# STAT5A Promotes Adipogenesis in Nonprecursor Cells and Associates With the Glucocorticoid Receptor During Adipocyte Differentiation

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**The differentiation of adipocytes is regulated by the activity of a variety of transcription factors, including peroxidase proliferator-activated receptor (PPAR)-γ and C/EBPα. Our current study demonstrates that ectopic expression of STAT5A, such as that of PPAR-γ and C/EBPα, promotes adipogenesis in two nonprecursor fibroblast cell lines. Using morphologic and biochemical criteria, we have demonstrated that STAT5A and the combination of STAT5A and STAT5B are sufficient to induce the expression of early and late adipogenic markers in BALB/c and NIH-3T3 cells. Yet, the ectopic expression of STAT5B alone does not induce the expression of adipocyte genes, but enhances the induction of these genes in cells also expressing STAT5A. This finding suggests that STAT5A and STAT5B do not function identically in adipocytes. In addition, these studies demonstrate that the phosphorylation of STAT5 proteins may play a role in adipogenesis. Moreover, we have shown that STAT5A is associated with the glucocorticoid receptor during adipogenesis in a highly regulated manner. *Diabetes* 52:308–314, 2003**

Adipocytes are highly specialized cells that play a major role in energy homeostasis in vertebrate organisms. Obesity is the primary disease of fat cells and the most common metabolic disorder in the industrial world. Obesity affects >30% of the adult population in the U.S. and is a major risk factor for the development of type 2 diabetes. Recent studies suggest that obesity and its related disorders may be linked to a breakdown in the regulatory mechanisms that control the expression of metabolic genes in adipocytes. Significant advances toward understanding these regulatory processes have been made by the identification of transcription factors that regulate the differentiation of fat cells and are involved in the induction and maintenance of adipocyte gene expression.

Signal transducers and activators of transcription (STATs) are a family of latent transcription factors that reside in the cytoplasm of resting cells. In response to various stimuli, STATs become tyrosine phosphorylated and translocate to the nucleus, where they mediate transcriptional regulation. STATs can be rapidly activated to regulate gene expression and represent a relatively unexplored paradigm in the transcriptional regulation of fat cells (1). Numerous studies suggest that STATs are involved in regulation of tissue-specific genes. Transgenic knockout experiments have revealed crucial roles for each known mammalian STAT (1), and cell-specific functions for STAT family members have been identified (2). However, the specificity of STAT activation and function is not completely understood. Specificity is determined, at least in part, by the receptor and the specific STAT protein. Other conditions such as serine phosphorylation, dimer composition, and the presence of other proteins associated with the STAT dimers may also confer STAT specificity (1). Moreover, STATs have additional levels of complexity. Two STAT family members, STAT5A and STAT5B, are highly related but are encoded by two separate genes (3). Murine STAT5A and STAT5B have a 96% sequence similarity, with the major difference occurring at the COOH-terminus, where the final eight residues are nonconserved. Also, STAT5B is 12 residues shorter than STAT5A. Interestingly, STAT5A and STAT5B have been shown to have both essential and nonessential roles in cytokine responses (4).

A number of studies suggest that STAT5 proteins may have an important role in fat cells. We have shown that STAT5A and STAT5B proteins are highly induced during adipocyte differentiation and that the expression of STAT5 proteins correlates with lipid accumulation and the expression of PPAR-γ and C/EBPα (5–7). STAT5 antisense oligonucleotides have been shown to inhibit the growth hormone (GH)-dependent differentiation of 3T3-F442A preadipocytes (8). In addition, the expression of a dominant-negative STAT5A in 3T3-L1 cells attenuates lipid accumulation (9). Animal studies also suggest that STAT5A and STAT5B may be important mediators of fat cell development (4). Transgenic knockout animals lacking either the *STAT5A* or *STAT5B* gene have a reduction in fat pad size, but mice lacking both STAT5A and STAT5B have fat pads that are approximately one-fifth the size of wild-type mice (4). Moreover, studies in these animals suggest that the lipolytic actions of GH involve STAT5 proteins (10).

The glucocorticoid receptor (GR) has also been shown

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DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GH, growth hormone; GPD, glycerol phosphate dehydrogenase; GR, glucocorticoid receptor; MDI, MIX, dexamethasone, insulin; MIX, methyl-isobutylxanthine; STATs, signal transducers and activators of transcription; TNF, tumor necrosis factor; TZD, thiazolidinedione.

to act as a transcriptional co-activator of STAT5 and enhance STAT5 dependent transcription (11). In the GR pathway, the receptor interacts with its steroid hormone ligand in the cytoplasm and undergoes an allosteric change that enables the hormone receptor complex to bind to a specific DNA-responsive element (glucocorticoid response element) and modulate transcription (12). Glucocorticoid excess frequently results in obesity, insulin resistance, glucose intolerance, and hypertension. Tissue sensitivity to glucocorticoids is regulated by the expression of GR isoforms and 11β-hydroxysteroid dehydrogenase type I–mediated intracellular synthesis of active cortisol from inactive cortisone. In mammary cells, GR can bind STAT5A and STAT5B homodimers and heterodimers (13). Interestingly, this complex does not appear to interact with glucocorticoid response elements or STAT5 binding sites within promoters. For example, when GR interacts with STAT5 proteins to regulate the β-casein promoter, binding occurs at sites that *do not* function in the absence of active STAT5 (14). In addition, STAT5 can inhibit GR regulation of transcription (15).

In this study, we examined the adipogenic capabilities of STAT5 proteins by ectopic expression in nonprecursor fibroblast (BALB/c and NIH-3T3) cell lines. This approach has been useful in examining other adipocyte transcription factors (16–20). In those experiments, ectopic expression of these transcription factors in nonadipogenic precursor cells resulted in the formation of lipid accumulating cells. Fibroblast cell lines do not normally undergo adipogenesis, even with the addition of the appropriate hormonal stimuli, and are significantly different from 3T3-L1 preadipocytes, which undergo adipogenesis with the same conditions (21). Our results clearly demonstrate that STAT5A is sufficient to confer adipogenesis in two fibroblast cell lines. We observed that ectopic expression of STAT5A alone, or in the presence of STAT5B, is adipogenic in BALB/c and NIH-3T3 cells, as judged by marker gene expression and lipid accumulation. Moreover, the adipogenesis of these cells occurs in the absence of thiazolidinediones (TZDs). STAT5B alone, unlike STAT5A, is *not* adipogenic. However, the presence of STAT5B enhances the adipogenic capabilities of STAT5A. Our studies also suggest that the tyrosine phosphorylation of STAT5 appears to be involved in the ability of STAT5A to confer adipogenesis. In addition, we have shown that association of STAT5A with the GR is highly regulated during differentiation and may contribute to the ability of STAT5 proteins to regulate adipocyte gene expression.

## RESEARCH DESIGN AND METHODS

**Materials.** Dulbecco's modified Eagle's medium (DMEM) was purchased from Life Technologies. Bovine and fetal bovine serum (FBS) were obtained from Sigma and Life Technologies, respectively. Murine tumor necrosis factor (TNF)-α was purchased from Biosource International. The non-phospho STAT antibodies were monoclonal IgGs purchased from Transduction Laboratories or polyclonal IgGs from Santa Cruz. A highly phospho-specific polyclonal antibody for STAT5 (tyrosine [$Y^{694}$]) was purchased from Upstate Biotechnologies. Anti–PPAR-γ was a mouse monoclonal (Santa Cruz). Anti-GR was a rabbit polyclonal (Santa Cruz). Darglitizone was generously provided by Pfizer (Groton, CT).

**Cell culture.** Murine 3T3-L1 preadipocytes were plated and grown to 2 days post-confluence in DMEM with 10% bovine serum. Medium was changed every 48 h. Cells were induced to differentiate by changing the medium to DMEM containing a standard induction cocktail of 10% FBS, 0.5 mmol/l 3-isobutyl-1-methylxanthine, 1 μmol/l dexamethasone, and 1.7 μmol/l insulin (MIX, dexamethasone, insulin [MDI]). After 48 h, this medium was replaced with DMEM supplemented with 10% FBS, and cells were maintained in this medium. NIH-3T3 and BALB/c fibroblast cell lines were obtained from the American Type Culture Collection (ATCC). Fibroblast cell lines ectopically expressing STAT5 genes were induced to differentiate with the standard (MDI) induction cocktail, in the presence or absence of 2.5 μmol/l darglitizone.

**Retroviral-mediated transfection in nonprecursor cells.** Retroviral-mediated stable expression of pBabe parental vector, pBabePPAR-γ2, pBabeSTAT5A, pBabeSTAT5B, and pBabeSTAT5A/B was generated in NIH-3T3 and BALB/c cell lines. Recombinant retroviruses were produced as described (22,23). Briefly, BOSC-23 packaging cells were transiently transfected with 20 μg of each retroviral construct. STAT5A/B combination transfections were carried out using 10 μg of each construct. At 8 and 24 h after transfection, the media were changed to DMEM and 10% calf serum, and at 48 h, the virus-containing media were collected, filtered (0.45 μm), and supplemented with 4 μg/ml polybrene. Target cells were incubated for 10 h with the retrovirus-containing solution. Puromycin (2.5 μg/ml) selection was initiated at 48 h and continued for 2 weeks.

**Preparation of whole-cell extracts.** Cell monolayers were rinsed with PBS and harvested in a nondenaturing buffer containing 150 mmol/l NaCl, 10 mmol/l Tris, pH 7.4, 1 mmol/l EGTA, 1 mmol/l EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 1 μmol/l phenylmethylsulfonyl fluoride, 1 μmol/l pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μmol/l leupeptin, and 2 mmol/l sodium vanadate. Samples were extracted for 30 min on ice and centrifuged at 15,000 rpm at 4°C for 15 min. Supernatants containing whole-cell extracts were analyzed for protein content using a BCA kit (Pierce) according to the manufacturer's instructions.

**Immunoprecipitations of GR from adipocyte extracts.** Cells were harvested under nondenaturing conditions, and the protein content of the whole-cell extracts was analyzed as described above. Protein extracts (1 mg/sample) were preincubated with protein A-Sepharose (RepliGen), and the resulting supernatant was incubated with polyclonal anti-GR antibody (2 μg) for 1 h at 4°C followed by incubation with protein A-Sepharose for an additional hour. The beads were rinsed twice with PBS, and immunoprecipitates were analyzed by SDS-PAGE followed by Western blotting with either STAT5A or GR antibodies.

**Oil Red-O staining.** Oil Red-O staining was performed as described by Green and Kehinde (21).

**Gel electrophoresis and immunoblotting.** Proteins were separated in 7.5% polyacrylamide (National Diagnostics) gels containing SDS according to Laemmli (24) and transferred to nitrocellulose (Bio-Rad) in 25 mmol/l Tris, 192 mmol/l glycine, and 20% methanol. After transfer, the membrane was blocked in 4% milk for 1 h at room temperature. Results were visualized with horseradish peroxidase–conjugated secondary antibodies (Sigma) and enhanced chemiluminescence (Pierce).

**RNA analysis.** Total RNA was isolated from cell monolayers with TriZOL (Invitrogen) according to the manufacturer's instructions, with minor modifications. For Northern blot analysis, 20 μg total RNA was denatured in formamide and electrophoresed through a formaldehyde-agarose gel. The RNA was transferred to Zeta Probe-GT (Bio-Rad), cross-linked, hybridized, and washed as previously described (25). Probes were labeled by random priming using the Klenow Fragment (Promega) and [$\alpha^{32}$P]dATP (Perkin Elmer, Life Sciences).

## RESULTS

To assess the role of STAT5 in adipogenesis, we generated a series of stable cell lines that ectopically express STAT5 proteins. We used retroviral-mediated transfection of nonprecursor fibroblast cells (BALB/c and NIH-3T3) to produce cell lines containing the pBabe vector alone, pBabeSTAT5A, pBabeSTAT5B, or the pBabe STATs 5A and 5B (STAT5A/B) expression vectors in combination. To test our hypothesis that STAT5 proteins are involved in adipogenesis, BALB/c cells ectopically expressing STAT5 genes were induced to differentiate at 2 days after confluence with the standard differentiation cocktail. Adipogenesis was judged by morphologic and biochemical criteria. As shown in Fig. 1, we assessed the adipogenesis of STAT5 BALB/c stable cell lines by examining the expression of PPAR-γ, C/EBPα, aP2, and glycerol phosphate dehydrogenase (GPD). Cells were induced to differentiate in the

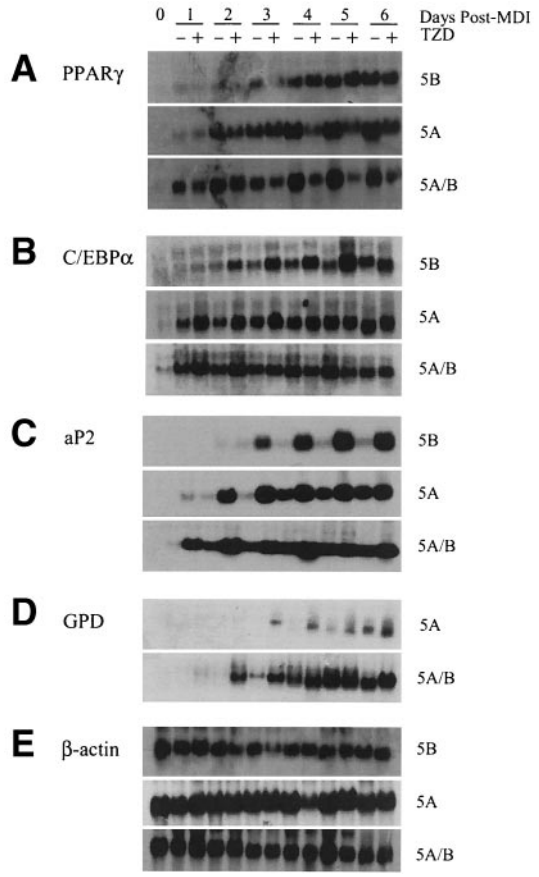


**FIG. 1. Induction of adipogenic markers in BALB/c STAT5B, STAT5A, and STAT5A/B cells. Total RNA was isolated at the indicated time points, in days, after the induction of differentiation in the absence (−) or presence (+) of 2.5 μmol/l darglitizone (TZD). Total RNA (15 μg) was electrophoresed, transferred to nylon, and subjected to Northern blot analysis for marker gene expression. β-Actin was included as a loading control. This was a representative experiment independently performed two times.**

absence or presence of darglitizone, and total RNA was isolated at the indicated times. Darglitizone (TZD) was added to ensure activation of any PPAR-γ expressed in the BALB/c or NIH-3T3 stable cell lines. Previous studies have shown that exogenous activators of PPAR-γ are necessary to obtain significant levels of adipogenesis in nonprecursor cell lines (18). As shown in Fig. 1*A*, we observed an induction in PPAR-γ mRNA in STAT5B cells at 3 days after the initiation of differentiation. Yet, the induction of PPAR-γ mRNA occurred at only 2 days after initiation of differentiation in STAT5A cells and occurred within 1 day in *STAT5A/B* cells. A similar pattern was observed for the induction of C/EBPα mRNA (Fig. 1*B*). In addition to examining two adipogenic transcription factors, PPAR-γ and C/EBPα, we examined the expression of aP2, a fat-specific lipid binding protein. As shown in Fig. 1*C*, the induction of aP2 mRNA occurred in STAT5B cells at day 3 and was expressed in a TZD-dependent manner. In STAT5A cells, aP2 mRNA was induced at day 2 in a TZD-dependent manner, and in STAT5A/B cells, this mRNA was induced at day 1 in a similar manner. Interestingly, the expression of aP2 in STAT5B cells always depended on the presence of TZD. Yet, in 5A cells or 5A/5B cells, the expression of aP2 mRNA was independent of TZD in the later stages of adipogenesis. The expression of

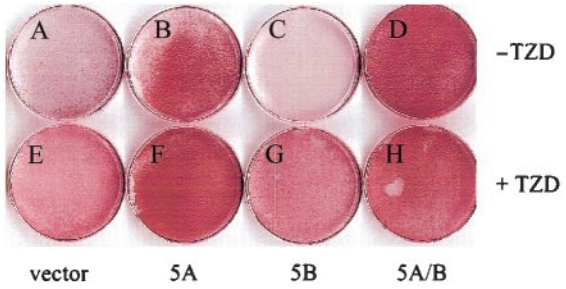


**FIG. 2. Oil Red-O staining of neutral lipids in BALB/c cell lines. Monolayers of BALB/c cells (vector, STAT5A, STAT5B, or STAT5A/B) were subjected to Oil Red-O staining at 96 h after exposure to the MDI differentiation cocktail. *A–D* were maintained in the absence of TZDs (−TZD). *E–H* were maintained in the presence of 2.5 μmol/l darglitizone (+TZD). This was a representative experiment independently performed two times.**

GPD, a late marker of adipogenesis, was induced 3 days after the initiation of differentiation in STAT5A cells and at 2 days after initiation of differentiation in STAT5A/B cells in the presence of TZD. However, GPD expression was independent of TZDs at the later stages of differentiation in STAT5A/B cells (days 5 and 6). GPD mRNA was never induced in 5B cells (data not shown).

The adipogenesis of BALB/c cells ectopically expressing STAT5 genes was also assessed by staining for neutral lipids. BALB/c cell lines were induced to differentiate in the absence or presence of darglitizone and subjected to Oil Red-O staining 96 h after the initiation of differentiation. As shown in Fig. 2, the presence of STAT5A alone or STAT5A and STAT5B in combination resulted in a substantial increase in Oil Red-O staining. We were able to detect some lipid accumulation in cells containing an "empty" vector or STAT5B in the presence of a TZD. However, there was no significant difference in Oil Red-O staining in these two cell types. In addition, the increased lipid accumulation observed in STAT5A cells and STAT5A/B cells was independent of TZDs.

The adipogenic capabilities of PPAR-γ2 and STAT5 proteins were compared by isolating RNA from BALB/c cells that stably expressed vector, PPAR-γ2, STAT5A, STAT5B, or STATs 5A and 5B. Each cell line was induced to differentiate in the absence or presence of darglitizone (TZD), and total RNA and whole-cell extracts were isolated 120 h later. The expression of adipsin, a late marker fat-specific gene, was examined by Northern blot analysis. As shown in Fig. 3*A*, adipsin mRNA was highly induced in PPAR-γ2, STAT5A, and STAT5A/B cells in the absence of TZD. In addition, adipsin mRNA was expressed at substantially lower levels in vector and 5B cells. The expression of adipsin has been shown to be repressed by TZDs (26).

Whole-cell extracts were also analyzed for the expression of PPAR-γ2 and STAT5A by Western blot analysis and compared with the levels of protein expression in 3T3-L1 adipocytes. As shown in Fig. 3*B*, PPAR-γ protein is readily detectable in all samples except the vector. In the PPAR-γ2 cells, there are three types of PPAR-γ proteins: PPAR-γ1, PPAR-γ2, and the ectopically expressed PPAR-γ2. The ectopic PPAR-γ2 protein migrates slowest, and the expression of endogenous PPAR-γ2 and PPAR-γ1 is induced in these cells, as we also observed in cells ectopically expressing STAT5 genes. The levels of PPAR-γ in the BALB/c cells are comparable to the level of PPAR-γ found

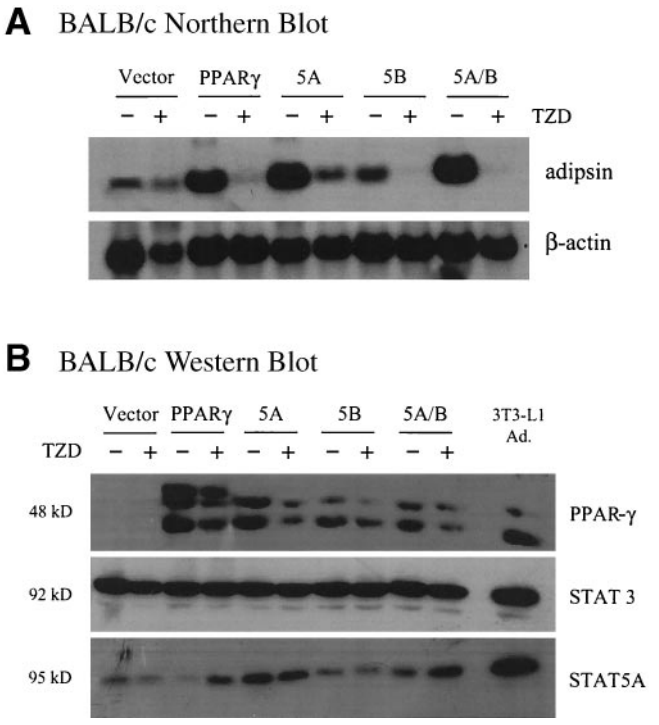


**FIG. 3. The expression of adipsin mRNA and PPAR-γ proteins in BALB/c stable cells. *A*: Total RNA was isolated 120 h after the induction of differentiation in the absence (−TZD) or presence (+TZD) of 2.5 μmol/l darglitizone. Total RNA (15 μg) was electrophoresed, transferred to nylon, and subjected to Northern blot analysis for adipogenic marker expression. β-Actin was included as a loading control. *B*: Whole-cell extracts were prepared at 120 h after induction of differentiation in the absence (−) or presence (+) of 2.5 μmol/l darglitizone. Then, 100 μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. The detection system was horseradish peroxidase–conjugated secondary antibodies (Sigma) and enhanced chemiluminescence (Pierce). Whole-cell extracts from fully differentiated 3T3-L1 adipocytes (3T3-L1 Ad.) was included for comparison of protein expression level, and STAT3 was included as a loading control. The BALB/c cell lines are vector alone (vector), PPAR-γ2, STAT5A (5A), STAT5B (5B), and STAT5A/B (5A/B). This was a representative experiment independently performed two times.**

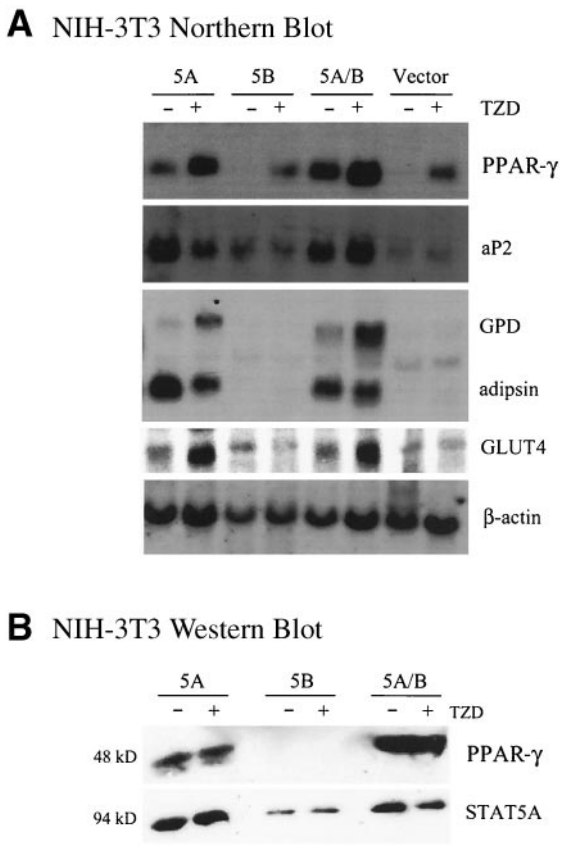


**FIG. 4. Expression of adipogenic markers in NIH-3T3 cells ectopically expressing STAT5A, STAT5B, or STAT5A/B. *A*: Total RNA was isolated 120 h after induction of differentiation in the absence (−) or presence (+) of 2.5 μmol/l darglitizone. Total RNA (15 μg) was electrophoresed, transferred to nylon, and subjected to Northern blot analysis for adipogenic marker expression. β-Actin was included as a loading control. The NIH-3T3 cell lines are vector alone (vector), STAT5A (5A), STAT5B (5B), and STAT5A/B (5A/B). *B*: Whole-cell extracts were prepared at 120 h after induction of differentiation in the absence (−) or presence (+) of 2.5 μmol/l darglitizone. Samples were processed, and results were visualized as described in the Fig. 3 legend. PPAR-γ1 and PPAR-γ2 were not resolved. This was a representative experiment independently performed three times.**

in 3T3-L1 adipocytes (Fig. 3*B*). The expression of STAT5A protein is detectable in vector and 5B cells, but the expression of this protein is induced in STAT5A cells and STAT5A/B cells. Also, the expression of STAT5A is elevated in PPAR-γ2 cells that were exposed to TZD treatment.

Because background levels of adipogenesis were observed in the BALB/c cells (Fig. 2*E*), we also examined the effect of ectopically expressed STAT5 genes in NIH-3T3 cells. The expression of various adipocyte markers was examined in NIH-3T3 cell lines ectopically expressing vector, STAT5A, STAT5B, or both STATs 5A and 5B. As shown in Fig. 4*A*, ectopic expression of STAT5A alone or STAT5A/B induced PPAR-γ mRNA expression. However, ectopic expression of STAT5B was insufficient to induce PPAR-γ mRNA in the NIH-3T3 cell lines above levels observed in vector cells. The induction of aP2 mRNA was slightly increased in a TZD-dependent manner in vector and 5B cells. However, there was a substantial induction of aP2 mRNA in STAT5A and STAT5A/B cells. Two late markers of adipogenesis, GPD and adipsin, were undetectable in vector or 5B cells. However, GPD was induced in a TZD-dependent manner in 5A and 5A/B cells, and adipsin was also significantly induced in these cells. Moreover, expression of GLUT4, the insulin-sensitive glucose transporter, was induced in a TZD-dependent manner in STAT5A and STAT5A/B cells. As an additional indicator of adipogenesis, we measured PPAR-γ protein levels in the NIH-3T3 cell lines. As shown in Fig. 4*B*, STAT5A or STAT5A/B cells induce expression of PPAR-γ, whereas STAT5B alone does not support PPAR-γ expression.

To study how STAT5 proteins are acting to influence the development of adipocytes, we examined the tyrosine phosphorylation of STAT5 during 3T3-L1 differentiation. These preadipocytes have the ability to differentiate into adipocytes that accumulated lipid, are insulin sensitive, and have the endocrine properties of native adipocytes. Whole-cell extracts were isolated over a time course of differentiation in 3T3-L1 cells and examined for STAT5 phosphorylation with a phospho-specific antibody that recognizes STAT5A and STAT5B when tyrosine$^{694}$ is phosphorylated. As shown in Fig. 5*A*, STAT5 proteins were tyrosine-phosphorylated early during adipogenesis and before the expression of PPAR-γ. The phosphorylation of both STATs 5A and 5B was confirmed by immunoprecipi-

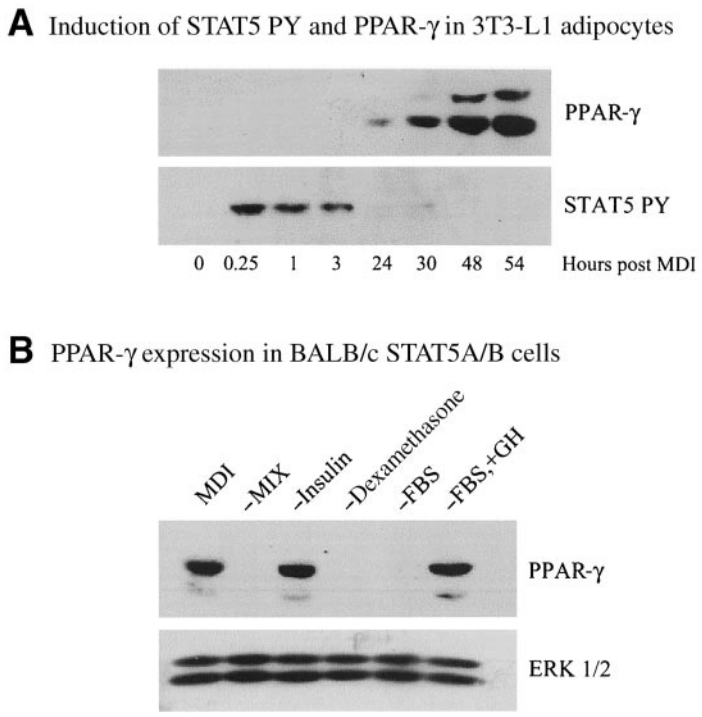


**FIG. 5. The activation of STAT5 proteins occurs early in adipogenesis and is associated with an induction of PPAR-γ expression. *A*: Whole-cell extracts were harvested at the indicated time points after induction of differentiation (hours after MDI) in 3T3-L1 preadipocytes. Then, 100 μg of each extract was analyzed by SDS-PAGE, followed by transfer to nitrocellulose and detection using enhanced chemiluminescence (Pierce). Tyrosine ($Y^{694}$)-phosphorylated STAT5 (STAT5PY) was detected using a phospho-specific polyclonal antibody. *B*: Whole-cell extracts were harvested at 96 h after the addition of the standard induction cocktail (MDI) or cocktails that lacked one component of the induction cocktail, as indicated at the top of the figure. Additional extracts were harvested after incubation with the standard MDI cocktail in the presence of 1% calf serum instead of FBS (−FBS) or 1% calf serum plus GH (−FBS, +GH). Extracellular-regulated kinase (ERK)-1/2 expression is included as a loading control. Samples were processed and results were visualized as described in the Fig. 4 legend. This was a representative experiment independently performed three times.**

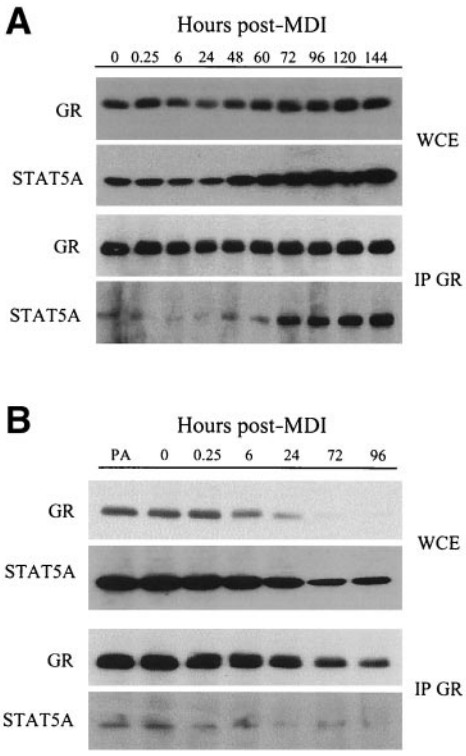


**FIG. 6. The association of STAT5A with the GR is highly regulated during adipogenesis of 3T3-L1 cells. *A*: Whole-cell extracts were harvested at the indicated time points after addition of the MDI differentiation cocktail to 3T3-L1 preadipocytes (hours post-MDI) and stored at −80°C until all of the samples were collected. Protein levels were quantified with the BCA kit (Pierce), and an equal amount of each extract was analyzed as whole-cell extracts (WCE) or immunoprecipitated (IP GR) with polyclonal anti-GR (Santa Cruz). The GR was detected using the polyclonal anti-GR antibody (Santa Cruz). STAT5A was detected using a polyclonal anti-STAT5A (Santa Cruz). *B*: Whole-cell extracts were isolated from 3T3-L1 preadipocytes (PA) that were pretreated for 15 min with 1 nmol/l TNF-α before the addition of the differentiation cocktail (MDI) at the 0 time point. Fresh TNF-α was added every 24 h. The samples were analyzed as described above. This was a representative experiment independently performed three times.**

tation with STAT5 antibodies and immunoblotting with an anti-phosphotyrosine antibody (data not shown). These results suggest that the tyrosine phosphorylation of STAT5 proteins has a role in adipogenesis. To address this question, we induced the STAT5A/B BALB/c cells to differentiate under conditions where a component of the induction cocktail was missing. We have previously shown that methyl-isobutyl-xanthine (MIX), insulin, or dexamethasone do not induce the tyrosine phosphorylation of STAT5 proteins in these cells (27). However, FBS treatment results in the tyrosine phosphorylation and nuclear translocation of STAT5 proteins, likely due to the presence of GH in FBS (27). Previous studies have demonstrated that MIX, DEX, and FBS are required for complete adipogenesis of 3T3-L1 cells (16) and nonprecursor cells ectopically expressing PPAR-γ or C/EBPs (17–20). Therefore, we examined the requirement of components of the induction cocktail (FBS, MIX, DEX, and insulin) in the differentiation of STAT5A/B BALB/c cells. The results in Fig. 5*B* demonstrate that the standard induction cocktail resulted in the induction of PPAR-γ expression in STAT5A/B BALB/c cells. Yet, in the absence of MIX, DEX, or FBS, these cells did not express PPAR-γ protein. However, if FBS was replaced with GH in 1% calf serum, the ability of STAT5A/B BALB/c cells to undergo adipogenesis and express PPAR-γ was restored. This regulation of PPAR-γ was identical in STAT5A BALB/c cells (data not shown).

Finally, we examined the interaction of STAT5A with GR. We chose to examine GR because it is highly associated with obesity and because the GR has been previously shown to associate with STAT5 in other cell types (11,13). In addition, the requirement of dexamethasone for expression of PPAR-γ in the STAT5A/B BALB/c cells suggests a role for GR in STAT5-mediated adipogenesis. To determine whether GR and STAT5A interact during adipogenesis, we harvested whole-cell extracts at various times during adipogenesis and performed Western blot analysis on whole-cell extracts as well as lysates from immunoprecipitations carried out against GR. As shown in Fig. 6*A*, GR levels are not regulated during adipogenesis, whereas STAT5A levels increase at 48 h after the initiation of differentiation. The results from immunoprecipitation experiments with anti-GR indicate that STAT5A associates with GR at 72 h after the initiation of differentiation. This interaction is maintained at 96, 120, and 144 h after the initiation of differentiation, when cells are fully differenti-

ated. Interestingly, this association was undetectable in confluent preadipocytes (time 0) or in the early stages of adipogenesis. To further examine the role of STAT5A/GR association during adipogenesis, confluent 3T3-L1 preadipocytes were induced to differentiate in the presence of TNF-α. TNF-α is known to inhibit adipogenesis (25). The results in Fig. 6*B* demonstrate that the STAT5A-GR interaction that occurs in the later stages of adipogenesis is inhibited by TNF-α. Furthermore, the levels of both GR and STAT5A decline in the presence of TNF-α.

## DISCUSSION

These studies demonstrate that STAT5A promotes adipogenesis in nonprecursor cells. Moreover, the adipogenesis of STAT5A cells or STAT5A/B cells was TZD independent, suggesting these cells are capable of making an endogenous PPAR-γ ligand. Our results also indicate that STAT5A is sufficient to induce adipogenesis in nonprecursor cells, and this adipogenesis is accompanied by an induction of PPAR-γ and C/EBPα in BALB/c cells. Although PPAR-γ is expressed at comparable levels in the BALB/c STAT5B cells, STAT5B is not adipogenic on its own (Figs. 1–4) but does enhance the adipogenic properties of STAT5A (Fig. 1*A*–*D*). STAT5A differs from STAT5B by eight amino acids at the COOH-terminus, and our results suggest that the COOH-terminal regions of STAT5A and STAT5B confer separate roles for these proteins in adipogenesis.

It is known that PPAR-γ promotes adipogenesis in C/EBPα-deficient cells, yet C/EBPα cannot promote adipogenesis in the absence of PPAR-γ (28). These recent findings suggest that C/EBPα and PPAR-γ participate in a single pathway of fat cell development, with PPAR-γ being the proximal effector of adipogenesis (28). We surmise that STAT5 proteins participate in this identified pathway of fat cell development and hypothesize it is likely that STAT5A is an early effector of adipogenesis. This is supported by our data demonstrating that ectopic STAT5A expression results in the induction of PPAR-γ expression in both BALB/c and NIH-3T3 fibroblasts (Figs. 1, 3, and 4) as well as in the early activation of STAT5 preceding the expression of PPAR-γ in 3T3-L1 adipocytes (Fig. 5*A*). The ability of STAT5A to induce adipogenesis in nonprecursor cells may be mediated by its ability to directly regulate PPAR-γ expression. Alternatively, the role of STAT5A in regulating adipogenesis could be mediated by its association with GR or other unidentified co-activators.

Interestingly, STAT5A and GR can associate in NIH-3T3 fibroblasts (13), but we have shown these two transcription factors are not associated in 3T3-L1 preadipocytes (Fig. 6*A*). The association of STAT5A and GR occurs late in adipogenesis, coincident with increasing levels of STAT5A, and may have an inhibitory role because STAT5 has been shown to inhibit GR-mediated regulation of transcription in other cell types (15). It seems unlikely that the interaction of STAT5A with GR depends on the concentration of STAT5A because NIH-3T3 and 3T3-L1 have similar levels of STAT5A expression (data not shown). Hence, we hypothesize that other factors contribute to the GR/STAT5A association that occurs in the later stages of adipogenesis.

Additionally, our data demonstrate that GR expression is not regulated during adipogenesis, but the association between GR and STAT5A is highly regulated during fat cell differentiation and that inhibition of differentiation is accompanied by an inhibition in the STAT5A/GR interaction (Fig. 6*B*). These results indicate that the association of STAT5A and GR may be important in regulating adipocyte gene expression, and future studies will focus on understanding this relationship. Furthermore, the effect of TNF-α on the association of STAT5A and GR suggests that the interaction of these transcription factors in adipocytes may play a role in insulin sensitivity. There is longstanding evidence that GR in the nervous system affects energy balance (29), but recent studies suggest that peripheral GR in adipose tissue also contributes to abdominal obesity (30). In particular, the effect of glucocorticoids produced in adipose tissue has been examined in animal studies. Transgenic mice overexpressing 11β-hydroxysteroid dehydrogenase type I selectively in adipose tissue exhibited pronounced insulin-resistant diabetes, hyperlipidemia, and hyperphagia (31). Taken together, these studies strongly suggest that there are GR-dependent pathways in adipocytes that contribute to systemic biology.

In summary, our findings suggest that STAT5A and STAT5B have nonredundant roles in adipogenesis and that STAT5A, alone or in the presence of STAT5B, is adipogenic in nonprecursor cells. These findings support recent studies demonstrating that STAT5 proteins are highly regulated in adipocytes (5,7), that they have the ability to influence adipogenesis (8,9), and that transgenic mice lacking either STAT5 gene have a reduction in fat pad size (4). Hence, it is not unexpected that STAT5A is adipogenic in nonprecursor cells. However, GH, a potent STAT5A and STAT5B activator, inhibits the expression of many adipocyte genes such as fatty acid synthase and GLUT4 in fully differentiated adipocytes (32). We predict that STAT5 proteins can exert both negative and positive effects on adipocyte gene expression, perhaps via their association with GR.

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