymethylbilane synthase; IBMX, 3-isobutyl-1-methylxanthine; IGFBP-3, IGF binding protein-3; mIGFBP-3, mouse IGFBP-3; MS, mouse serum; PPARγ, peroxisome proliferator-activated receptor-γ; pSmad, phosphoSmad; qRT-PCR, quantitative RT-PCR; RXR, retinoid X receptor; siRNA, small interfering RNA; TBS-T, Tris-buffered saline with Tween 20; TβRI, type I TGFβ receptor.

IGF binding protein-3 (IGFBP-3) is a secreted glycoprotein that has multiple roles in mammalian physiology (14). In the circulation, it transports the anabolic and mitogenic peptides IGF-I and IGF-II and regulates their access to target tissues (15). At the tissue level, IGFBP-3 affects IGF signaling through the IGF-I receptor, both by directly binding the IGFs (14) and by an IGF-binding-independent mechanism (16). IGFBP-3 also modulates the signaling activity of other cell surface receptors in both malignant and nonmalignant cell types, including the type I TGFβ receptor (TβRI)/TβRII system in human breast cancer (17) and intestinal smooth muscle cells (18) and the epidermal growth factor receptor in phenotypically normal mammary epithelial cells (16). It can cause apoptosis by both inducing Bax (19) and binding to it in mitochondria (20). Further, IGFBP-3 is known to translocate to the nucleus in some cell types (21) and, by interacting with the retinoid X receptor (RXR) (22) and other nuclear receptors (23), can modulate transcriptional activity.

We have previously reported that human IGFBP-3 (hIGFBP-3), administered either as the exogenous recombinant protein or by transfection of cDNA, inhibits the differentiation of murine 3T3-L1 preadipocytes to mature adipocytes (24). Because IGFBP-3 was shown to bind to PPARγ, block PPARγ-RXRα heterodimer formation, and inhibit PPARγ ligand-activated transcriptional activity, this was proposed as a likely inhibitory mechanism (24). An additional mechanism is suggested (25) by the observation that IGFBP-3 activates TGFβ receptor signaling in a variety of cell models (17, 18, 26), initiating Smad-dependent pathways known to potently inhibit adipogenesis (11). Our original study showing the inhibitory effect of IGFBP-3 on adipogenesis (24) did not reveal the role of endogenous IGFBP-3 in preadipocyte differentiation, which is relevant because adipose tissue is known to express IGFBP-3 (27). We now describe experiments suggesting that endogenous IGFBP-3 exerts a tonic inhibitory role on 3T3-L1 adipogenesis, which involves interaction with the TGFβ signaling pathway.

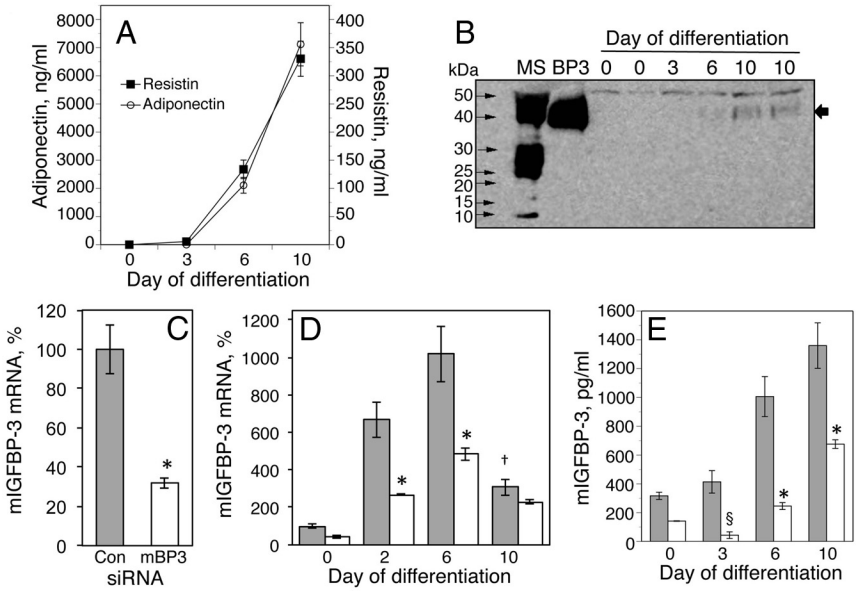

FIG. 1. Expression of mIGFBP-3 by 3T3-L1 preadipocytes over 10 d of adipogenic differentiation. A, Time course of the appearance of resistin (*squares*) and adiponectin (*circles*) measured by ELISA in the medium conditioned by 3T3-L1 preadipocytes at d 0, 3, 6, and 10 after the initiation of differentiation. Mean values ± SEM pooled from two independent experiments, each in duplicate. B, Western immunoblot for mIGFBP-3 in 5-μl samples of medium conditioned by 3T3-L1 cells at d 0, 3, 6, and 10 after the initiation of differentiation. Normal mouse serum (MS) (2 μl) and recombinant hIGFBP-3 (BP3) (20 ng) were run on the same blot for comparison. *Left arrows* indicate molecular weight markers, and *right arrow* indicates IGFBP-3. C, Expression of mIGFBP-3 mRNA at d 0 of differentiation, determined by qRT-PCR, in 3T3-L1 cells treated with control siRNA (*shaded bars*) or mIGFBP-3 siRNA (*white bars*). D, Expression of mIGFBP-3 mRNA over 10 d of differentiation in 3T3-L1 cells treated with control siRNA (*shaded bars*) or mIGFBP-3 siRNA (*white bars*). Means ± SEM of triplicates from a single experiment, representative of three similar experiments. E, Detection of mIGFBP-3 by ELISA in medium from differentiating 3T3-L1 cells treated 48 h before the initiation of differentiation with control siRNA (*shaded bars*) or mIGFBP-3 siRNA (*white bars*). Mean values ± SEM from two experiments in duplicate. Overall effect of siRNA by ANOVA: $F = 40.575$, $P < 0.001$ (for mRNA); $F = 1186.8$, $P < 0.001$ (for protein). §, $P < 0.005$ and *, $P < 0.001$ by ANOVA *vs.* control siRNA at the same day; †, $P < 0.001$ *vs.* control siRNA at d 6.

Materials and Methods

Materials

Fetal bovine serum (FBS) used for cell culture was purchased from Thermo Trace (Noble Park, Victoria, Australia). Penicillin, streptomycin, 3-isobutyl-1-methylxanthine (IBMX), insulin from bovine pancreas, dexamethasone, and biotin were from Sigma-Aldrich (St. Louis, MO). Mouse IGFBP-3 (mIGFBP-3) antibody was purchased from R&D Systems (Minneapolis, MN) (specificity in Western blotting not stated; ~5% cross-reactivity with hIGFBP-3 by ELISA). Phosphorylated Smad2 antibody (reactive only with Smad2 phosphorylated at both Ser465 and Ser467) and total Smad2 antibody (also reactive with Smad3) were from Cell Signaling Technology (Beverly, MA). NuPAGE products and electrophoresis equipment and reagents were from Invitrogen (Carlsbad, CA). Recombinant hIGFBP-3 was expressed in human 911 retinoblastoma cells as previously described (28). Recombinant hTGFβ1 protein was purchased from Austral Biologicals (San Ramon, CA). mIGFBP-3, adiponectin, and resistin ELISA kits were from R&D Systems. PhosSTOP phosphatase inhibitors were from Roche (Castle Hill, New South Wales, Australia).

3T3-L1 cell culture and differentiation

3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were

maintained in DMEM (Thermo Scientific, Waltham, MA) containing 4.5 g/liter D-glucose and 4 mM L-glutamine, and supplemented with 10% heat-inactivated FBS at 37 C in 5% CO_2. Cells were passaged before reaching 50% confluence. For differentiation experiments, 2 d after confluence or after transfection (d 0), 3T3-L1 cells were induced to differentiate for 3 d with the addition of 0.5 mM IBMX, 0.1 μg/ml dexamethasone, 0.7 μM insulin, 0.4 μM biotin, and penicillin-streptomycin in DMEM supplemented with 10% FBS. The cells were then maintained in DMEM supplemented with 10% FBS, 20 μM insulin, and penicillin-streptomycin for a further 3 d and then changed to DMEM containing 10% FBS and penicillin-streptomycin for another 4 d, for a total of 10 d. The effect of TGFβ on differentiating cells was examined by the addition of recombinant hTGFβ1 protein at indicated concentrations with every media change during the 10-d differentiation process. Conditioned media were collected at indicated time points during the differentiation process. At d 10, cells were harvested and processed for gene expression analysis as described below. For short-term signaling experiments, cells were treated with IGFBP-3 and TGFβ1 at the indicated concentrations and time and then processed for immunoblotting as described below.

Transient transfections

Two small interfering RNA (siRNA) duplexes (QIAGEN, Valencia, CA) targeting the endogenous mIGFBP-3 gene, siRNA#1 (Mm_Igfbp3_1 HP) and siRNA#2 (Mm_Igfbp3_3 HP), were used to achieve knockdown, and AllStars Negative Control siRNA (QIAGEN) was used as the control. At 24 h after plating, 1 μg of siRNA was transfected into 1×10^5 cells using HiPerfect transfection reagent (QIAGEN) according to the manufacturer's instructions. Differentiation or signaling experiments were then performed 48 h after transfection. For overexpression studies, the hIGFBP-3 cDNA (29) was subcloned into pcDNA4/TO (Invitrogen). 3T3-L1 fibroblast cells were transfected using AMAXA Nucleofector system (Lonza, Cologne, Germany) according to the method recommended by the manufacturer. In brief, cells were harvested, and 1×10^6 cells were resuspended in 100 μl of AMAXA Nucleofector solution V. The cell suspension was mixed with 1 μg of siRNA or 4 μg of IGFBP-3 DNA plasmid for siRNA knockdown and IGFBP-3 overexpression experiments, respectively. Subsequently, the cells were nucleofected using program T-030 and mixed with prewarmed media before plating.

RNA isolation and real-time PCR

Cells were washed with PBS, and total cellular RNA was extracted using TRIzol reagent (Invitrogen) and precipitated with the addition of isopropanol. Samples were treated with deoxyribonuclease I enzyme (QIAGEN), and RNA was quantitated using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA (1 μg) was RT to cDNA using Superscript III First Strand Synthesis Supermix kit (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR analysis using TaqMan probes (Applied Biosystems, Foster City, CA) for mIGFBP-3 (Mm01187817_m1), hIGFBP-3 (Hs00181211_m1), murine adiponectin (Mm00456425_m1), and murine resistin (Mm00445641_m1) was performed in an ABI 7900HT (Applied Biosystems). The comparative cycle method was used for gene expression analysis by using hydroxymethylbilane synthase (HMBS) (Mm00660260_m1) as the endogenous control.

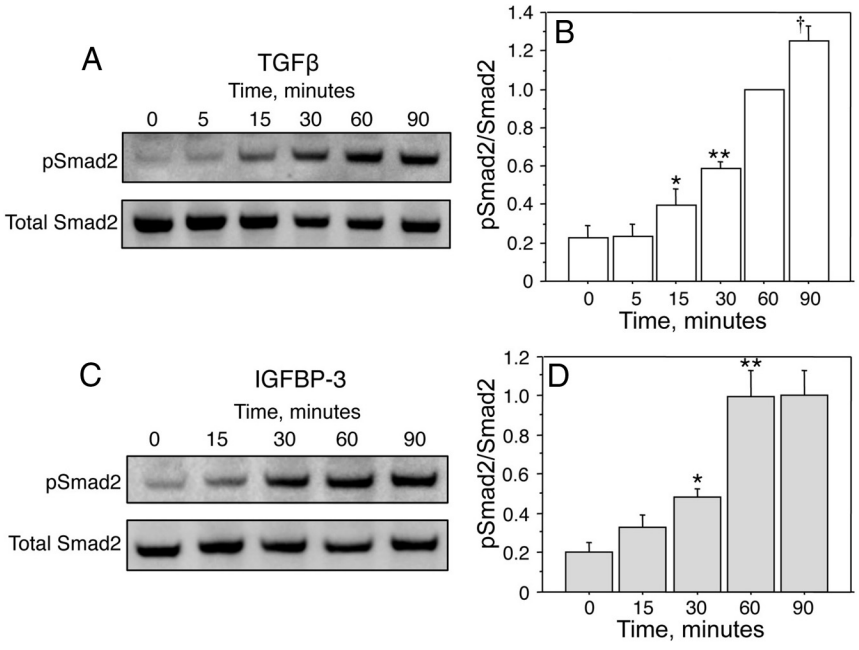

FIG. 2. Stimulation of Smad2 phosphorylation by TGFβ1 and IGFBP-3 in 3T3-L1 preadipocytes. A, Representative Western blotting showing the time course of increase in phospho-Smad2 (Ser465/467) after addition of 1 ng/ml human TGFβ1. B, Pooled pixel count data from three experiments, each in duplicate, showing the ratio of phospho/total Smad2, expressed relative to the 60-min ratio. *Error bars* indicate SEM of pooled data. Analysis by two-factor ANOVA: for the overall effect of time, F = 60.686, $P < 0.001$. *, Significant stimulation compared with time 0 ($P < 0.05$); **, all later time points showed significant stimulation compared with time 0 ($P < 0.001$); †, significant stimulation compared with 60 min ($P = 0.004$). C, Representative Western blotting showing the time course of increase in phospho-Smad2 after addition of 500 ng/ml recombinant hIGFBP-3. D, Pooled pixel count data from three experiments, each in duplicate, showing the ratio of phospho/total Smad2, expressed relative to the 60-min ratio. *Error bars* indicate SEM of pooled data. Analysis by two-factor ANOVA: for the overall effect of time, F = 15.957, $P < 0.001$. Stimulation approached significance at 30 min (*, $P = 0.052$) and was highly significant at 60 and 90 min (**, $P < 0.001$).

Immunoassays and Western immunoblotting

The protein levels in the conditioned media of differentiating cells were measured using mIGFBP-3, TGFβ, adiponectin, and resistin ELISA kits (R&D Systems) according to the manufacturer's instructions. Secreted hIGFBP-3 was measured by in-house RIA. To measure Smad2 activation by immunoblot, 2 d after confluence or after transfection, cells were treated with TGFβ1 and/or IGFBP-3 in duplicate for the indicated times. Cells were washed with PBS and lysed in Laemmli sample buffer [62.5 mM Tris-HCl (pH 6.8), containing 2% wt/vol sodium dodecyl sulfate, 10% vol/vol glycerol, 0.01% wt/vol bromophenol blue, and 50 mM dithio-

threitol] containing phosphatase inhibitors (1×), and sonicated. Prepared lysates and conditioned media samples were separated on 10% NuPAGE Bis-Tris gels under reducing conditions. Proteins were transferred to Hybond C-Extra nitrocellulose membranes (GE Healthcare, Little Chalfont, Bucks, UK) and subsequently blocked in Tris-buffered saline with Tween 20 (TBS-T) [10 mM Tris, 150 mM NaCl (pH 7.4), containing 0.1% vol/vol Tween 20] containing 5% skim milk powder for 1 h, then probed with phosphorylated Smad2 (Ser465/467), total Smad2, or mIGFBP-3 primary antibodies diluted in TBS-T containing 5% skim milk powder overnight at 4 C. Membranes were washed in cold TBS-T and incubated with the appropriate horseradish peroxidase-labeled secondary antibodies (Amersham Biosciences, Piscataway, NJ) for a further 2 h at room temperature. Membranes were again washed and developed using enhanced chemiluminescence plus Western Blot Detection System (GE Healthcare), and bands were visualized using a LAS3000 imaging system (FujiFilm, Tokyo, Japan) and quantitated using MultiGuage 3.0 software (Science Lab; FujiFilm).

Nile red staining

Ten days after differentiation, cells were fixed with 10% formalin (Sigma-Aldrich), stained with Nile red at a final working concentration of 1 μg/ml, and viewed under an Olympus IX70 inverted fluorescent microscope (Olympus Corp., Tokyo, Japan) at 485 nm excitation/525 nm emission.

Statistics

All experiments were performed independently at least three times each in duplicate or triplicate. Statistical analysis by ANOVA, as indicated in the text or figure legends, was performed using Statview version 5 (Abacus Concepts, Berkeley, CA) or SPSS version 19 (SPSS, Inc., Chicago, IL). For comparisons of time series, repeated measures ANOVA was used to examine overall effects. All ANOVA analyses were conducted on combined data from multiple experiments and included a factor for experiment number in addition to the treatment factor(s). *Post hoc* testing was by Fisher's least significant difference test. $P < 0.05$ was considered statistically significant.

Results

Among the growing number of adipokines that are recognized to communicate a variety of paracrine and endocrine functions of adipocytes (30), adiponectin and resistin are easily measured secreted proteins that serve as useful markers of adipogenesis. Figure 1A demonstrates the appearance of these adipogenic markers, measured by ELISA in the culture medium of 3T3-L1 preadipocytes over the 10-d course of differentiation induced by the addition of insulin, IBMX, and dexamethasone. Although mature white adipose tissue has a high level of IGFBP-3 gene expression (27), lower expression might be expected in preadipocytes, because we have shown that IGFBP-3 is inhibitory to adipogenesis (24). Endogenous mIGFBP-3, detected by immunoblot in culture medium conditioned by differentiating 3T3-L1 cells, was not visible before the initiation of differentiation (d 0), or on d 3, and was first detected at d 6 as the expected diffuse 40–45 kDa doublet band, increasing at d 10 (Fig. 1B). For comparison, mIGFBP-3 immunoreactivity in 2 μl of normal mouse serum (MS) and 20 ng of recombinant hIGFBP-3 are also shown. The MS contains both intact IGFBP-3 (40–45 kDa) and proteolysed IGFBP-3 (25–30 kDa).

To evaluate the role of endogenous mIGFBP-3, its expression was down-

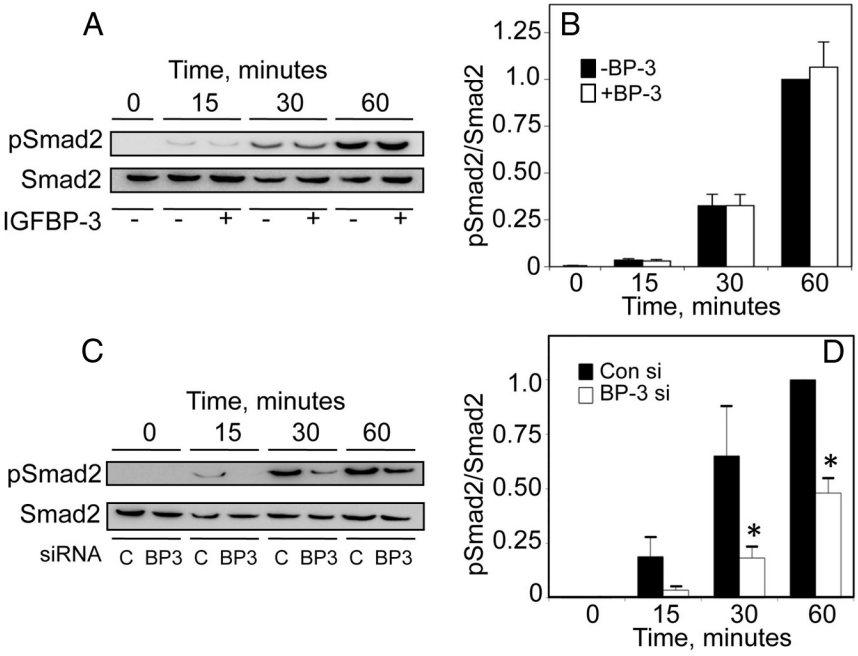

FIG. 3. Interaction between TGFβ1 and IGFBP-3 in the stimulation of Smad2. A, Representative Western blotting showing the time course of increase in phospho-Smad2 (Ser465/467) after addition of 0.25 ng/ml rhTGFβ1, without or with the addition of 500 ng/ml IGFBP-3 as indicated. B, Pooled pixel count data from three Western blot experiments, in which phospho-Smad2 was measured at the indicated times in response to 0.25 ng/ml TGFβ1 alone (*black bars*, −BP-3), or together with 500 ng/ml IGFBP-3 (*white bars*, +BP-3). Data represent mean ± SEM of pooled data. Adding IGFBP-3 did not enhance the TGFβ1 effect ($P = 0.77$ by ANOVA). C, Representative Western blotting showing the time course of increase in phospho-Smad2 after addition of 0.25 ng/ml rhTGFβ1 in cells that had been treated 48 h previously with control siRNA or IGFBP-3 siRNA#2 as indicated. D, Pooled data from three Western blot experiments, in which phospho-Smad2 was measured at the indicated times in response to 0.25 ng/ml TGFβ1 in cells treated 48 h previously with control (Con) siRNA (*black bars*), or IGFBP-3 siRNA (*white bars*). Data represent mean ± SEM of pooled data. The effect of down-regulating IGFBP-3 was significant overall by repeated measures ANOVA ($F = 9.164$, $P = 0.013$). Two-factor ANOVA: *, $P < 0.001$ for control *vs.* IGFBP-3 siRNA at the indicated time points.

regulated using a specific siRNA, with a nonsilencing siRNA used as a control, and the extent of down-regulation was evaluated by measuring mIGFBP-3 mRNA by quantitative RT-PCR (qRT-PCR). Down-regulation of endogenous mIGFBP-3 mRNA at d 0 of differentiation, *i.e.* 48 h after siRNA transfection, was typically 60–70% compared with cells treated with the nonsilencing control siRNA (Fig. 1C). Over 10 d of differentiation, there was a highly significant effect of both time and siRNA treatment on mIGFBP-3 expression (both $P < 0.001$ by ANOVA). Substantial down-regulation at the gene expression level was evident at d 2 and 6 of differentiation ($P < 0.001$), but the difference between control and siRNA treatments was lost by d 10 (Fig 1D). Interestingly, in three repeat experiments, a decline in mIGFBP-3 mRNA was observed between d 6 and 10 of differentiation. In contrast, a monotonic increase in secreted protein levels measured by ELISA was seen over 10 d (Fig. 1E), perhaps reflecting the stability of accumulating IGFBP-3 in the culture medium despite a decline in the rate of new synthesis after d 6. In cells treated with mIGFBP-3 siRNA before differentiation, a significant decrease in secreted IGFBP-3 was observed over the 10-d time course ($P < 0.001$ by repeated measures ANOVA) and at individual d 3, 6, and 10 (Fig. 1E).

To determine whether IGFBP-3 might signal through the TGFβ receptor system in 3T3-L1 cells, as shown for some other cell types, Smad2 phosphorylation was measured by immunoblotting in response to recombinant TGFβ1 and IGFBP-3. Figure 2A shows the time course of Smad2 activation by 1 ng/ml (0.023 nmol/liter) TGFβ in 3T3-L1 preadipocytes. Determined as an increase in the ratio of phosphoSmad2 (pSmad2) to total Smad2, averaged over three experiments (Fig. 2B), there was a significant increase in Smad2 phosphorylation within 15 min of exposure to TGFβ ($P < 0.05$) with a 5-fold increase, compared with time 0, seen at 60 min ($P < 0.001$). Beyond 60 min, a further small increase in phosphorylation was seen at 90 min ($P = 0.004$ compared with 60 min). Comparable results were seen when IGFBP-3 was used instead of TGFβ. Recombinant hIGFBP-3 was used at a concentration of 500 ng/ml (~12 nmol/liter) after preliminary experiments showed that higher doses gave little or no further stimulation (data not shown). A similar concentration has been used previously to study TGFβ receptor activation in other cell types (17, 18, 26). Figure 2C shows the time course of Smad2 activation by 500 ng/ml IGFBP-3. Determined as an increase in the ratio of pSmad2 to total Smad2, averaged over three experiments (Fig. 2D), the earliest increase in the pSmad/Smad ratio was seen at 30 min of exposure to IGFBP-3 ($P = 0.052$) with a 5-fold increase, compared with time 0, at 60 min ($P < 0.001$). Beyond 60 min, there was no further increase in Smad2 phosphorylation in response to IGFBP-3.

The interaction between IGFBP-3 and TGFβ on Smad2 activation in 3T3-L1 preadipocytes was tested in two ways: by adding exogenous recombinant proteins together and by testing TGFβ when endogenous IGFBP-3 was down-regulated by siRNA. As shown in Fig. 3A and analyzed for three independent experiments in Fig. 3B, there was a no significant additional effect on Smad2 activation of adding 500 ng/ml IGFBP-3 together with TGFβ1, above the effect of TGFβ alone, indicating that the stimulatory effect of TGFβ alone was maximal. In contrast, a considerable decrease in the stimulation of Smad2 phosphorylation by TGFβ was observed when endogenous IGFBP-3 was down-regulated (Fig. 3C), significant over the full time course ($P = 0.013$, repeated measures ANOVA) and at the 30- and 60-min time points ($P < 0.001$), but not at 15 min ($P = 0.184$), when analyzed for three independent experiments (Fig. 3D). A second IGFBP-3 siRNA also attenuated TGFβ1-stimulated Smad2 activation (data not shown). Together, these data indicate that exogenous TGFβ and IGFBP-3, at their respective optimal doses, can activate TGFβ receptor signaling to a similar extent in 3T3-L1 preadipocytes, and endogenous IGFBP-3 contributes to the maximal stimulatory effect of TGFβ on Smad2 activation.

Newly synthesized TGFβ exists as a latent complex, which can be activated by proteolysis and other mechanisms to release the active growth factor (31). To assess the relationship between the exogenous TGFβ concentration (1 ng/ml) used to stimulate Smad2 phosphorylation and endogenous TGFβ in 3T3-L1 preadipocytes, the total (latent + active) and active TGFβ1 pools were measured by ELISA. As shown in Fig. 4A for one experiment of four, active TGFβ1 levels in conditioned media were low (undetectable in some experiments) in preadipocytes and in-

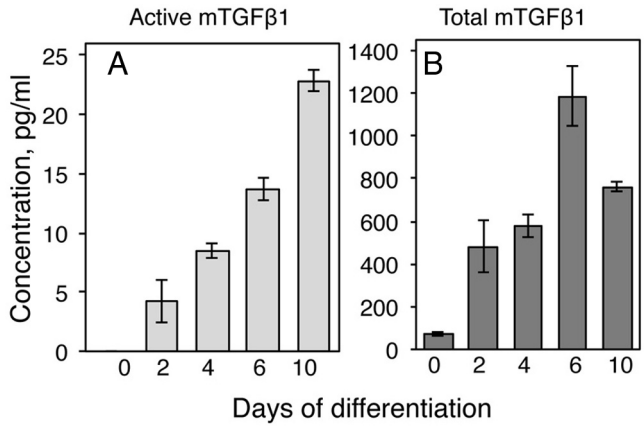

FIG. 4. Time course of mTGFβ1 concentration in culture media conditioned by differentiating 3T3-L1 cells. A, Active mTGFβ1 was measured by ELISA in conditioned medium collected at the indicated time points. B, Total mTGFβ1 was measured in the same samples after transient acidification with 0.17 M HCl. Data, corrected for the active or total TGFβ1 content of the FBS in unconditioned media, are mean values ± SEM from a single experiment, representative of four.

creased as early as d 2 after the initiation of differentiation, reaching 20–70 pg/ml by d 10. Calculated over four independent experiments, the mean concentration of active TGFβ1 at d 0 was 10.0 ± 4.8 pg/ml (SEM), rising to 49.5 ± 7.9 pg/ml at d 10, a concentration inhibitory to 3T3-L1 differentiation (32). However, the potential pool of active TGFβ1 is much greater, because the total TGFβ1 pool exceeded 1 ng/ml by d 6, falling slightly at d 10 (Fig. 4B). Calculated over four independent experiments, the mean concentration of total TGFβ1 at d 0 was 199 ± 75 pg/ml (SEM), rising to 1190 ± 165 pg/ml at d 10. This suggests that the endogenous pool of latent TGFβ1 exceeds that required to generate active TGFβ1 capable of fully stimulating the Smad pathway and inhibiting adipogenesis.

We previously reported that exogenous IGFBP-3, or stably transfected IGFBP-3, was inhibitory to 3T3-L1 preadipocyte differentiation (24) and in this study confirmed similar inhibition when IGFBP-3 was expressed transiently, 48 h before the induction of differentiation. Figure 5A shows the ectopically expressed hIGFBP-3 mRNA levels were high at the initiation of differentiation but declined rapidly and had almost disappeared by d 6. Corresponding levels of immunoreactive hIGFBP-3 in the culture medium, determined by RIA, were 42.8 ± 10.1 ng/ml at d 0, 20.5 ± 4.4 ng/ml at d 3, and 8.1 ± 0.9 ng/ml at d 6 (means ± SEM from three experiments in duplicate). No change in total or active TGFβ1, measured by ELISA, was seen in response to IGFBP-3 overexpression (data not shown). Staining for lipid droplets using Nile red (Fig. 5B) indicated substantial lipid accumulation at the end of the induction period (d 10), which was significantly attenuated when IGFBP-3 was overexpressed before d 0. Gene expression of the adipogenic markers adiponectin (Fig. 5C) and resistin (Fig. 5D) was undetectable in 3T3-L1 preadipocytes but increased many thousand-fold over the 10-d differentiation period in cells transfected with empty vector. When IGFBP-3 was overexpressed by transfection, the level of adiponectin induction was significantly reduced by approximately 50% ($P = 0.007$). Similarly, the induction of resistin mRNA was inhibited by approximately 75% ($P < 0.001$) when IGFBP-3 was overexpressed.

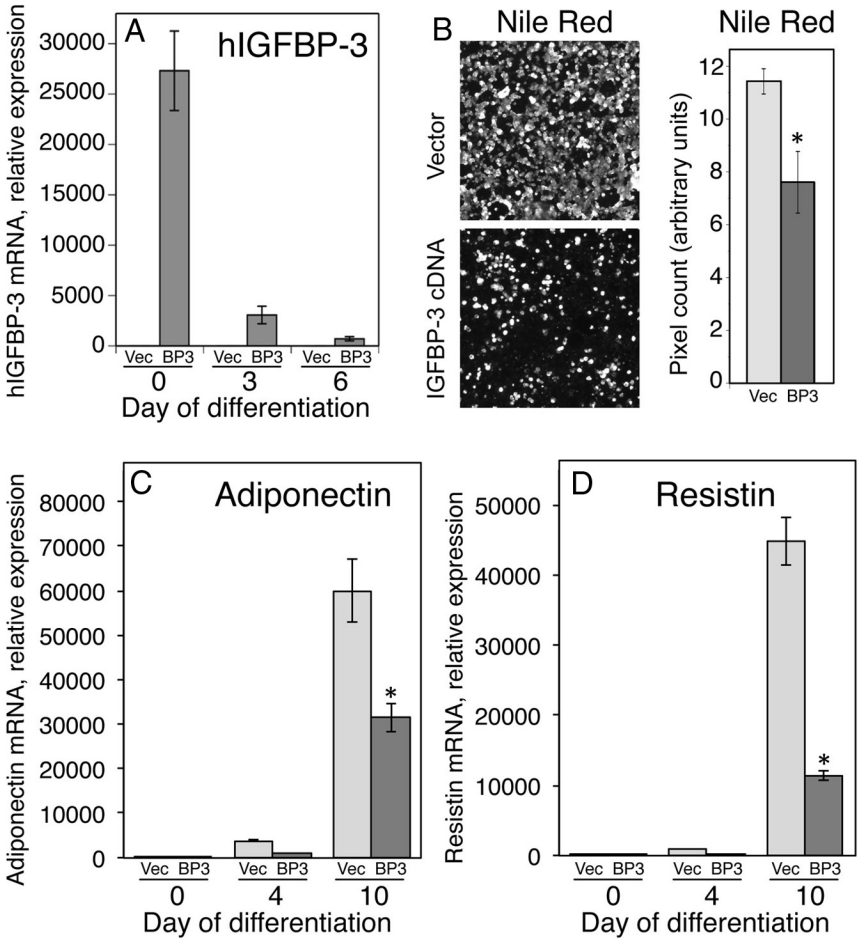

FIG. 5. Effect of hIGFBP-3 overexpression on the differentiation of 3T3-L1 cells. Cells were transiently transfected to express hIGFBP-3, 48 h before the initiation of differentiation. A, hIGFBP-3 mRNA measured by qRT-PCR in cells expressing vector (Vec) alone or IGFBP-3 (BP3) cDNA at the days indicated; mean values ± SEM from two experiments in duplicate. B, Lipid droplets were stained with Nile red (*left*) and quantitated by densitometry in arbitrary units (*right*) from four fields each of duplicate wells of cells expressing vector alone (*light shading*) or IGFBP-3 cDNA (*dark shading*) at d 10. *, Effect of treatment significant by ANOVA, $F = 32.63$, $P < 0.001$. Adiponectin (C) and resistin (D) mRNA was measured by qRT-PCR in cells expressing vector alone (*light shading*) or IGFBP-3 cDNA (*dark shading*) at the days indicated. Data are means ± SEM, normalized to the expression of the housekeeping gene HMBS, and expressed as relative increase compared with uninduced (d 0) cells. *, The inhibitory effect of IGFBP-3 expression was significant by ANOVA on both adiponectin ($P = 0.007$) and resistin ($P < 0.001$) mRNA levels.

Activation of Smad2 signaling by TGFβ is known to inhibit preadipocyte differentiation (33, 34). Because TGFβ1 and IGFBP-3 appear to act similarly in initiating Smad2 activation in 3T3-L1 preadipocytes (Fig. 2), their interaction in the inhibition of 3T3-L1 preadipocyte differentiation was examined. To evaluate whether endogenous IGFBP-3 might tonically influence differentiation, mIGFBP-3 was down-regulated approximately 70% using siRNA#2 (measured at 48 h, equivalent to d 0 of differentiation). Unexpectedly, this had little effect on the time course or magnitude of adiponectin induction (Fig. 6A) and no effect on resistin induction (Fig. 6B) over the subsequent 10 d of

3T3-L1 differentiation, suggesting at first sight that endogenous IGFBP-3 might have no role in the regulation of preadipocyte differentiation.

However, the possibility remained that endogenous IGFBP-3 might be involved through interaction with TGFβ receptor signaling. We tested this using two independent mIGFBP-3 siRNA. Compared with siRNA#2, shown in Fig. 3D, siRNA#1 was somewhat less effective, decreasing TGFβ-stimulated Smad2 phosphorylation at 30 min by approximately 50% (data not shown). Recombinant TGFβ1 inhibited the induction of adipocyte differentiation markers markedly at 0.5 ng/ml (Fig. 6, C and D). In these experiments, down-regulation of IGFBP-3 by siRNA#2, but not siRNA#1, increased the induction of adiponectin, but not resistin, in the absence of TGFβ. In the presence of 0.25 ng/ml TGFβ, both siRNA stimulated adiponectin induction (Fig. 6C) but not resistin (Fig. 6D). In contrast, in the presence of 0.5 ng/ml TGFβ, both siRNA significantly attenuated its inhibitory effect, effectively preventing the inhibition of both adiponectin induction (Fig. 6C) and resistin induction (Fig. 6D) caused by 0.5 ng/ml TGFβ1. These observations indicate that endogenous IGFBP-3 does indeed have the ability to influence the extent of preadipocyte differentiation, by affecting 3T3-L1 sensitivity to inhibition by TGFβ.

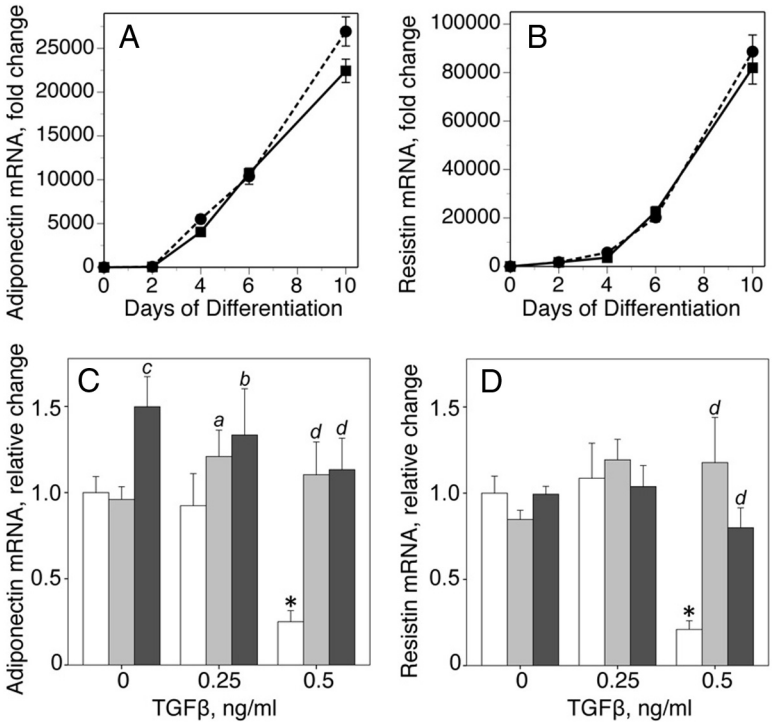

FIG. 6. Effect of endogenous IGFBP-3 down-regulation on the differentiation of 3T3-L1 cells. Cells were transiently transfected with a control siRNA or IGFBP-3 siRNA, 48 h before the initiation of differentiation. The time course of induction of adiponectin mRNA (A) and resistin mRNA (B) in cells expressing control siRNA (*solid line*) or IGFBP-3 siRNA#2 (*dashed line*) over 10 d of differentiation. Data are means ± SEM of triplicates from one experiment out of three. C and D, Inhibition of adiponectin induction (C) or resistin induction (D) at d 10 of differentiation by exogenous TGFβ1, in cells transfected with either control siRNA (*open bars*) or IGFBP-3 siRNA#1 (*gray bars*) or siRNA#2 (*dark bars*). Pooled data (means ± SEM) from two independent experiments in duplicate. All mRNA levels were measured by qRT-PCR, normalized to the expression of the housekeeping gene HMBS, and expressed relative to levels in cells treated with nonsilencing siRNA in the absence of TGFβ. Analysis by two-way ANOVA: overall effect of treatment, $F = 17.02$, $P < 0.001$ for adiponectin; $F = 9.817$, $P < 0.001$ for resistin. *, $P < 0001$ *vs.* value for nonsilencing siRNA in the absence of TGFβ. a, $P = 0.03$; b, $P = 0.003$; c, $P = 0.001$; d, $P < 0.001$, *vs.* value for nonsilencing siRNA at the same TGFβ concentration.

Discussion

We have previously reported that 3T3-L1 preadipocytes stably expressing hIGFBP-3 are significantly inhibited in their ability to differentiate to mature adipocytes, as indicated by decreased induction of PPARγ and resistin gene expression, and decreased appearance of intracellular lipid staining by Nile red (24). However, the role of endogenous IGFBP-3 in regulating adipogenic differentiation *in vivo* has been difficult to elucidate. Mice with a targeted deletion of the *igfbp3* gene show a small but significant increase in their fat mass as a percentage of body weight compared with controls (35), consistent with an inhibitory effect of endogenous IGFBP-3 on fat mass development. In contrast, the detection, by Oil Red O staining, of fat cells in bone marrow cell cultures subjected to an adipogenic differentiation stimulus did not differ between cultures from IGFBP-3 knockout and wild-type mice (36). Expression of the adipogenic marker C/EBPα was significantly elevated in bone marrow from IGFBP-3-deficient mice compared with controls, again consistent with an inhibitory effect of endogenous IGFBP-3, but paradoxically, expression of the marker aP2 was significantly lower in the knockout animals, and expression of PPARγ was unaffected compared with controls (36). These disparate results do not allow an unambiguous conclusion as to the role of endogenous IGFBP-3 on fat cell development.

Our previous studies have shown that IGFBP-3 can activate signaling through the TGFβ receptor (TβRI/TβRII) pathway in breast cancer cells, inducing phosphorylation of the receptor-regulated Smads, Smad2 and Smad3 (17), a finding subsequently confirmed by others in cell lines of intestinal (18) and renal (26) or-

igin. The canonical ligands of this signaling system are several distinct proteins of the TGFβ family, including TGFβ1, TGFβ2, and TGFβ3, all members of a much broader TGFβ superfamily (37). Of these, both TGFβ1 and TGFβ2 are reported to be secreted by 3T3-L1 cells (38). Because Smad2/3 activation is well recognized as being inhibitory to adipogenesis (10, 39), this raised the question of the possible interaction between IGFBP-3 and TGFβ signaling in the regulation of adipogenic differentiation. Measuring Smad2 as representative of the TGFβ-activated receptor-regulated Smads, IGFBP-3 was found to activate Smad2 phosphorylation in 3T3-L1 preadipocytes to the same extent and with a similar time course as TGFβ, when both were used at their optimal concentrations. However, although recombinant TGFβ1 was maximally active at a concentration of 1 ng/ml or less, within the range of total immunoreactive TGFβ1 secreted by these cells during the differentiation process, recombinant IGFBP-3 required a much higher concentration (500 ng/ml) than the 1–2 ng/ml secreted by the cells. A possible explanation is that exogenous hIGFBP-3 has lower bioactivity in this cell system than endogenously produced mIGFBP-3. This may be unrelated to the species difference, because a similar difference in apparent activity between exogenous and endogenous hIGFBP-3 in some human cell lines has been described previously (40), but to date, there is no clear explanation. It does not appear to be an intrinsic problem of the IGFBP-3 preparation itself, because the same preparation induces significant biological effects in the MCF-10A human mammary cell line at 10 ng/ml, and even 1 ng/ml (16). hIGFBP-3 circulates at about 2000–4000 ng/ml (15), and IGFBP-3 in the murine circulation has been reported at about 2000 ng/ml (41), so a concentration of 500 ng/ml is physiologically plausible if the IGFBP-3 is serum derived.

Although the explanation for the apparently greater bioactivity of endogenous mIGFBP-3 remains unclear, our study supports the concept that it has a role in regulating adipocyte sensitivity to signaling through the TGFβ pathway. Activation of Smad2 phosphorylation by 0.25 ng/ml TGFβ was significantly attenuated by IGFBP-3 silencing, and the inhibitory effect of TGFβ on the induction of adipogenic markers was essentially blocked when IGFBP-3 was down-regulated. The findings imply that endogenous IGFBP-3 has a permissive role for TGFβ action in 3T3-L1 differentiation.

We previously published evidence that the inhibitory effect of IGFBP-3 on adipogenic differentiation involved its interaction with PPARγ, inhibition of PPARγ-RXRα heterodimer formation, and suppression of PPARγ ligand-activated transcriptional activity (24). Because TGFβ and its signaling intermediate Smad3 down-regulate C/EBP-mediated PPARγ expression (11), the current work implicates IGFBP-3 inhibitory effects on PPARγ expression through TGFβ signaling and C/EBP. Combined with our earlier study (24), it reflects that IGFBP-3 inhibits fat cell differentiation through more than one cellular mechanism, which may converge on PPARγ. Further, TGFβ and PPARγ signaling pathways have been shown to intersect at several levels in other systems, particularly in the context of fibrotic disease: for example, TGFβ down-regulates PPARγ expression (42, 43) and, conversely, PPARγ inhibits TGFβ-dependent, Smad-mediated transcriptional activity, without preventing Smad2/3 phosphorylation (44, 45). Therefore, IGFBP-3 might stimulate Smad2/3 signaling both directly through activation of the TβRI (17) and indirectly through inhibition of PPARγ. In conclusion, we have demonstrated in 3T3-L1 preadipocytes that TGFβ/Smad signaling, known to be a potent regulator of fat cell differentiation, can be activated by IGFBP-3 and that endogenous IGFBP-3 in these cells is able to regulate cell sensitivity toward exogenous TGFβ. This study provides further evidence that IGFBP-3 has the potential to play a significant role in the regulation of adipogenesis.

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