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PPARγ Ligand-Dependent Induction of STAT1, STAT5A, and STAT5B during Adipogenesis

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We have recently demonstrated that STAT1, STAT5A, and STAT5B are induced during adipogenesis of cultured preadipocytes in a differentiation-dependent manner. Members of the C/EBP and PPAR families of transcription factors have also been shown to be induced during adipocyte differentiation and to play a significant role in the regulation of fat-specific genes. In this investigation, we have examined the ability of C/EBPs and PPARs to contribute to STAT protein expression during conversion of non-precursor fibroblasts to functionally mature adipocytes. For this study, NIH-3T3 fibroblasts engineered to ectopically co-express C/EBPβ and C/EBPδ under the control of a tetracycline-responsive, inducible expression system were utilized to assess STAT expression during controlled adipogenesis. Data presented here demonstrate that STAT1, STAT5A, and STAT5B, but not STAT3 and STAT6, were induced in a tetracycline-responsive manner during the differentiation of these engineered fibroblasts. The STAT protein accumulation resulting from C/EBP expression was tightly coupled to the morphological conversion of fibroblasts to adipocytes and represents an expression profile identical to that reported for mature adipocytes *in vivo*. Data are also presented demonstrating that STAT protein accumulation and adipocyte conversion occurred only during controlled conditions leading to the expression of PPARγ and that the expression of these three STATs was tightly regulated in a PPARγ ligand dose-response fashion. These data illustrate that the cascade of transcriptional events leading to adipogenesis regulate the STAT family of transcription factors and that the differentiation-dependent upregulation of STAT protein expression is regulated downstream of PPARγ in a ligand-dependent manner.

The STAT (*S*ignal *T*ransducers and *A*ctivators of *T*ranscription) family of mammalian transcription factors is comprised of seven proteins (STATs 1, 2, 3, 4, 5A, 5B and 6) which are localized within the cytoplasm of numerous cell types. Stimulation of various cell surface receptors, primarily those related to cytokines, results in phosphorylation of tyrosine residues within the STAT proteins and subsequent translocation to the nucleus where they function to regulate gene transcription. Each STAT family member shows a distinct pattern of activation and exhibits specificity for regulating gene expression (1). Since the distribution and function of each STAT is unique, the regulation of tissue specific genes may be a physiological role for these proteins (2). Transgenic knockout experiments have revealed crucial roles for each STAT family member and cell specific functions for STAT proteins have been identified (1). We have recently demonstrated that some STAT family members that are expressed in rat adipose tissue *in vivo* are highly induced during the differentiation of murine 3T3-L1 cells from fibroblasts to adipocytes (3). Importantly, these studies demonstrated that STAT1, STAT5A and STAT5B are clearly regulated at the level of their expression as well as by their cytokine-mediated phosphorylation and nuclear translocation during adipocyte differentiation. Although it is hypothesized that STATs play a regulatory role in adipocyte gene expression, their specific functions during adipogenesis or in the mature adipocyte are largely unknown.

To date, two families of transcription factors, the CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptors (PPARs), have been shown to be induced during adipogenesis and to play a significant role in the regulation of fat specific genes. Transgenic mice lacking both C/EBPβ and C/EBPδ or C/EBPα alone have defective adipocyte differentiation (4–6) and ectopic expression of various C/EBPs and PPARs has been shown to convert non-precursor fibroblastic cell lines into functionally ma-

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ture adipocytes (7–13). Of particular interest, co-expression of C/EBPβ and C/EBPδ in the presence of differentiation medium containing dexamethasone results in the endogenous expression of PPARγ (12, 13) suggesting a cascade regulation between different families of transcription factors during the course of adipocyte differentiation. In this regard, we hypothesized that C/EBPs and PPARs may also be involved in the upregulation of STAT expression during adipogenesis. Although the activation of STATs by tyrosine phosphorylation is well studied, there is no information on how these transcription factors are induced or regulated at the level of their protein expression.

To examine the induction and regulation of STAT expression by various transcription factors during adipocyte differentiation, we utilized a previously described NIH-3T3 fibroblast cell line engineered to ectopically express C/EBPβ and C/EBPδ under the control of an inducible expression system (11–13). This stable cell line, designated "$\beta\delta$ cells", was created based on the bacterial tetracycline resistance operator/repressor originally described by Gossen and Bujard (14). Previous reports demonstrated that ectopic gene expression in these fibroblasts is repressed in the presence of tetracycline and that removal of this antibiotic from the culture medium results in the controlled, co-expression of C/EBPβ and C/EBPδ. When chemically induced to differentiate in the presence of a synthetic ligand for PPARγ activation, these C/EBP expressing non-precursor fibroblasts have been shown to completely convert to functionally mature adipocytes with coordinate changes in gene expression and acquisition of the fat laden phenotype (13).

In this investigation, we demonstrate that differentiation of $\beta\delta$ cells into mature adipocytes was accompanied by significant induction of STAT1, STAT5A and STAT5B protein expression. The time course and profile of STAT induction was kinetically similar to that reported for differentiating 3T3-L1 preadipocytes suggesting that these *trans*-acting factors may play an important role during adipogenesis and/or maintenance of the adipocyte phenotype. In addition, the data presented here clearly illustrate that STAT protein expression tightly correlated with lipid accumulation and PPARγ expression. Moreover, induction of these STAT proteins was regulated in a PPARγ ligand-dependent manner. These studies provide evidence that PPARγ, directly or indirectly, plays a regulatory role in the induction of STAT expression during adipocyte differentiation.

EXPERIMENTAL PROCEDURES

Stable cell lines and cell culture. The NIH-3T3 cell line ectopically expressing C/EBPβ and C/EBPδ under the control of a tetracycline operator was created and described previously (13). This stable cell line, designated "$\beta\delta$ cells", was propagated in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; Hyclone) in the presence of tetracycline (1 μg/ml) which has been shown to repress the ectopic expression of both C/EBPs. Tetracycline was removed from the growth medium at near confluence and at approximately two days post-confluence, the growth medium was replaced with differentiation medium containing DMEM supplemented with 10% FBS, 0.5 mM 3-isobutyl 1-methylxanthine, 1 μM dexamethasone, and 1.7 μM insulin (MDI). After 48 h, cells were maintained in DMEM containing 10% FBS and 0.4 μM insulin throughout the remaining time course of experimentation. Maintenance medium was changed every 48 h until the cells were utilized for experimentation. Cells were differentiated in the presence of 10 μM ciglitazone (Upjohn) unless otherwise noted. Throughout the study, time "0" refers to postconfluent cells immediately before chemical induction of differentiation with the addition of MDI to the culture medium. The term "post-MDI" refers to the time elapsed since the addition of MDI to the culture medium.

Protein analysis. Cells were rinsed with phosphate-buffered saline (PBS) and harvested in a buffer containing 25 mM Tris (pH 7.4), 50 mM NaCl, 0.5% sodium deoxycholate, 2% NP-40, 0.2 % SDS, 1 μM PMSF, 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin and 10 μM leupeptin. Protein content for whole cell extracts was determined using a BCA kit (Pierce) according to the manufacturer's instructions. Following quantitation, proteins were separated in 7.5% polyacrylamide gels (National Diagnostics) containing sodium dodecyl sulfate (SDS) according to Laemmli (15) and transferred to nitrocellulose (Biorad) in 25 mM Tris, 192 mM glycine and 20% methanol. Following transfer, the membrane was blocked in 4% milk for 1 hour at room temperature. Membranes were probed with STAT monoclonal IgG antibodies (Transduction Laboratories) and the results were visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma) and enhanced chemiluminescence (Pierce).

RNA analysis. Total RNA was isolated from cell monolayers with Trizol (Gibco BRL) according to manufacturer's instruction with minor modifications. For Northern blot analysis, 20 μg of total RNA was denatured in formamide and electrophoresed through formaldehyde/argarose gels. The RNA was transferred to Hybond-N (Amersham), crossed-linked, hybridized and washed as described previously (16). Probes were labeled by random priming using the Klenow fragment of DNA polymerase I (New England Biolabs Inc., Beverly, MA) and [α-^{32}P]dCTP (Dupont-NEN, Boston, MA). Hybridization to the ribosomal 18S subunit was used to quantitate equal loading.

Oil Red O staining. Adipocyte conversion was assessed with lipid accumulation stained with Oil Red O according to the procedure described by Green and Kehinde (17) with minor modifications as described previously (13).

RESULTS

Previous studies have demonstrated that ectopic co-expression of C/EBPβ and C/EBPδ in non-precursor NIH-3T3 fibroblasts results in conversion to functional adipocytes in a PPARγ ligand-dependent manner (13). Since several STAT family members are induced during adipocyte differentiation (3), we utilized the diagnostic potential of these $\beta\delta$ cells to mechanistically investigate the regulation of STAT expression during adipogenesis. Initially, we examined the regulation of STAT expression as a function of tetracycline which has been shown to regulate, by repression, the inducible expression system (14). Cells were propagated in the presence of 1 μg/ml tetracycline until approximately 80% confluent, then cultured in growth medium

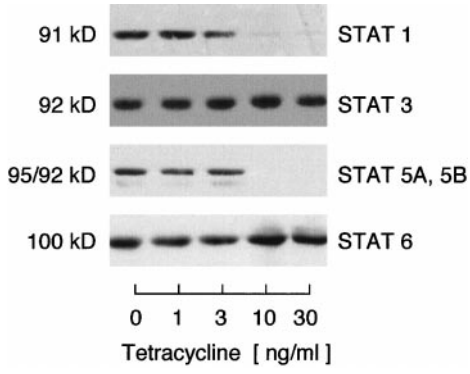

FIG. 1. Tetracycline dose-dependent expression of STAT1 and STAT5 in $\beta\delta$ cells. At two days post-confluence, cells were induced to differentiate by replacing growth medium with differentiation medium containing FBS, MDI, ciglitazone and varied concentrations of tetracycline as described in Experimental Procedures. Throughout the experiment, indicated tetracycline concentrations were maintained during each change of the culture medium. Whole cell extracts were prepared 6 days after the induction of differentiation. Fifty μg of protein were separated by SDS-PAGE, transferred to nitrocellulose and subjected to Western blot analysis of STAT protein expression. The 95 kDa and 92 kDa bands of STAT5A and STAT5B, respectively, were illustrated. In this experiment, STAT5B was lightly exposed.

containing varied concentrations of tetracycline to regulate the ectopic C/EBP expression as reported previously (11–13). At two days post-confluence (day 0), the cells were chemically induced to differentiate with medium containing FBS, MDI and ciglitazone as described in Experimental Procedures. With each medium change, the varied concentrations of tetracycline were maintained. On day 6, cells were harvested and whole cell extracts were subjected to Western blot analysis to examine STAT protein expression. As shown in Fig. 1, the expression of STAT1, STAT5A and STAT5B (lightly exposed) were reciprocally regulated and induced in a tetracycline dose-dependent fashion. Induction of these STAT family members tightly correlated with the concentration of tetracycline permissive for C/EBP expression and the acquisition of the fat laden phenotype (data not shown). The specificity of the increased expression of these three STATs was clearly demonstrated by the complete lack of effect on the expression of STAT3 and STAT6. These data suggest that members of the C/EBP family of transcription factors play a role upstream of STAT expression during the known sequence of transcriptional events mediating adipocyte differentiation.

It is well documented that differentiation of 3T3-L1 preadipocytes involves a temporal pattern of gene expression that includes the immediate activation of C/EBPβ and C/EBPδ which are succeeded by expression of C/EBPα and PPARγ occurring 2–3 days following induction of differentiation (18–20). To gain further insight into the potential role of these *trans*-acting factors in the regulation of STAT expression during adipogenesis, the kinetics of STAT protein accumulation were examined over a time course of $\beta\delta$ cell differentiation. Whole cell lysates were harvested every 24 h following the induction of differentiation and subjected to Western blot analysis. As illustrated in Fig. 2, the protein expression of STAT1, STAT5A and STAT5B was substantially induced 4 days following the induction of differentiation. Consistent with the specificity depicted in Fig. 1, the protein expression of STAT3 and STAT6 did not change at any point during the course of differentiation. Interestingly, the onset of lipid accumulation (data not shown) closely correlated with the kinetics STAT protein expression. The temporal coupling of these processes was identical when comparing the differentiation of these engineered $\beta\delta$ fibroblasts and 3T3-L1 preadipocytes (3). The data illustrated in Figs. 1 and 2 also demonstrate that the effect on STAT protein expression resulted from the expression of C/EBPβ and C/EBPδ and not due to the chemical inducers since the maintained presence of tetracycline (i.e., suppressing ectopic C/EBP expression) in cells chemically induced to differentiate completely eliminated the induction of STAT expression.

The 4 day delay of STAT protein accumulation following C/EBPβ and C/EBPδ expression suggested that other adipogenic transcription factors may mediate the C/EBP effect on STAT expression. Since these C/EBPs have been shown to induce PPARγ expression in $\beta\delta$ cells (12), we hypothesized that the induction of STAT1 and STAT5 may be mediated by PPARγ rather than by C/EBPβ and/or C/EBPδ. In $\beta\delta$ cells, this effect could not be due to C/EBPα which has been shown, by independent investigations, to be repressed in NIH3T3 fibroblasts (8, 12). To address this hypothesis, we cultured $\beta\delta$ cells under the following diagnostic conditions: 1) presence of chemical inducers (i.e., MDI and ciglitazone) and tetracycline (i.e., repressed ectopic C/EBP expression); 2) absence of chemical inducers and tetracycline (i.e., only ectopic C/EBP expression); 3) MDI and ectopic C/EBP expression and 4) MDI, ectopic C/EBP expression plus ciglitazone. Following 6 days of conditional treatment, cells were analyzed for STAT protein expression (Fig. 3A), adipocyte conversion marked by lipid accumulation (Fig. 3B), and the expression of C/EBPβ, C/EBPδ and PPARγ mRNA (Fig. 3C). As depicted in Fig. 3A, the full complement of chemical inducers in the absence of ectopic C/EBPs was not sufficient for the induction of STAT expression (lane 1). Similarly, STAT protein accumulation did not occur with ectopic expression of C/EBPβ and C/EBPδ in the absence of chemical inducers (lane 2). Conversely, however, ectopic co-expression of these C/EBPs in the presence of MDI resulted in an increased protein accumulation of STAT1, STAT5A and STAT5B (lane 3) and this effect was significantly enhanced with the addition of the PPARγ specific ligand, ciglitazone (lane 4). To correlate the induction of STAT protein expression with adipocyte conversion, lipid accumulation was

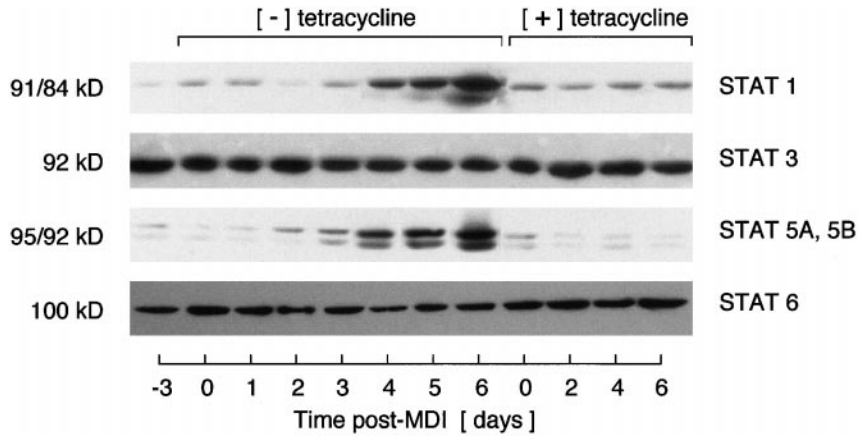

FIG. 2. Time course of STAT1 and STAT5 protein expression during the differentiation of $\beta\delta$ cells. Whole cell extracts were prepared at various times following the induction of differentiation in the absence [−] and presence [+] of 1 μg/ml tetracycline. The term "post-MDI" refers to the time elapsed since the addition of MDI to the culture medium as described in Experimental Procedures. Extracts from subconfluent, proliferating cells propagated in the presence of tetracycline (−3 days) were included. Fifty μg of protein were separated by SDS-PAGE, transferred to nitrocellulose and subjected to Western blot analysis.

quantified by Oil-Red-O staining and the results illustrated in Fig. 3B. Plates 1-4 correspond to identical treatment conditions depicted in lanes 1–4 of Fig. 3A. Under conditions that supported little or no STAT1, STAT5A and STAT5B protein expression (lanes 1 and 2; Fig. 3A) there was no detectable lipid accumulation (plates 1 and 2; Fig. 3B). Ectopic C/EBP expression in the presence of MDI which elicited a modest increase in STAT protein accumulation (lane 3; Fig. 3A) resulted in 15–25% morphological conversion of fibroblasts to adipocytes (plate 3; Fig. 3B). More notably, differentiating $\beta\delta$ cells in the presence of ciglitazone which produced maximal STAT protein expression (lane 4; Fig. 3A) resulted in 90–100% adipocyte conversion (plate 4; Fig. 3B). To confirm the expression of PPARγ, accumulation of transcription factor mRNA was also assessed under the four diagnostic conditions described above. Ectopic co-expression C/EBPβ and C/EBPδ is depicted in lane 2 of Fig. 3C. Consistent with the effect on STAT expression and adipocyte conversion, PPARγ mRNA modestly increased with ectopic C/EBP expression when cells were chemically induced to differentiate with MDI (lane 3; Fig. 3C). Interestingly, the addition of ciglitazone to the induction cocktail resulted in a significant increase in PPARγ mRNA accumulation (lane 4; Fig. 3C) which correlated with the maximal increase in STAT protein expression and adipocyte conversion described above.

To further explore the specificity of PPARγ ligand-dependent induction of STAT protein expression, $\beta\delta$ cells were chemically induced to differentiate with MDI and varied concentrations of ciglitazone. On day 6, cells were harvested and whole cell extracts were subjected to Western blot analysis to examine STAT protein expression. As illustrated in Fig. 4, STAT1, STAT5A and STAT5B proteins accumulated in a ciglitazone dose-dependent fashion. Moreover, increasing concentrations of ciglitazone resulted in adipocyte conversion (data not shown) which correlated with the induction of STAT protein expression. The specificity of these results is demonstrated by the expression of STAT3 and STAT6 which were unaffected by the presence of the PPARγ ligand. Similar data were obtained with pioglitazone and BRL 4953 (data not shown) demonstrating that these results were not solely due to a specific PPARγ ligand.

DISCUSSION

This investigation presents a molecular mechanism coupling the expression of the STAT family of transcription factors with the sequential pattern of transcriptional events known to mediate the developmental processes of adipogenesis. First, we demonstrate that differentiation of non-precursor fibroblasts engineered to ectopically co-express C/EBPβ and C/EBPδ results in STAT protein accumulation with an expression profile identical to that reported previously for mature adipocytes. Second, data are presented demonstrating that protein accumulation of STAT1, STAT5A and STAT5B resulting from C/EBP expression is tightly coupled to the morphological conversion of fibroblasts to adipocytes. Third, the differentiation-dependent upregulation of STAT protein expression is regulated downstream of PPARγ in a ligand-dependent manner. Collectively, these data demonstrate that the cascade of transcription factors that mediate adipogenesis also regulate the expression of the STAT family of transcription factors providing additional evidence for sequential activities and interactions of structurally diverse *trans*-acting factors that, collectively, may mediate the complex process of adipocyte differentiation.

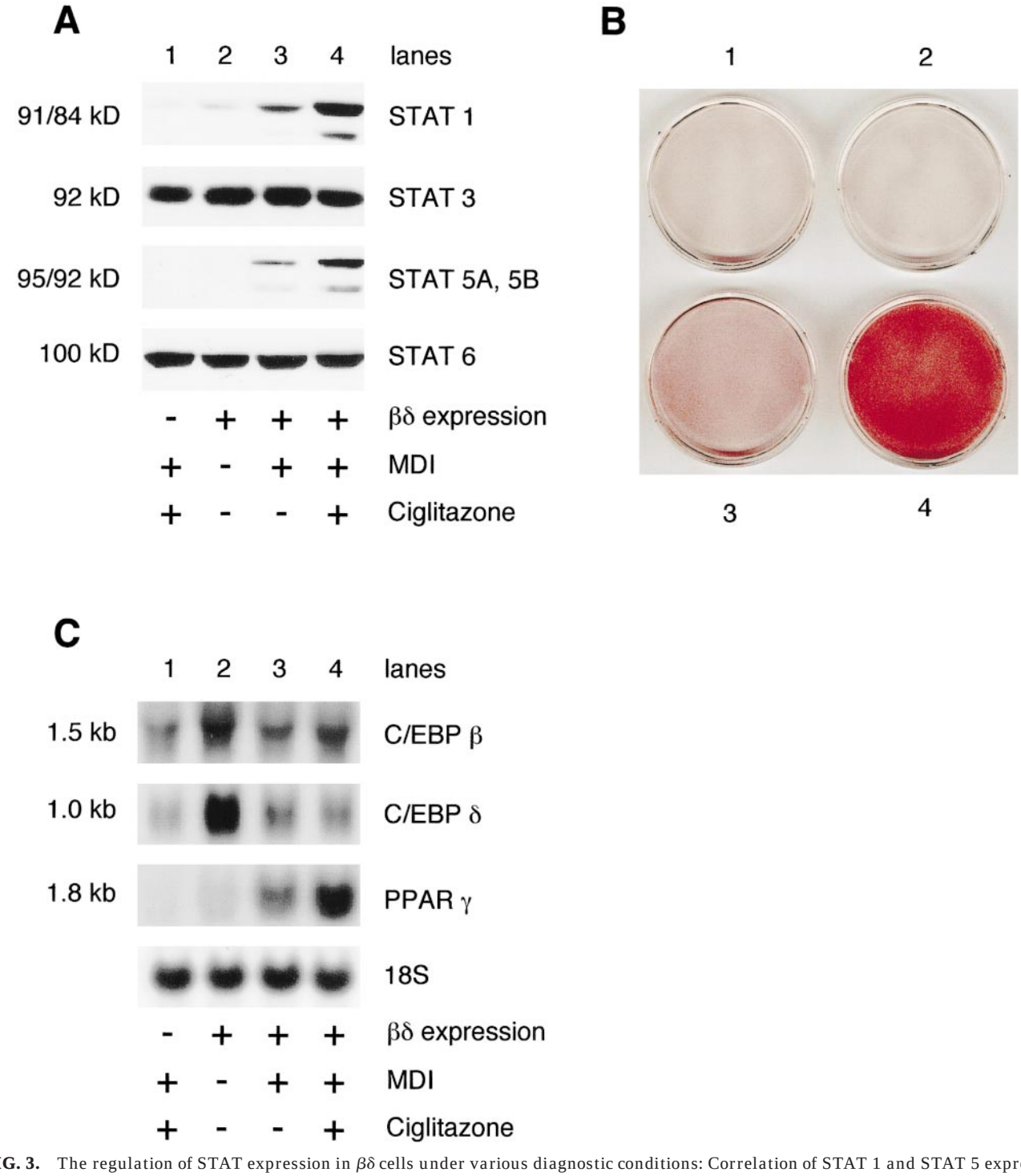

FIG. 3. The regulation of STAT expression in *βδ* cells under various diagnostic conditions: Correlation of STAT 1 and STAT 5 expression with lipid accumulation and PPARγ expression. (A) *βδ* cells were cultured for 6 days under various diagnostic conditions depicted below the illustrated data. Fifty μg of whole cell extracts were separated by SDS-PAGE, transferred to nitrocellulose and subjected to Western blot analysis for STAT protein expression. (B) Cells cultured under each diagnostic condition depicted in panel A were analyzed for adipocyte conversion by examining lipid accumulation stained with Oil Red O. (C) Total RNA was isolated following 6 days of conditional treatment identical to that presented in panel A. Twenty μg of total RNA was electrophoresed, transferred to nylon and subjected to Northern blot analysis for C/EBP and PPAR mRNA expression. Hybridization to the ribosomal 18S subunit was examined for quantitation of equal loading.

Although the specific roles for STATs in developing/mature adipocytes are largely unknown, the data presented in this investigation suggest that these transcription factors are important for transactivating gene expression related to adipocyte function. In support of this notion, it is important to compare STAT protein

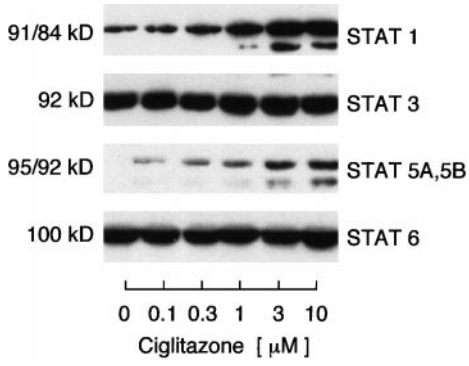

FIG. 4. PPARγ ligand dose-dependent induction of STAT1 and STAT5 during differentiation of βδ cells. Tetracycline was removed from the culture medium as cells approached confluence. At two days post confluence, growth medium was changed to differentiation medium containing FBS, MDI and varied concentrations of ciglitazone. Throughout the experiment, ciglitazone concentrations were maintained during each change of culture medium. Whole cell extracts were prepared 6 days after the induction of differentiation as described in Experimental Procedures. Fifty μg of protein were separated by SDS-PAGE, transferred to nitrocellulose and subjected to Western blot analysis of STAT protein expression.

expression during differentiation of engineered non-precursor fibroblasts presented here and the 3T3-L1 preadipocyte cell line presented elsewhere (3). Albeit extremely diverse in their developmental origins, differentiation of these two cell lines results in identical kinetics of STAT protein expression that tightly correlate with the appearance of the fat-laden phenotype. Moreover, the expression profile following differentiation of these cell lines is identical to that observed in the mature adipocyte, *in vivo*. The conserved regulation of STAT expression within these diverse systems of adipocyte differentiation is likely to represent an important event leading to the functionally mature adipocyte.

One hypothesis which has developed from previous studies is that a potential function of STAT1 is the regulation of genes involved with insulin sensitivity. We have shown that STAT1 protein is markedly decreased in TNFα induced insulin resistance (3). While glucose metabolism is an important function of the mature adipocyte, it is unlikely that STAT1 is essential for the morphological fat-laden phenotype. Observations of STAT1 deficient mice have led others to report that the only critical function of STAT1 is to regulate a set of genes which collectively provide innate immunity (21, 22). Although these mice have no overt developmental abnormalities (i.e., deformed or absence fat pads), the regulation of gene expression in the adipocytes of these animals has not been investigated. In contrast to STAT1, the contributions of STAT5A and STAT5B to adipocyte morphology appear to be much greater. Recent studies have demonstrated that transgenic mice lacking STAT5A or STAT5B have significantly smaller fat pads. Furthermore, transgenic animals lacking both STAT5A and STAT5B have fat pads only one-fifth the size of wild type animals (23). Collectively, these studies support the notion that these three STAT family members may be important regulators of gene expression mediating adipocyte function and that STAT5A and STAT5B are likely to play essential transactivating roles during adipocyte differentiation.

The data presented in this investigation clearly demonstrate that STAT1, STAT5A and STAT5B are regulated, directly or indirectly, by PPARγ. We find this idea particularly exciting for two reasons. First, a largely fat-specific transcription factor (i.e., PPARγ) may be involved in regulating the expression of other transcription factors (i.e., STATs) whose tissue-specific functions may include the induction and/or maintenance of fat-specific gene expression. Second, this is the first study to suggest the involvement of any *trans*-acting factors in the induction of STATs in adipocytes. The recent cloning and analysis of the murine genomic promoter of STAT1 has revealed a functional retinoic acid (RA) response element which preferentially interacts with the RA receptor β and the retinoic X receptor α (RXRα) (24). Members of the PPAR family of transcription factors heterodimerize with RXRα (25) and PPARγ is known to interact with RXRα when it is bound to DNA (26). These studies strongly suggest that PPARγ may play a direct role in regulating the STAT1 promoter via heterodimerization with RXRα. We are currently conducting studies to demonstrate if PPARγ is directly involved in the regulation of STAT1, STAT5A and STAT5B gene expression.

A current model of adipogenesis suggest that a cascade expression of C/EBPβ and C/EBPδ leads to the expression of C/EBPα and PPARγ which precede and regulate the expression of many genes representative of the mature adipocyte. The data presented here demonstrate that STATs represent additional *trans*-acting factors that are regulated downstream of PPARγ and precede or coincide with the expression of adipocyte specific genes. The conserved regulation of STAT protein expression in diverse systems of adipocyte differentiation suggests that these transcription factors may also play a role in regulating functional adipocyte gene expression. Moreover, recent studies have demonstrated that STAT5B plays a role in modulating PPARα in other cell types (27) suggesting the possibility that STATs may have the potential to regulate upstream transcriptional activity as well as downstream gene expression in the mature adipocyte. While the data presented here narrow the focus for mechanisms regulating STAT expression during adipocyte differentiation, it should be noted that recent studies have implicated a synergy between various C/EBPs and PPARγ in regulating adipocyte gene expression. In this regard, it is possible that a role for C/EBPα, which is repressed in NIH3T3 fibroblasts, may be imparted by the ectopic expression of C/EBPβ and/or C/EBPδ in

mediating the effect on STAT protein expression. Determining the function of STATs during adipogenesis and deciphering the complex interactions of these and other adipogenic transcription factors will provide a better understanding of the molecular processes of adipocyte differentiation.

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