TG-interacting factor is required for the differentiation of preadipocytes

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Abstract The accumulation of visceral adipose tissue is closely associated with insulin resistance and metabolic syndrome. Therefore, it is important to identify genes that are required for adipocyte differentiation. To identify genes that are required for the differentiation of 3T3-L1 preadipocytes into mature adipocytes, we used retrovirus insertion-mediated random mutagenesis to generate 3T3-L1 cell lines that lose their ability to differentiate into mature adipocytes. One of the genes identified was TG-interacting factor (TGIF), a DNA binding homeodomain protein that has been demonstrated to suppress Smad-mediated activation of transforming growth factor β (TGF-β)-regulated transcription. In the TGIF-disrupted clone of 3T3-L1 preadipocytes, the rate of differentiation into mature adipocytes was clearly reduced compared with that in the wild-type clone. Suppression of TGIF by lentivirus-mediated RNAi also inhibited the differentiation of 3T3-L1 cells. Insulin specifically increased the abundance of TGIF protein, primarily by enhancing its stability. In addition, insulin caused the rapid accumulation of TGIF in the nuclei. Forced expression of exogenous TGIF repressed both endogenous and overexpressed Smad2/3-mediated promoter activity in 3T3-L1. These findings suggest that insulin specifically antagonizes TGF-β signaling in preadipocytes by stabilizing the putative Smad transcriptional corepressor TGIF and regulates adipocyte differentiation.—Horie, T., K. Ono, M. Kinoshita, H. Nishi, K. Nagao, T. Kawamura, Y. Abe, H. Wada, A. Shimatsu, T. Kita, and K. Hasegawa. **TG-interacting factor is required for the differentiation of preadipocytes. *J. Lipid Res.* 2008.** 49: **1224–1234.**

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A growing epidemic of obesity is affecting many countries worldwide. Obesity is defined as an increased mass of adipose tissue, which confers a higher risk of cardiovascular and metabolic disorders such as diabetes, hyperlipidemia, and coronary heart disease (1, 2). Obesity is the result of an expansion of individual adipocytes and an increase in the number of adipocytes. Therefore, it is important to identify genes that are responsible for adipocyte differentiation.

3T3-L1 preadipocyte cells, which are fibroblastic cells determined to the adipocyte lineage, are widely used for investigating the mechanism of the differentiation of adipocytes (3). Adipocyte differentiation can be imitated by 3T3-L1 preadipocytes in vitro, in response to adipogenic inducers. To identify genes that are required for the differentiation of 3T3-L1 preadipocytes into mature adipocytes, we used retrovirus insertion-mediated random mutagenesis to generate 3T3-L1 cell lines that lose their ability to differentiate into mature adipocytes (4–6). One of the genes identified was 5′ TG 3′-interacting factor (TGIF), a homeobox protein that belongs to an expanding three-amino-acid loop extension superclass of atypical homeodomains (7). TGIF has been shown to function as an important negative regulator in the transforming growth factor β (TGF-β) signaling pathway (8).

TGF-β initiates a response by contacting two types of transmembrane serine/threonine kinases called receptors I (TβRI) and II (TβRII), and promotes the activation of TβRI by TβRII kinase (9, 10). The activated TβRI then propagates the signal to the nucleus by phosphorylating Smad2 and Smad3 at their extreme C termini on two serine residues in an "SSXS" motif, and this is facilitated by an adaptor protein called SARA (Smad anchor for receptor activation) (10). The phosphorylation of Smad2 is also under regulation by the cytoplasmic promyelocytic leukemia protein (cPML), a tumor suppressor that plays a crucial role in TGF-β signaling by mediating the association of SARA with Smad2 (11). Once phosphorylated, Smad2 and Smad3 are complexed with Smad4 and translocated to the nucleus, where they regulate the expression of TGF-β target genes through interactions with transcriptional cofactors (10, 12).

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The mechanism of the TGIF-mediated inhibition of TGF-β signaling has been attributed primarily to the ability of TGIF to recruit to Smad2, a transcriptional repressor complex that contains histone deacetylases (8). Recently, it has been shown that TGIF functions in TGF-β signaling by preventing Smad2 phosphorylation, and this event occurs by a mechanism that is independent of its association with Smad2 (13). It has been reported that TGIF can also associate with the E3 ubiquitin ligase TGIF-interacting ubiquitin ligase 1 to target Smad2 for ubiquitin-dependent degradation (14). The finding that TGIF facilitates the degradation of Smad2 is consistent with the traditional function of TGIF as a repressor in TGF-β signaling. These observations suggest that TGIF has multiple regulatory pathways for the silencing of TGF-β signaling.

TGF-β has potent effects on a wide variety of cell types. It inhibits the proliferation of epithelial cell lines while stimulating the proliferation of some mesenchymal cells (15–17). The effects of TGF-β on the differentiation of various tissues of mesenchymal and epithelial origin include both the prevention and induction of expression of specific phenotypes. It has also been observed that TGF-β inhibits adipocytogenesis of the murine 3T3-F442A cell line by signaling through Smad3 (18). Moreover, TGF-β/Smad3 has been shown to inhibit the induction of CCAAT/enhancer binding protein α (C/EBP-α) and peroxisome proliferator-activated receptor γ (PPAR-γ) in murine 3T3-F442A and NIH3T3 cells (18, 19). Despite its ability to inhibit adipocyte differentiation, TGF-β is expressed in cultured adipocytes and adipose tissue (20, 21). Therefore, we hypothesized that there may be endogenous mechanisms that affect TGF-β signaling in adipocyte differentiation.

In the present study, we demonstrated that insulin specifically antagonizes TGF-β signaling in preadipocytes by stabilizing the putative Smad transcriptional corepressor TGIF and regulates adipocyte differentiation.

MATERIALS AND METHODS

Cell lines, reagents, and antibodies

The mouse cell line 3T3-L1 was obtained from the American Type Culture Collection (Rockville, MD) and cultured under the recommended conditions in DMEM containing 10% FBS. For the differentiation experiment, medium was replaced with DMEM containing 10% FBS, 10 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 μM dexamethasone 2 days postconfluence. After another 2 days, the medium was changed to DMEM containing 10 μg/ml insulin and 10% FBS, and replaced every 2 days. Differentiation-resistant cell lines derived from 3T3-L1 preadipocyte cells were established by retroviral infection. The antibodies used were a mouse monoclonal anti-TGIF antibody (sc-17800; Santa Cruz Biotechnology, Santa Cruz, CA), a mouse monoclonal anti-PPAR-γ antibody (sc-7273; Santa Cruz Biotechnology), and a mouse monoclonal anti-β actin antibody (A5441; Sigma-Aldrich, St. Louis, MO). After pDisrup retroviral infection, cells were treated with 5 μg/ml of blasticidin S·HCl (Invitrogen; Carlsbad, CA) to select clones whose genes had been disrupted. After isolation and expansion of each single clone, differentiation was induced by IBMX, dexamethasone, and insulin in 24-well plates. The level of adipogenesis was determined at 8 days after the initiation of adipogenesis using the triglyceride-specific dye Oil Red O (Sigma-Aldrich, St. Louis, MO) according to a previously published method (22), and differentiation-resistant clones were picked up.

Retroviral production and cell infection

The pDisrup retroviral vector was provided by Dr. Jiahuai Han (Department of Immunology, Scripps Research Institute). Viruses were produced at 32°C, and virus-containing medium was collected 24 h posttransfection and filtered through a 0.45 μm filter. 3T3-L1 cells were plated in six-well plates at a density of 5×10^5 cells/well. One round of retroviral infection was performed by replacing the medium with 2 ml of pDisrup virus (containing 8 μg/ml of Polybrene, Sigma-Aldrich, St. Louis, MO), followed by centrifugation of the plates at 2,500 rpm for 30 min at 30°C.

3′-RACE

The portion of the endogenous gene that was fused with the *blasticidin* gene was amplified by the 3′ rapid amplification of cDNA ends (RACE) technique, as described previously (4).

siRNA-mediated knockdown of TGIF

The oligonucleotides used for small interfering RNA (siRNA) of TGIF were TGIF RNAi1: sense (ACACAGATACAACGCCTAT) and antisense (ATAGGCGTTGTATCTGTGT); TGIF RNAi2: sense (ATCTGGACCAAGTACGAAT) and antisense (ATTCGTACTTGGTCCAGAT).

Randomly shuffled forms of the TGIF RNAi1 sequence were used as a control. Every siRNA construct was made using the pSINsi-mU6 vector (Takara Bio., Inc., Shiga, Japan), and the siRNA-producing constructs were introduced to the lentivirus vector plasmid pLenti6/V5-D-TOPO vector (Invitrogen) and transduced to 3T3-L1. One round of lentiviral infection was performed by replacing the medium with 2 ml of virus-containing medium, followed by centrifugation of the plates at 2,500 rpm for 30 min at 30°C, as was done for the retrovirus.

Western blot analysis

Cell lysates were prepared as described and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by standard Western blotting procedures (23).

Quantification of mRNA by real-time RT-PCR

Real-time RT-PCR was performed with a 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Five micrograms of total RNA was reverse-transcribed in a reaction volume of 20 μl using Superscript III reverse transcriptase and random primers (Invitrogen). The product was diluted to a volume of 400 μl, and 5 μl aliquots were used as templates for amplification using SYBR Green PCR amplification reagent (Applied Biosystems) and gene-specific primers. The primers used for the assay were: PPAR-γ sense, 5′AGG CCG AGA AGG AGA AGC TGT TG3′; PPAR-γ antisense, 5′TGG CCA CCT CTT TGC TCT GCT C3′; aP2 sense, 5′GTG ATG CCT TTG TGG GAA CCT GGA AG3′; aP2 antisense, 5′TCA TAA ACT CTT GTG GAA GTC ACG CC3′; LPL sense, 5′GCA GAC TCT GTG TCT AAC TGC CAC3′; LPL antisense, 5′CAA TCA CAC GGA TGG CTT CTC CAA T3′; CEBP-β sense, 5′GCA AGA GCC GCG ACA AG3′; CEBP-β antisense, 5′GGC TCG GGC AGC TGC TT3′; TGIF sense, 5′ AT GAA AAG CAA GAA GGG TCT TGT TGC AG3′; TGIF antisense, 5′AGC TGT GAG TTT GGC CTG AAG CTC CAT C 3′; PAI-1 sense, 5′TCA GCC CTT GCT TGC CTC AT3′; PAI-1 antisense, 5′GCA TAG CCA GCA CCG AGG A3′; GAPDH sense, 5′TTG CCA

TCA ACG ACC CCT TC3′; and GAPDH antisense, 5′TTG TCA TGG ATG ACC TTG GC 3′.

Immunocytochemistry

The mouse cell line 3T3-L1 was grown in a flask-style chamber with glass slides. Immunocytochemical staining was performed by the indirect immunoperoxidase method as described previously (24). Briefly, the cells were fixed with Bouin's solution for 10 min at room temperature and subsequently autoclaved for 10 min at 121°C in 10 mM citrate, pH 6.0, for heat-induced antigen retrieval. The cells were incubated with anti-TGIF antibody (Santa Cruz Biotechnology) at a dilution of 1:100. TGIF signals were detected with anti-mouse FITC-conjugated secondary antibody at a dilution of 1:75 for 45 min.

DNA transfection and luciferase assay

The reporter construct p3TP-Lux, Smad2 and Smad3 expression vectors, and TGIF expression vectors (pHA-TGIF) were provided by Dr. J. Massague (Memorial Sloan-Kettering Cancer Center, New York, NY) (25–27). The p3TP-Lux construct contains three tetradecanoyl phorbol acetate (TPA) response elements and a TGF-β-responsive segment of the human plasminogen activator inhibitor-1 (PAI-1) promoter cloned upstream of a luciferase reporter gene. pHA-TGIF was created within a modified pCMV5, which contained an initiation codon together with two copies of an HA epitope. Smad2 and Smad3 were expressed from within pCMV5 (10). 3T3-L1 cells were transfected with p3TP-Lux (1.0 μg), with or without Smad2 (0.5 μg) and/or Smad3 (0.5 μg) expression vectors, and pHA-TGIF (0.5 μg) expression vector. A fixed amount (0.1 μg) of internal control reporter *Renilla reniformis* luciferase driven under thymidine kinase (TK) promoter (pRL-TK; Promega, Madison, WI) was also cotransfected to normalize the transfection efficiency. After transfection with Lipofectamine 2000 reagent (Invitrogen), the cells were incubated for an additional 48 h in the absence or presence of 2 ng/ml TGF-β. *Photinus* and *Renilla* luciferase activities were measured with a dual luciferase kit (PG-DUAL-SP; Toyo Ink Co., Tokyo, Japan). The relative luciferase activity of each construct (arbitrary units) was reported as the fold induction after normalizing for transfection efficiency. All experiments were repeated at least three times to ensure reproducibility.

Lentiviral-mediated TGIF expression

TGIF-expressing vector plasmid [pLenti6/V5-D-TOPO vector (Invitrogen)] or empty vector were used for lentivirus production and transduced to 3T3-L1.

Statistical analysis

Data are presented as means ± SE. Statistical comparisons were performed using unpaired two-tailed Student's *t*-tests or an ANOVA with Scheffe's test where appropriate. A probability value of <0.05 was taken to indicate significance.

RESULTS

TGIF was disrupted by pDisrup insertion in differentiation-resistant 3T3-L1 line

We used retrovirus insertion-mediated mutagenesis in 3T3-L1 preadipocytes coupled with adipocyte induction to select differentiation-resistant cell lines that were generated by mutagenesis (**Fig. 1**). The retroviral vector was designed so that the *blasticidin* gene was fused to the sequence of the exon that was at the 3′ end of the viral insertion site. The identities of the disrupted genes in various differentiation-resistant cell lines were determined by 3′-RACE of the fused *blasticidin* mRNA (Fig. 1).

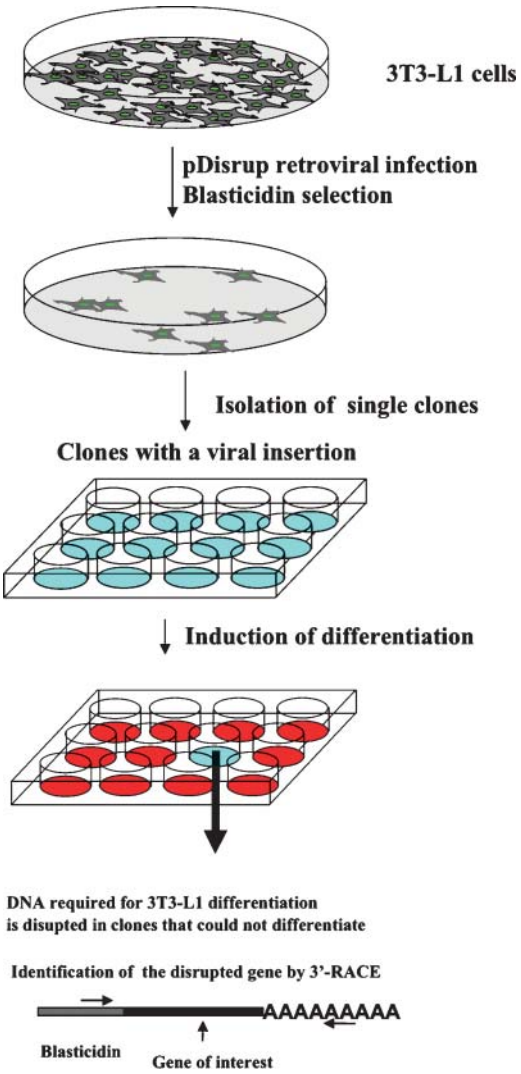

Fig. 1. Overview of our experimental procedure. After pDisrup retroviral infection, cells were treated with blasticidin S·HCl to select clones whose genes had been disrupted. After isolation and expansion of each single clone, differentiation was induced by 3-isobutyl-1-methylxanthine, dexamethasone, and insulin. As expected, some of the blasticidin-resistant clones could not differentiate into mature adipocytes (indicated as blue). The identities of the disrupted genes in various differentiation-resistant cell lines were determined by 3′ rapid amplification of cDNA ends (3′-RACE) of the fused *blasticidin* mRNA.

The gene disrupted in one of the differentiation-resistant cell lines was identified as TGIF. A partial sequence of the fused gene product generated by retroviral insertion in this line (called $TGIF^{mut}$) is shown in **Fig. 2A**. Northern blot analysis using a *blasticidin* probe indicated that there is a single viral insertion in the $TGIF^{mut}$ line (Fig. 2B). The size of the *blasticidin*–TGIF fusion mRNA was consistent with the predicted length.

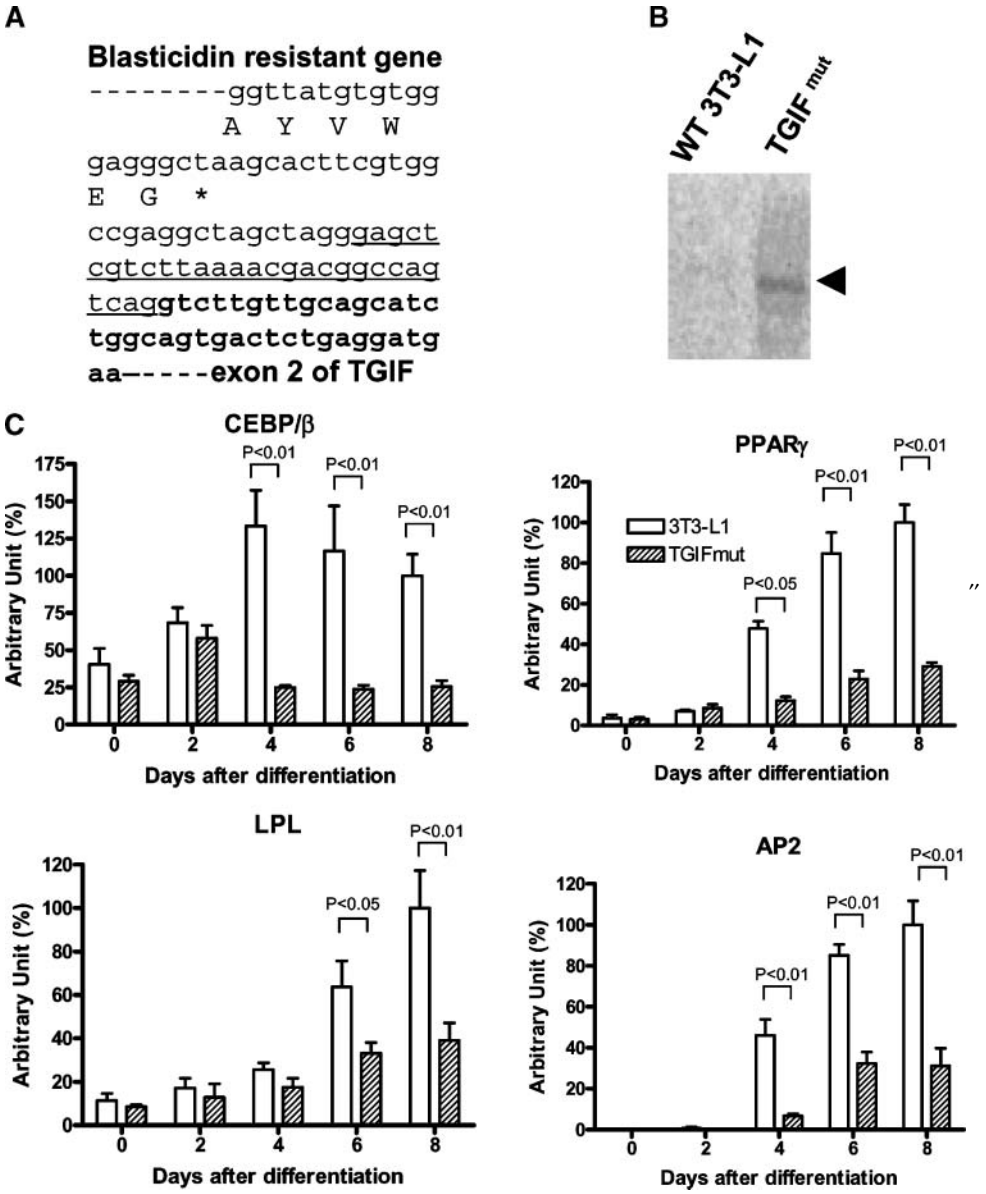

Fig. 2. TG-interacting factor (TGIF) is involved in adipocyte differentiation in 3T3-L1. A: The fused mRNA of *blasticidin* and an endogenous gene in a differentiation-resistant 3T3-L1 cell line was amplified by 3′-RACE. The junction sequence of the fused cDNA is shown, which reveals that viral insertion occurred at the 5′ end of exon 2 of the TGIF gene. The amino acid sequence at the C terminus of *blasticidin* is shown beneath the mRNA sequence. The sequence introduced by the viral vector is shown in lowercase. The underlined sequence is splicing donor. B: Total RNA was prepared from TGIF^{mut} and wild-type 3T3-L1. *Blasticidin* mRNA levels were examined by Northern blot analysis using a ^{32}P-labeled double-stranded *blasticidin* probe. A single *blasticidin* fusion mRNA was detected in TGIF^{mut} cells, as indicated by the triangle. A ^{32}P-labeled double-stranded GAPDH probe was used to determine whether the same amount of RNA was loaded. C: Expression levels of adipogenic marker genes by real-time RT-PCR in TGIF^{mut} cells and wild-type 3T3-L1. The mean expression level of wild-type 3T3-L1 at 8 days after differentiation was defined as 100%. Error bars indicate ± SEM.

We determined the expression levels of adipogenic marker genes by real-time RT-PCR in TGIF^{mut} cells and wild-type 3T3-L1. The mean expression level at 8 days after differentiation was defined as 100%. The expression levels of C/EBP-β, PPAR-γ, lipoprotein lipase (LPL), and adipocyte fatty acid-binding protein (aP2) decreased in TGIF^{mut} cells, indicating that the reduction of TGIF expression inhibits adipogenesis of 3T3-L1 cells (Fig. 2C). We also measured the expression level of a myoepithelial marker, α-smooth muscle actin (α-SMA), during adipocyte differentiation in TGIF^{mut} cells and wild-type 3T3-L1. However, there was no difference in the α-SMA expression level throughout adipogenesis between these lines (data not shown).

Requirement of TGIF for adipocyte differentiation

To evaluate whether resistance to the differentiation-inducing agents of the TGIF^{mut} line was due to the lack of TGIF expression in these cells, we stably expressed a cDNA encoding TGIF under the control of a cytomegarovirus promoter in the TGIF^{mut} cell line (designated reconstituted). Western blotting analysis using a monoclonal antibody that recognizes both phosphorylated and unphosphorylated forms of TGIF showed that there was a reduction in TGIF immunoreactivity in the TGIF^{mut} cell line compared with wild-type 3T3-L1 and reconstituted cells (**Fig. 3A**).

Next, we assessed the requirement of TGIF for adipocyte differentiation. Resistance to differentiation induction in the TGIF^{mut} line was shown by the expression of PPAR-γ protein and the relative levels of triglyceride accumulation by the use of a triglyceride-specific dye, Oil Red O. As shown in Fig. 3B, the expression of PPAR-γ in wild-type 3T3-L1 and reconstituted lines was higher than that in the TGIF^{mut} cell line transfected with empty vector at 4 and 8 days after the initiation of adipogenesis. Relative levels of triglyceride accumulation were also reduced in the vector cell line compared with wild-type 3T3-L1 and

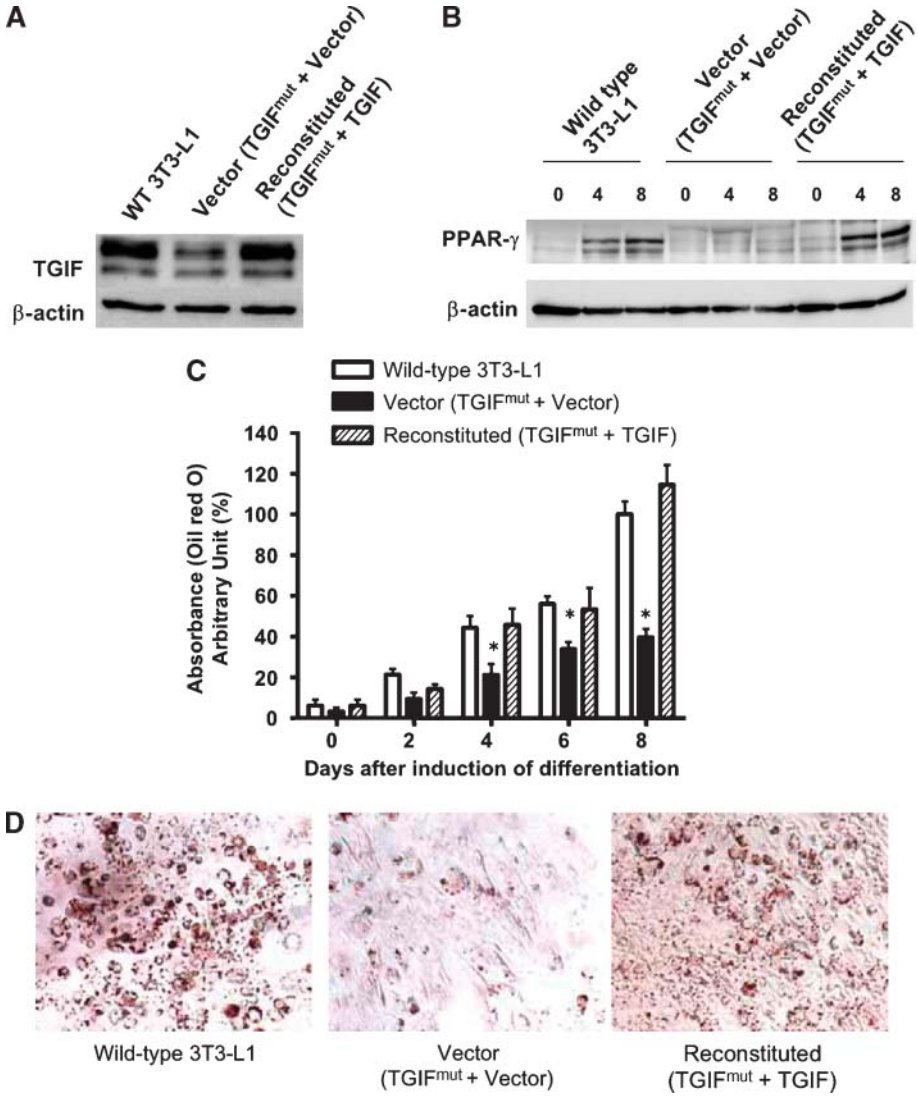

Fig. 3. Reconstitution of TGIF in TGIF^{mut} cells restores resistance to adipocyte differentiation. A: Stable cell lines were generated from TGIF^{mut} cells by transfection with either a wild-type TGIF expression vector (reconstituted) or the empty vector (vector). Expression levels of TGIF were determined by Western blotting. B: Wild-type 3T3-L1 cells and these cell lines were treated with adipogenic inducers, and expression levels of peroxisome proliferator-activated receptor γ (PPAR-γ) were determined by Western blotting at 0, 4, and 8 days after treatment. C: Wild-type 3T3-L1, vector, and reconstituted cells were treated with adipogenic inducers for various periods of time as indicated. Adipogenesis was measured at baseline and 2, 4, 6, and 8 days after the initiation of adipogenesis using the triglyceride-specific dye Oil Red O. Triglyceride accumulation was then expressed in outer diameter (OD) units. The mean value of wild-type cells on day 8 was set to 100%, and the other measurements were expressed relative to this value. Values are the means ± SE of three independent experiments. * $P < 0.05$ versus wild-type 3T3-L1 and reconstituted cells. D: Triglyceride accumulation is enhanced in the wild-type 3T3-L1 and reconstituted cells compared with vector cells at 8 days after the initiation of adipogenesis.

reconstituted lines (Fig. 3C). Triglyceride accumulation is enhanced in wild-type 3T3-L1 and reconstituted cells compared with vector cells at 8 days after the initiation of adipogenesis (Fig. 3D).

TGIF expression during adipogenesis and the effect of knockdown of TGIF on adipocyte differentiation

We measured the mRNA expression level of TGIF during adipocyte differentiation by real-time PCR. As shown in **Fig. 4A**, TGIF expression was significantly increased after 4 days of adipogenic induction. Concomitant with the increase in the mRNA level, the protein level of TGIF was also increased during adipocyte differentiation (Fig. 4B). Figure 4C shows the effect of lentiviral-mediated RNAi on TGIF expression. The transfection efficiency of our lentivirus experiments is always over 90% (data not shown). We examined the effects of two different RNAi constructs on the expression level of TGIF. The expression levels of TGIF in 3T3-L1 cells transduced with these RNAis were reduced to less than 10% of those transduced with control RNAi. We also measured relative levels of triglyceride accumulation by staining with Oil Red O after differentiation was induced in TGIF-knockdown cells and control cells. Knockdown of TGIF by two different RNAi

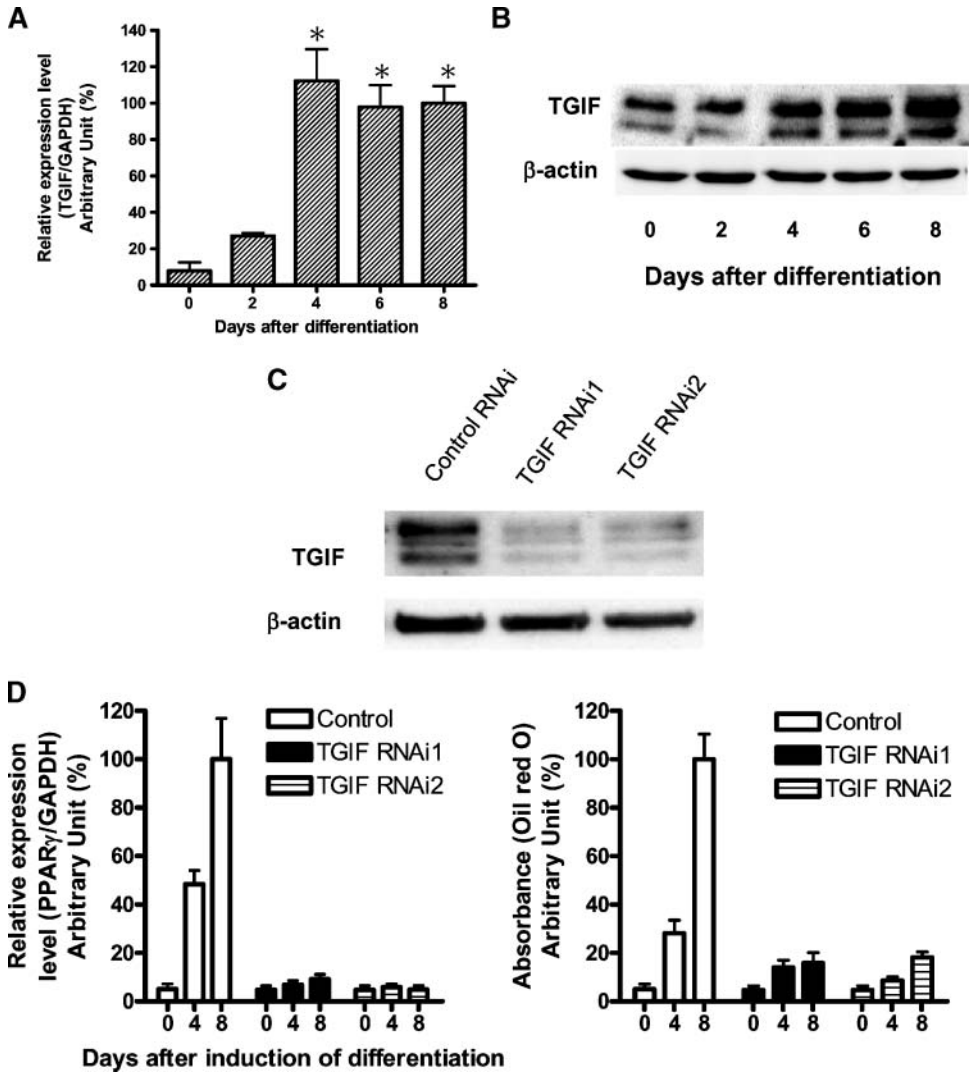

Fig. 4. TGIF expression level is enhanced during adipocyte differentiation, and knockdown of TGIF reduced the differentiation efficiency in 3T3-L1. A: Real-time PCR showed that the expression level of TGIF is enhanced after the initiation of adipocyte differentiation. The mean expression level at 8 days after differentiation was defined as 100%. Values are the means ± SE from three independent experiments. * $P < 0.05$ versus wild-type 3T3-L1 (day 0). B: TGIF protein expression level was determined by immunoblotting. It was also enhanced during adipocyte differentiation. C: TGIF expression was reduced in cells that had been transduced with TGIF RNAi by lentivirus. TGIF RNAi1 and TGIF RNAi2 have almost the same effect on the TGIF expression level. D: Wild-type 3T3-L1 cells that had been transduced with control RNAi or TGIF RNAi1 and RNAi2 were treated with adipogenic inducers for various periods of time, as indicated. Adipogenesis was measured at baseline and 4 and 8 days after the initiation of adipogenesis. Relative expression levels of PPAR-γ were measured by real-time PCR methods. Triglyceride accumulation was then expressed in OD units. The mean level of wild-type 3T3-L1 at 8 days after differentiation was defined as 100%. Values are the means ± SE from three independent experiments.

constructs significantly inhibited PPAR-γ expression levels and triglyceride accumulation at 4 and 8 days after adipogenic induction (Fig. 4D). On the other hand, overexpression of TGIF further enhanced the differentiation efficiency in 3T3-L1 cells, as demonstrated by increased levels of PPAR-γ expression and triglyceride accumulation at 4 and 8 days after adipogenic induction (Fig. 4E).

Insulin increases TGIF by promoting its stability in 3T3-L1

We next assessed the effects of insulin on the expression of TGIF in 3T3-L1. As shown in **Fig. 5A**, insulin increased the amount of TGIF protein, which peaked as early as 30 min after insulin stimulation without affecting the TGIF mRNA level. The elevated TGIF level in the absence of an increased rate of transcription implies that the turnover rate for TGIF may decrease in response to insulin, therefore increasing its stability. To examine this possibility, 3T3-L1 cells were treated with the protein synthesis inhibitor cycloheximide (CHX) in a time-course experiment (for 0, 5, 15, 30, 45, 60, 90, and 120 min) and cell lysates were analyzed by immunoblotting. The results of this experiment show that the TGIF protein level decreased to ~50% within 60 min of treatment with CHX. To determine the effects of insulin on TGIF stability, 3T3-L1 cells were treated with insulin and CHX in a time-course experiment similar to that described above. Figure 5C shows that insulin decreased intracellular TGIF turnover. Figure 5D shows that the level of TGIF decreased to ~50% after 60 min of CHX exposure and to 20% at 120 min.

TGIF degradation by the proteasome in 3T3-L1

The decreased turnover rate in insulin-treated 3T3-L1 cells implies that TGIF may become more resistant to degradation. Proteins with regulatory functions, such as cyclins and transcription activators (28, 29), are commonly degraded by the proteasome through a ubiquitin-dependent pathway. Therefore, we sought to determine whether TGIF might be proteolytically degraded by this pathway. 3T3-L1 cells were treated with the proteasome inhibitor MG-132 in the presence or absence of insulin for 4 h. As shown in **Fig. 6A**, MG-132 caused TGIF accumulation in 3T3-L1. Treatment with insulin also caused the accumu-

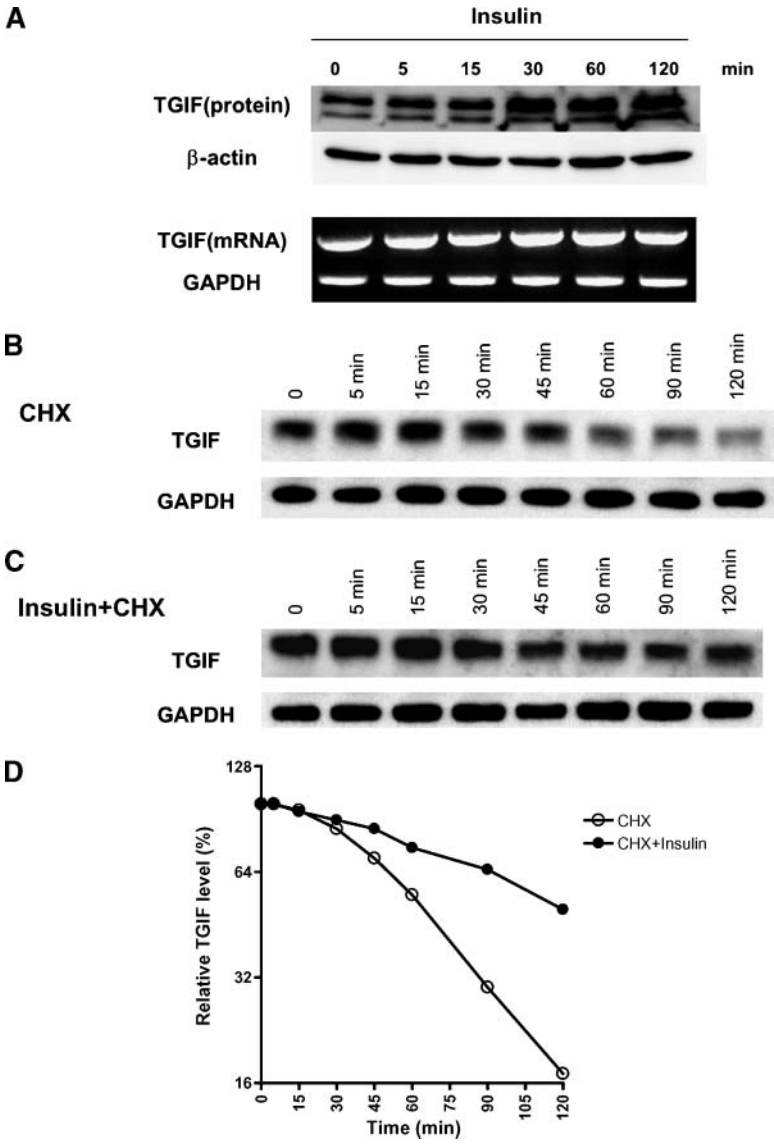

Fig. 5. Insulin increases the amount of TGIF by promoting its stability in 3T3-L1. A: TGIF protein and mRNA levels were determined by Western blotting and RT-PCR in 3T3-L1 cells that had been treated with insulin for various periods of time, as indicated. Insulin increases the TGIF protein level without affecting the mRNA level. B, C: Stabilization of TGIF in 3T3-L1 cells exposed to insulin. 3T3-L1 cells were treated with 5 μg/ml CHX or with insulin (10 μg/ml) and CHX over a 120 min period. Cells were lysed, and total lysates were immunoblotted with anti-TGIF antibodies. D: The immunoblots were scanned, and the intensity of the protein band was quantified and plotted on a semi-log graph, with the value obtained for cells that were not treated with CHX set as 100%. The values were normalized with those of GAPDH.

lation of TGIF. It has been previously reported that the ERK or PI3K signaling pathway may be involved in the regulation of protein stability (30, 31). We sought to determine whether signaling mediated by these pathways may have a role in promoting TGIF stability. Figure 6B shows that the insulin-induced increase in TGIF protein depended on ERK1/2 and PI3K activation. Pretreatment with PD 98056 (20 μM) or LY 294002 (50 μM) for 1 h significantly inhibited the basal and insulin-induced TGIF protein levels, suggesting that ERK and PI3K activation are needed to mediate TGIF stabilization.

TGIF protein induced by insulin is predominantly localized in the nuclei

To examine the localization of endogenous TGIF in response to treatment with insulin in 3T3-L1 cells, we performed an immunofluorescence microscopic analysis. As shown in **Fig. 7A**, TGIF induced by insulin was predominantly localized in the nuclei, as demonstrated by immunofluorescence staining.

TGIF inhibits Smad activation induced by TGF-β in 3T3-L1

To investigate the functional significance of increased TGIF expression in TGF-β signaling, we examined the effects of TGIF on Smad-mediated gene transcription by the transient transfection of TGIF. 3T3-L1 cells were transfected with p3TP-Lux, a TGF-β-responsive luciferase reporter plasmid that has been widely used to examine TGF-β responsiveness (26, 27). The p3TP-Lux construct contains three TPA response elements and a TGF-β-responsive segment of the human PAI-1 promoter cloned upstream of a luciferase reporter gene. As shown in Fig. 7B, TGF-β significantly increased the luciferase reporter activity of p3TP-Lux. Cotransfection with TGIF repressed both basal and TGF-β-induced luciferase activity, suggesting that increased TGIF can abrogate endogenous Smad-mediated gene transcription in 3T3-L1. When Smads were overexpressed in 3T3-L1 cells after the transient transfection of both Smad2 and Smad3 expression vectors, luciferase activity of p3TP-Lux reporter was increased in the absence or presence of TGF-β1 (Fig. 7B), suggesting that Smad plays an important role in mediating the promoter activity. Finally, ectopic expression of TGIF also suppressed the gene transcription activated by overexpressed Smad2/3 in 3T3-L1 cells with or without TGF-β1 stimulation. We further evaluated the effect of TGIF on a TGF-β downstream target. As shown in Fig. 7C, forced expression of exogenous TGIF in 3T3-L1 cells repressed both basal and

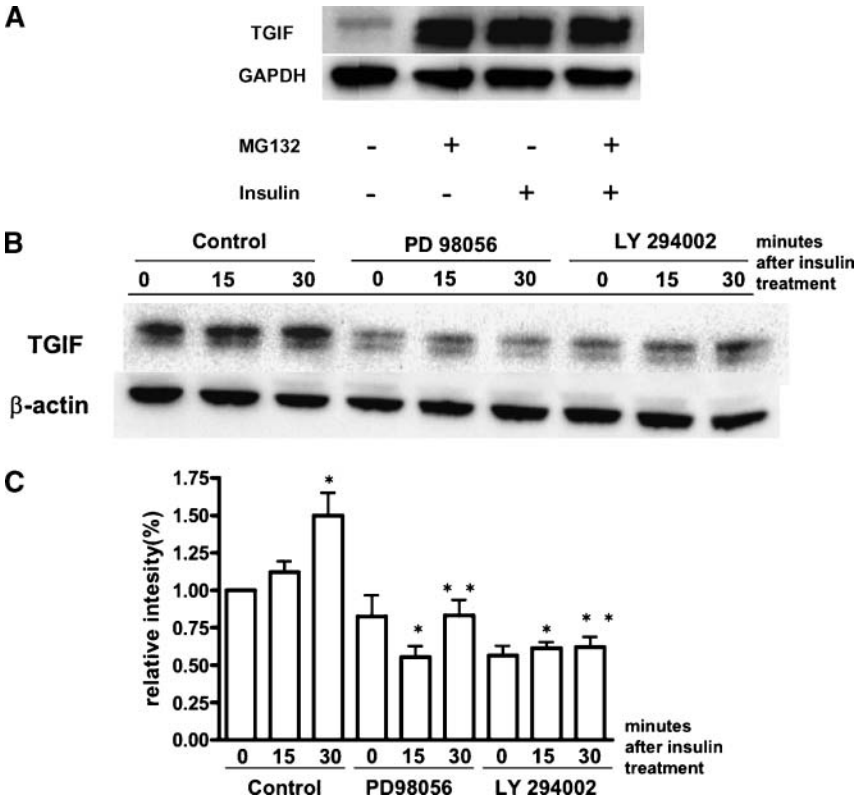

Fig. 6. Accumulation of TGIF is induced by inhibitors of ubiquitin-proteasome, ERK1/2, and PI3K pathways. A: 3T3-L1 cells were exposed to the proteasome inhibitor MG-132 (1 μM) with or without insulin (10 μg/ml) for 4 h, and total cell lysates were prepared and analyzed by immunoblotting with anti-TGIF antibody. B: The insulin-induced increase in the amount of TGIF protein depended on ERK1/2 and PI3K activation. Pretreatment with PD 98056 (20 μM) or LY 294002 (50 μM) for 1 h inhibited the insulin-induced increase in the TGIF protein level. C: TGIF intensity was normalized to β-actin and was expressed as mean ± SEM. * $P < 0.05$ versus control cells at 15 min after insulin treatment. ** $P < 0.05$ versus control cells at 30 min after insulin treatment.

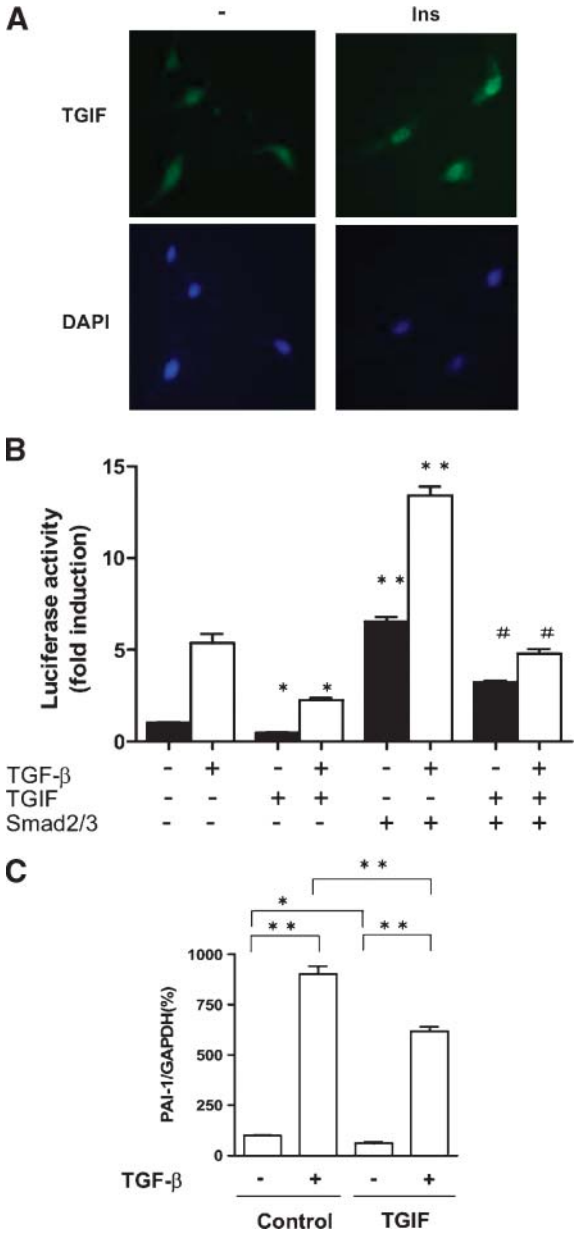

Fig. 7. TGIF blocks Smad-mediated gene transcription in 3T3-L1. A: Immunofluorescence staining shows the nuclear localization of TGIF induced by insulin in 3T3-L1. Cells were treated with or without insulin for 15 min. B: Forced expression of exogenous TGIF represses both endogenous and overexpressed Smad-mediated promoter activity. 3T3-L1 cells were transiently cotransfected with p3TP-Lux reporter plasmid together with TGIF expression vector, Smad2/3. Luciferase activity was determined after the transfected cells were incubated for 36 h in the absence or presence of 2 ng/ml transforming growth factor β1 (TGF-β1). Relative luciferase activity (fold induction) is presented as the mean ± SE of three independent experiments. * $P < 0.05$ versus controls without TGIF; ** $P < 0.01$ versus controls without Smad; # $P < 0.01$ versus groups with Smad but without TGIF. C: Forced expression of exogenous TGIF represses both basal and TGF-β-induced levels of PAI-1 mRNA. TGIF-expressing vector plasmid (pLenti6/V5-D-TOPO vector) or empty vector were used for lentivirus production and transduced to 3T3-L1. Levels of PAI-1/GAPDH were determined after the transfected cells were incubated for 48 h in the absence or presence of 2 ng/ml TGF-β1. Mean expression level of PAI-1/GAPDH in control cells without TGF-β was set as 100%. Error bars indication ± SEM. * $P < 0.05$; ** $P < 0.01$.

TGF-β-induced levels of PAI-1 mRNA, suggesting that TGIF can abrogate endogenous TGF-β-mediated gene transcription in these cells.

DISCUSSION

Understanding the balance between positive and negative regulators of adipogenesis has important health-related implications for anti-obesity medical therapy and lipodystrophy. To provide new insights into the mechanism of adipocyte differentiation, we used a functional gene identification procedure based on the poly-A trap technique. The combination of a potentially strong splice acceptor and an effective polyadenylation signal ensures complete disruption of the function of the trapped gene (4–6). After isolation and expansion of each single clone, we discovered that TGIF, which is a functional antagonist that specifically represses TGF-β1-mediated Smad signaling (7, 8), is required for adipocyte differentiation in 3T3-L1.

It has been previously shown that the overexpression of TGF-β1 in adipose tissue in transgenic mice results in a dramatic reduction in total body fat (32). However, TGF-β has been shown to be increased in adipose tissue in obese mice (21). Therefore, the expression and function of the components of the TGF signaling pathway during adipocyte differentiation, and the role of endogenous TGF-β expression and TGF-β responsiveness in the regulation of adipocyte differentiation, have been matters of interest. Previous reports have indicated that the cell-surface availability of TGF-β receptors strongly decreases during adipocyte differentiation and interferes with the activation of Smad2/3, which are the downstream effectors of TGF-β (18). Because TGF-β inhibits adipocyte differentiation, the repression of TGF-β receptor availability may enable the cells to withdraw from the autocrine, differentiation-inhibitory activity of TGF-β. Other mechanisms, in addition to receptor availability, may also be involved. Rahimi et al. (33) reported that differentiation of preadipocytes is accompanied by a block in the activation of autocrine latent TGF-β. Our results indicate that there is yet another mechanism that counteracts the activity of TGF-β in preadipocytes. In the TGIF-disrupted clone of 3T3-L1 preadipocytes, the rate of differentiation into mature adipocytes was clearly reduced compared with that in the wild-type clone. It is well known that C/EBP-β is expressed immediately upon the induction of differentiation, and triggers a transcriptional cascade by transcriptionally inducing the expression of C/EBP-α and PPAR-γ (34–37). In our experiment, the changes in the levels of adipogenic marker genes, including C/EBP-β, were apparent from day 4 after differentiation induction. These results suggest that TGIF is an important factor that affects the fate of preadipocytes from an early step in adipocyte differentiation. Suppression of TGIF by lentivirus-mediated RNAi also inhibited the differentiation of 3T3-L1 cells. Because the TGIF protein level was specifically increased by insulin treatment and the forced expression of exogenous TGIF

repressed both endogenous and overexpressed Smad2/3-mediated promoter activity in 3T3-L1, TGIF is an important target of insulin during adipocyte differentiation.

Our present results suggest that the upregulation of TGIF by insulin probably occurs at posttranscriptional levels by stabilizing its protein against degradation. This notion is supported by the rapid induction of TGIF after stimulation with insulin. Such posttranscriptional regulation of TGIF, in contrast to de novo expression, makes it possible for insulin to control TGF-β1 signaling in an expeditious manner. Although the precise mechanism by which insulin stabilizes TGIF in 3T3-L1 cells remains unknown, it depends on ERK and PI3K activation. It has been previously reported that ERK activation can directly phosphorylate TGIF in a human keratinocyte cell line (25). Such phosphorylation of TGIF promotes its stabilization and the formation of Smad2/TGIF complexes in response to TGF-β1 (25). Because PD98056 and LY294002 blocked the insulin-induced increase in the TGIF protein level, the stabilization of TGIF by insulin may be mediated, at least in part, through ERK and PI3K activation. TGIF induced by insulin may suppress the ability of Smad to initiate the transcription of TGF-β1-responsive genes and override the inhibitory action of TGF-β1 on the differentiation of 3T3-L1 cells.

TGIF may exert its repressive activity by mechanisms other than binding to activated Smad to make it transcriptionally inactive (38). Others have suggested that TGIF might function to sequester in the nucleus components of the TGF-β signaling pathway that facilitate Smad2 phosphorylation by TGF-β receptor (14). TGIF also acts as a component of a ubiquitin ligase complex that mediates the degradation of Smad2 in response to TGF-β signaling (11).

Further studies are needed to clarify whether a single or multiple mechanisms are involved in mediating TGIF repression of TGF-β-activated gene expression in adipogenesis.

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