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Research Article

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PPARγ Induces the Insulin-dependent Glucose Transporter GLUT4 in the Absence of C/EBPα During the Conversion of 3T3 Fibroblasts Into Adipocytes

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Abstract

To define the molecular mechanisms that control GLUT4 expression during adipogenesis, NIH-3T3 fibroblasts ectopically expressing different adipogenic transcription factors (C/EBPβ, C/EBPδ, C/EBPα, and PPARγ) under the control of a tetracycline-responsive inducible (C/EBPs) or a constitutive retroviral (PPARγ) expression system were used. Enhanced production of C/EBPβ (β2 cell line), C/EBPβ together with C/EBPδ (β/δ39 cell line), C/EBPα (α1 cell line), or PPARγ (Pγ2 cell line) in cells exposed to dexamethasone and the PPARγ ligand ciglitazone (a thiazolidinedione) resulted in expression of GLUT4 mRNA as well as other members of the adipogenic gene program, including aP2 and adipsin. Focusing our studies on the β/δ39 cells, we have demonstrated that C/EBPβ along with C/EBPδ in the presence of dexamethasone induces PPARγ, adipsin, and aP2 mRNA production; however, GLUT4 mRNA is only expressed in cells exposed to ciglitazone. In addition, enhanced expression of a ligand-activated form of PPARγ in the β/δ39 fibroblasts stimulates synthesis of GLUT4 protein and gives rise to a population of adipocytic cells that take up glucose in direct response to insulin. C/EBPα is not expressed in the β/δ39 cells under conditions that stimulate the adipogenic program. This observation suggests that PPARγ alone or in combination with C/EBPβ and C/EBPδ is capable of activating GLUT4 gene expression. (*J. Clin. Invest.* 1998. 101:22–32.) Key words: adipogenesis • transcription factors • insulin action • glucose transporter 4 • antidiabetic drugs

Introduction

Transport of glucose across an impermeable cell membrane is facilitated by a family of glucose transporters (GLUT1-5 and GLUT7) that are expressed in a tissue-restricted manner (1, 2). The insulin-dependent glucose transporter, GLUT4, is expressed exclusively in adipose tissue, cardiac muscle, and skeletal muscle (3–7), where it functions to regulate glucose uptake into these tissues in response to elevated levels of insulin in the circulation. GLUT1 is also involved in transporting glucose into adipose tissue and muscle, but its expression is not limited to these sites since it has been identified in nearly all cell types where it is thought to account for basal uptake of glucose (2). The molecular mechanisms regulating the tissue-restricted expression of GLUT4 are essentially unknown.

Both GLUT4 and GLUT1 have been shown to be differentially expressed during development of striated muscle and brown adipose tissue (8). In the fetal and early postnatal stages of mouse development, GLUT1 is the predominant transporter. After birth, there is an extensive increase in GLUT4 expression in striated muscle and brown adipose tissue with a corresponding decrease in GLUT1 expression. This developmental pattern of glucose transporter gene expression is mimicked to some extent in various mouse cell lines that give rise to either adipocytes or myocytes in culture. GLUT4 is not produced in preadipocytes or myoblasts, but its expression is enhanced manyfold during differentiation of each of these cell types into adipocytes and myocytes, respectively (7, 9, 10). The observation that a different set of transcription factors regulates the differentiation of these two cell types raises the following question: is the expression of the GLUT4 gene in adipocytes and myocytes regulated by a corresponding set of tissue-restricted transcription factors, or is it regulated by other factors that are not involved in determining the phenotype of these specific tissues? In the case of muscle-specific expression of GLUT4, it has been suggested that myocyte enhancer factor 2 (MEF2) and thyroid hormone receptor α1 (TRα1) play a direct role in its regulation (10, 11). The promoter of the GLUT4 gene contains several putative binding sites for many regulatory proteins, including an MEF2 and TRα1 site. There are also several E box elements that have the potential of binding the MyoD family of transcription factors; however, there is no data to suggest that these myogenic factors are involved in regulating GLUT4 expression in muscle.

There is significantly less information on the mechanisms responsible for the extensive induction of GLUT4 during adipogenesis. Differentiation of preadipocytes into adipocytes is regulated by at least two families of transcription factors: the PPARs[1] (peroxisome proliferator-activated receptors) and the C/EBPs (CCAAT/enhancer binding proteins). The PPARs (PPARα, PPARγ, and PPARδ/NUC1) are members of the nuclear hormone receptor superfamily that includes receptors for the retinoid, thyroid, and steroid hormones (12–14). There are varying levels of these PPARs in many tissues where their function is to regulate the metabolism of lipids by activating expression of key enzymes (15). Of the known members of the

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1. *Abbreviations used in this paper:* C/EBP, CCAAT/enhancer binding protein; DEX, dexamethasone; GPD, glycerol phosphate dehydrogenase; MIX, methylisobutyl xanthine; PPAR, peroxisome proliferator-activated receptors; TZD, thiazolidinediones.

PPAR family, PPARγ appears to be primarily involved in adipogenesis (16). Expression of PPARγ is high in adipose tissue where it is induced quite early during differentiation of preadipocytes into adipocytes (17, 18). In this regard, ectopic expression of PPARγ in nonadipogenic fibroblasts promotes their conversion into fat-laden adipocyte-like cells after their exposure to a PPAR activator or ligand (18). As with other nuclear hormone receptors, the ability of PPARγ to function as a transcription factor is dependent on its binding to a ligand. Although the biological ligand for PPARγ has not yet been identified, recent studies have demonstrated that 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 (15d-J_2) and the thiazolidinediones can act as direct ligands (19–21). The thiazolidinediones that include ciglitazone, pioglitazone, and troglitazone among others are a synthetic class of insulin-sensitizing drugs that can lower blood glucose when given to diabetic animals (22–24).

The C/EBP family consists of several members, the most notable being α, β, and δ, which are expressed at specific times during adipogenesis in a manner that is consistent with a regulatory role for each protein during the differentiation process (25). In contrast to PPARγ, however, none of the C/EBPs is expressed in an exclusively tissue-restricted fashion. C/EBPα is expressed in high abundance in adipose tissue and liver (26). Its expression is induced during adipogenesis in culture after the induction of PPARγ, but preceding the synthesis of many of the enzymes and proteins characteristic of the fully differentiated adipogenic phenotype. In fact, C/EBPα may play a direct role in establishing and maintaining the terminally differentiated state of adipocytes, since constitutive expression of an antisense C/EBPα RNA in 3T3-L1 preadipocytes blocks the expression of several adipogenic genes, including GLUT4, and prevents accumulation of cytoplasmic fat droplets (27). The relatively late induction of C/EBPα during adipogenesis, however, suggests that it is not a master regulator of adipogenesis. Nevertheless, forced overexpression of C/EBPα in some fibroblastic cell lines can induce their conversion into fat-laden adipocytic cells (28). Recently, McKnight and coworkers suggested that C/EBPβ and C/EBPδ may play an early catalytic role in differentiation of 3T3-L1 preadipocytes, relaying the effects of dexamethasone (DEX) and methylisobutylxanthine (MIX), respectively, in a cascade-like fashion that ultimately leads to induction of C/EBPα (29). Our recent studies also demonstrate that conditional ectopic expression of C/EBPβ and C/EBPδ in NIH-3T3 fibroblasts (β/δ39 cell line) induces expression of PPARγ in the presence of glucocorticoids, and their subsequent exposure to a PPAR activator, 5, 8, 11, 14-eicosatetraynoic acid (ETYA), stimulates their conversion into fat-laden adipocytic cells (30).

Since GLUT4 is abundantly expressed in fully mature adipocytes, it is quite likely that PPARγ and/or the C/EBPs play a principal role in controlling its expression during the differentiation of preadipocytes into adipocytes. The circumstantial evidence mentioned previously, where the expression of an antisense C/EBPα RNA in preadipocytes blocked GLUT4 expression, suggests that C/EBPα is involved in regulating this gene (27). In addition, earlier studies on the GLUT4 gene promoter have identified a C/EBP binding site that may serve to facilitate C/EBP-dependent activation of the GLUT4 promoter/CAT gene construct in in vitro transient transfection assays (31). Other investigations have shown that the PPARγ ligand pioglitazone enhances expression of several adipogenic genes including GLUT4 during the differentiation of 3T3-L1 and 3T3-F442A preadipocytes into adipocytes (32, 33). Despite these observations, however, there is no direct evidence indicating whether any of the C/EBPs or PPARγ are responsible for inducing expression of GLUT4 during adipogenesis.

To define the molecular mechanisms that control GLUT4 gene expression during conversion of mesenchymal stem cells into adipocytes, NIH-3T3 fibroblasts ectopically expressing different combinations of the adipogenic transcription factors C/EBPβ, C/EBPδ, C/EBPα, and PPARγ using either a tetracycline-responsive inducible (C/EBPs) or a constitutive retroviral (PPARγ) expression system (18) were used. The cell lines were induced to differentiate, and expression of GLUT4 mRNA and protein were analyzed. The results demonstrate that conditional ectopic expression of C/EBPβ and C/EBPδ together in NIH-3T3 cells exposed to glucocorticoids induces expression of PPARγ. Subsequent treatment of these cells with the PPARγ ligand ciglitazone results in their extensive conversion into fat-laden adipocytes and induction of GLUT4 mRNA and protein, as well as an increase in insulin-sensitive glucose uptake. There appears to be no direct involvement of C/EBPα in inducing GLUT4 mRNA or protein expression in these cells.

Methods

Plasmids and cell lines. The tetracycline-responsive expression system that comprises two plasmids—pUHD15-1 (tTA) and pUHD10-3—was obtained from H. Bujard (Zentrum für Molekulare Biologie, University of Heidelberg, Germany; 34). To construct a tetracycline-dependent C/EBPα expression vector, the 3.5 kb of KpnI-Xba restriction fragment of the rat C/EBPα gene (35), which corresponds to 125 bp 5′-upstream from the AUG start codon to 1.0 kb of the 3′-downstream untranscribed region of the C/EBPα gene, was subcloned into the pUHD 10-3 vector. The resultant plasmid, Tet-O-C/EBPα, is driven by the CMV promoter containing the tet operator upstream of the C/EBPα gene in response to coexpression of tTA from the pUHD15-1 plasmid.

The stable NIH-3T3 cell lines—tA, β2, β/δ39, and δ23—were created as described previously (30, 36). The Pγ2 cell line that overexpresses PPAPγ2 by viral infection was kindly provided by Dr. Bruce Spiegleman (Dana Farber Institute, Harvard Medical School; 18). The NIH-3T3 α1 cell line was generated by transfecting Tet-O-C/EBPα expression vector along with a pBabe-puromycin plasmid into the tA cell line. Transfection was performed using lipofectAMINE following the procedure outlined by the supplier (GIBCO BRL, Gaithersburg, MD). The transfected cells were selected in DMEM containing 10% FBS (GIBCO BRL), 0.4 mg/ml G418 (GIBCO BRL), 1.5 μg/ml puromycin (Sigma Chemical Co, St. Louis, MO) and 1 μg/ml of tetracycline for 10–14 d. The resistant clones were subsequently isolated and propagated into cell lines.

Cell culture. 3T3-L1 preadipocytes were grown to confluence in DMEM with 10% normal calf serum (Intergen Co., Purchase, NY) and then induced to differentiate as described previously (37). The NIH-3T3 stable cell lines were passaged in DMEM with 10% FBS in the presence of tetracycline (1 μg/ml). Tetracycline was withdrawn from the medium to induce expression of the transfected genes. The cells were allowed to grow for an additional 48 h in the absence of tetracycline, during which time they reached confluence. At this stage, designated as day 0, differentiation was initiated by exposure of the cells to a cocktail containing 0.5 mM MIX, 1 μM DEX, 5 μg/ml insulin, and 10% FBS in DMEM for 48 h. The cells were then maintained in DMEM containing 10% FBS, 5 μg/ml insulin, and 10 μM ciglitazone (Upjohn Co., Kalamazoo, MI) unless otherwise indicated. Cells were refed every 2 d.

RNA analysis. Total RNA was extracted from the NIH-3T3 cell lines and the 3T3-L1 cells following the procedure of Chomczynski

and Sacchi (38). In brief, cells were lysed with guanidine thiocyanate and extracted with acidic phenol/chloroform, followed by isopropanol precipitation overnight at −20°C. Total RNA (20–25 μg) from each sample was subjected to Northern blot analysis as described previously (39). Probes were labeled by random priming using the Klenow fragment of DNA polymerase I (New England Biolabs Inc., Beverley, MA) and [α-^{32}P]dCTP (DuPont-NEN, Boston, MA). cDNAs used as probes were as follows: C/EBPα, C/EBPβ, and C/EBPδ (25); 422/aP2 (40, 41); PPAPγ (17); adipsin (42); glycerol phosphate dehydrogenase (GPD; 43); and GLUT4 and GLUT1 (glucose transporter 4 and 1; 7).

Western blot (immunoblot analysis). Total membrane proteins were isolated from NIH-3T3 β/δ39 and 3T3-L1 adipocytes (44, 45). In brief, cells were lysed in ice-cold buffer A (250 mM sucrose, 20 mM Hepes, 1 mM EDTA, and 1 mM PMSF). Cell lysates were placed in a homogenization vessel and immediately homogenized with a motor-driven Teflon pestle on ice. Total membrane proteins were recovered by centrifugation of the homogenates at 55,000 rpm in a 60Ti rotor for 90 min at 4°C. The total membrane pellets were dissolved in buffer B (20 mM Hepes, 1 mM EDTA, and 1 mM PMSF) and quantified using a BCA protein assay kit (Pierce, Rockford, IL). Total membrane proteins from β/δ39 cells (300 μg) and 3T3-L1 adipocytes (150 μg) were fractionated by 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH), which was then subjected to Western blot analysis as described previously (46). The primary antibody for GLUT4 protein was obtained from Dr. Paul Pilch at Boston University School of Medicine (46). The secondary antibody for GLUT4 was a goat anti–mouse polyvalent consisting of the immunoglobulins IgA, IgM, IgG (1:1,000 dilution; Sigma Chemical Co., St. Louis, MO).

2-Deoxy-D-glucose uptake. Glucose uptake was performed following the protocol described previously (47). In brief, cells were stimulated with 100 nM insulin at 37°C for 8 min before the assay. The glucose transport assay was then carried out in a cocktail containing Krebs-Ringer Hepes (KRH) buffer (pH 7.4) with 90 μM of D-glucose and 1 μCi/ml of [^{3}H]2-deoxyglucose (1 ml per 35-mm dish; DuPont-NEN, Boston, MA) for 3 min at room temperature. The assay was terminated by two washes with KRH buffer. The cells were then lysed by incubation in digitonin release buffer (0.25 M mannitol, 17 mM potassium Mops, 2.5 mM EDTA, and 8 mg/ml of digitonin, pH 7.4; 1 ml/well). An aliquot (0.3 ml) from each lysate was used to determine the amount of radioactivity by liquid scintillation counting. Nonspecific diffusion was determined in the presence of 5 μM cytochalasin B (Sigma Chemical Co.). Measurements were performed in triplicate and normalized by protein concentrations in the lysates. They were corrected by subtracting the nonspecific diffusion. The final results were presented as pmol/min/mg protein.

Oil Red O staining. Oil Red O staining was performed following the procedure described by Green and Kehinde (48) with minor modifications. In brief, cells were washed twice with PBS and fixed with 10% formalin in PBS for 15 min. After two washes in PBS, cells were stained for at least 1 h in freshly diluted Oil Red O solution (six parts Oil Red O stock solution and four parts H_2O; Oil Red O stock solution is 0.5% Oil Red O in isopropanol). The stain was then removed and the cells were washed twice with water, with or without counterstain (0.25% giemsa for 15 min) and then photographed.

Results

Temporal pattern of expression of adipogenic genes during the differentiation of 3T3-L1 preadipocytes. To determine the temporal pattern of expression of the adipogenic transcription factors and other adipocyte-specific genes relative to each other, we analyzed adipogenic gene expression during the conversion of 3T3-L1 fibroblasts into adipocytes by Northern blot hybridization. Fig. 1 shows that C/EBPβ and C/EBPδ are expressed in proliferating preadipocytes (*P*). Exposure of a confluent population of these cells to the adipogenic inducers (DEX, MIX, insulin, and FBS) results in a transient increase in both C/EBPβ and C/EBPδ within the first 48 h. The levels of these mRNAs decrease to ∼ 30–50% of the original prestimulatory amounts between 6 and 8 d. This peak of C/EBPβ and C/EBPδ mRNA expression precedes the induction of both PPARγ and C/EBPα which are activated at 2 d, reaching their maximum levels of expression by days 3 and 4, respectively. The downstream adipogenic genes are activated in a sequential manner after induction of both the C/EBPα and PPARγ transcription factors. The fatty acid–binding protein, aP2, is induced early in the differentiation process, immediately after PPARγ and C/EBPα expression. GPD is induced at day 3, the insulin-dependent glucose transporter (GLUT4) at day 4, and adipsin at day 5. The ubiquitous glucose transporter, GLUT1, is expressed in proliferating preadipocytes and is gradually downregulated during the differentiation process.

Ectopic expression of C/EBPs and PPARγ in NIH-3T3 fibroblasts induces the terminal phase of adipogenesis as indi-

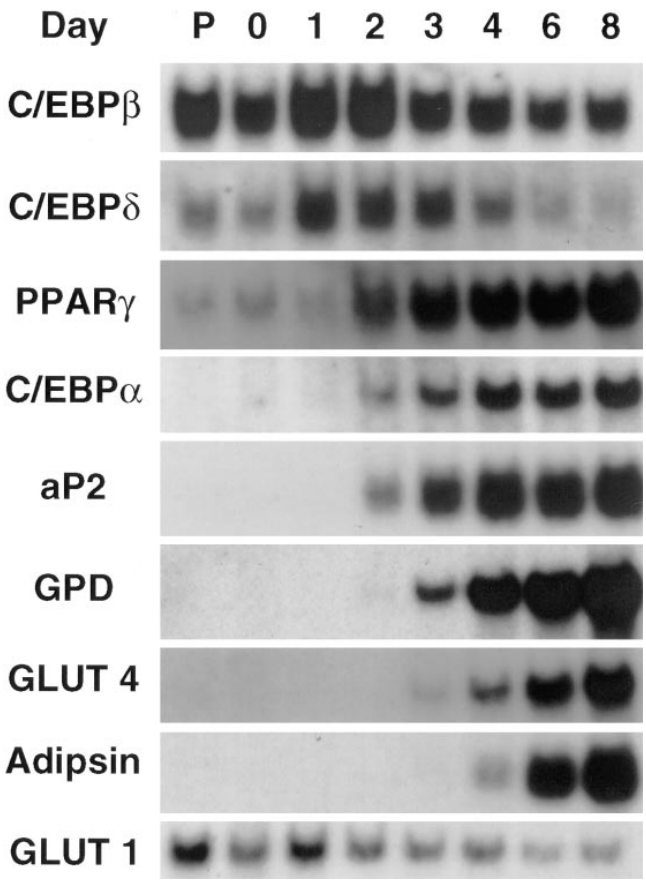

Figure 1. Sequential activation of gene expression during the differentiation of 3T3-L1 preadipocytes into adipocytes. Subconfluent 3T3-L1 preadipocytes were grown and passaged in DMEM with 10% calf serum. Confluent 3T3-L1 preadipocytes (day 0) were exposed to a differentiation cocktail containing 1 μM DEX, 0.5 mM MIX, 10 μg/ml insulin, and 10% FBS in DMEM for 48 h. The cells were then maintained in DMEM with 2.5 μg/ml insulin and 10% FBS and replenished with this medium every 2 d. Total RNA (20 μg) extracted at various times as indicated during the differentiation process was analyzed by Northern blot hybridization using the following ^{32}P-labeled cDNA probes: C/EBPβ, C/EBPδ, PPARγ, C/EBPα, aP2, GPD, GLUT4, adipsin, and GLUT1.

cated by expression of the insulin-responsive glucose transporter (GLUT4). As shown in Fig. 1, the C/EBPs and PPARγ are expressed before other adipogenic genes during differentiation of 3T3-L1 preadipocytes. It is conceivable, therefore, that these transcription factors are directly responsible for activating the adipocyte genes (i.e., GLUT4) that are indicative of the mature adipocyte phenotype. Previous investigations have shown that a number of adipose-specific gene promoters and/or enhancers [GLUT4 (31), aP2, SCD1 (steroyl CoA desaturase 1; 49), and PEPCK (phosphoenol pyruvate carboxykinase; 50–52)] contain binding sites for C/EBP and PPARs, and in some cases these factors are capable of transactivating the corresponding genes in in vitro assays. In addition, overexpression of each of these transcription factors—C/EBPβ, C/EBPα, or PPARγ—in nonadipogenic cells induces their conversion into fat-laden cells that express some of the markers of the terminally differentiated phenotype, i.e., aP2, adipsin, and GPD (18, 28, 29, 36, 53). Although these cells accumulate fat droplets and turn on part of the adipogenic gene program, it is not known whether they can function as mature adipocytes. In these studies, we chose to define the transcriptional events that induce expression of genes that correlate with the terminal stages of adipogenesis (as illustrated in Fig. 1) with an emphasis on the insulin-responsive glucose transporter GLUT4. To achieve this goal, we conditionally expressed C/EBPs in NIH-3T3 fibroblasts using the tetracycline-responsive system as previously described by us (36). The resulting stable cell lines, δ23, β2, β/δ39, and α1, can be induced to ectopically express C/EBPδ, C/EBPβ, C/EBPβ in combination with C/EBPδ, and C/EBPα, respectively. The stable cell line Pγ2, shown in Fig. 2, constitutively overexpresses PPARγ2 by retroviral infection (18). To determine which adipogenic genes are expressed in each of these cell lines, differentiation was induced by culturing the cells in tetracycline-free medium (when appropriate) followed by exposure of the cells to a cocktail of the adipogenic inducers DEX, MIX, insulin, FBS, and a PPAR ligand, ciglitazone. Northern blot analysis of total RNA extracted from the various cell lines at day 8 after differentiation is presented in Fig. 2. The tA cells that ectopically produce only the tetracycline activator protein from the pUHD15-1 plasmid express low levels of both C/EBPβ and C/EBPδ, equivalent to the levels observed in the parent NIH-3T3 cell line (data not shown). Furthermore, these control cells show no sign of adipocyte-specific gene expression. The δ23 cells respond to depletion of tetracycline from the culture medium by enhancing C/EBPδ mRNA expression, but they do not express the adipocyte-specific mRNAs. In contrast, induction of C/EBPβ in the β2 cells results in expression of PPARγ, GPD, aP2, and adipsin, as observed previously (36), and these β2 cells express GLUT4 mRNA. Ectopic expression of C/EBPβ and C/EBPδ together in the β/δ39 cells results in a similar pattern of adipogenic gene expression as the β2 cells, but to a significantly greater extent. Retroviral-mediated expression of PPARγ in NIH-3T3 cells has no effect on C/EBPβ or C/EBPδ mRNA synthesis; however, it is capable of transactivating the endogenous PPARγ gene and stimulating terminal differentiation of these cells as illustrated by the production of GPD, aP2, adipsin, and GLUT4 mRNAs. The level of GLUT4 expression is as high in this cell line as it is in the β/δ39 cells, even though expression of PPARγ mRNA, including both the exogenous and endogenous forms, is several times lower in the Pγ2 cells compared with its level in the β/δ39 cells.

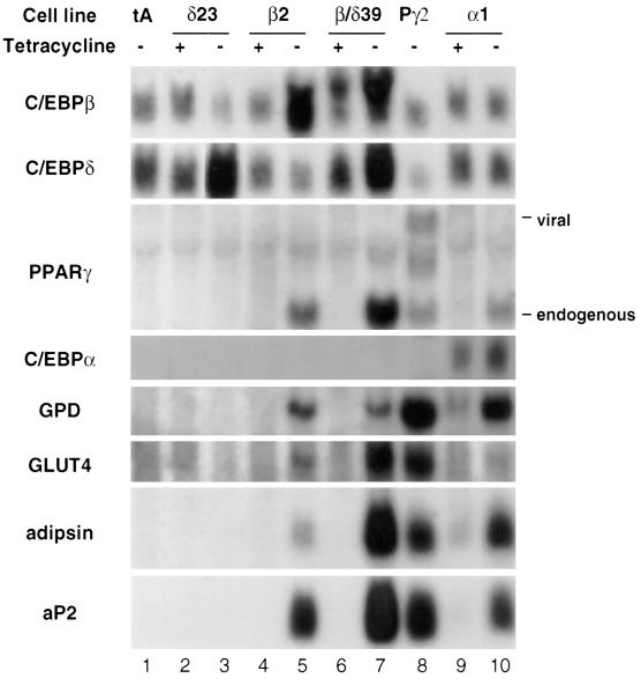

Figure 2. Induction of the adipogenic genes in various NIH-3T3 stable cell lines expressing C/EBPs or PPARγ. NIH-3T3 fibroblasts were cultured as described in Methods. 3 d before differentiation, half the cultures of the δ23, β2, β/δ39, and α1 were maintained in medium containing tetracycline, while the remaining cells were cultured in tetracycline-free medium. Upon reaching confluence, the cells were induced to differentiate by exposure to 1 μM DEX, 0.5 mM MIX, 5 μg/ml insulin, and 10% FBS in DMEM for 48 h. They were subsequently maintained in DMEM containing 5 μg/ml insulin and 10% FBS, as well as a synthetic PPARγ ligand, ciglitazone (10 μM), for an additional 6 d. Total RNA was extracted, and 25 μg of each RNA was subjected to Northern blot analysis. The following cDNAs were used as probes: C/EBPβ, C/EBPδ, PPARγ, C/EBPα, GPD, GLUT4, adipsin, and aP2. tA, tetracycline activator cell line; δ23, cell line expressing C/EBPδ; β2, cell line expressing C/EBPβ; β/δ39, cell line expressing both C/EBPβ and C/EBPδ; Pγ2, cell line expressing PPARγ2; α1, cell line expressing C/EBPα.

It is of interest that none of these cell lines (β2, β/δ39, and Pγ2) expresses C/EBPα mRNA, suggesting that one aspect of adipogenesis is not induced in NIH-3T3 cells at least in response to PPARγ, C/EBPβ, and/or C/EBPδ. To determine whether C/EBPα is also capable of stimulating terminal differentiation in NIH-3T3 cells (i.e., GLUT4 expression), we constructed an additional cell line that ectopically produces C/EBPα in response to culture in tetracycline-depleted medium. Unlike the other cell lines, (δ23, β2, and β/δ39), the α1 cells express a low level of C/EBPα mRNA, even in the presence of a dose of tetracycline (1 μg/ml) that completely suppresses transcription of the exogenous genes in the other engineered cells. Despite this low level of leakiness, the α1 cells respond to the withdrawal of tetracycline by inducing a level of PPARγ expression equivalent to that of the endogenous PPARγ mRNA observed in the Pγ2 cells. This event results in activation of GPD, adipsin, and aP2 mRNA expression, but is only modestly capable of enhancing GLUT4 expression. It appears, therefore, that the level of GLUT4 expression correlates with the total

amount of PPARγ mRNA (endogenous and retroviral in the case of the Pγ2 cells) expressed in each cell line, suggesting that PPARγ plays an important role in activating the GLUT4 gene. To further dissect the role of PPARγ in regulating GLUT4, we chose to study the β/δ39 cells since the level of PPARγ and GLUT4 expression in these cells is relatively high, and the adipogenic gene program can be readily switched on and off by the presence or absence of tetracycline in the culture medium.

Sequential expression of the adipogenic genes during the conversion of the β/δ39 cells into adipocytes. As observed in Fig. 1, there is a sequential activation of gene expression during 3T3-L1 preadipocyte differentiation. To determine whether the pattern of gene expression in the β/δ39 cells resembles that of the 3T3-L1 preadipocytes during normal adipogenesis, we analyzed the expression of select adipocyte mRNAs in these engineered cells after stimulating them to differentiate (Fig. 3 *A*). Proliferating β/δ39 cells were switched to tetracycline-free medium for 48 h, during which time they reached confluence and expressed maximum amounts of C/EBPβ and C/EBPδ mRNAs. At confluence (day 0) when they express C/EBPβ and C/EBPδ but negligible amounts of PPARγ and the other adipogenic mRNAs, they were exposed to the differentiation cocktail containing DEX, MIX, and insulin for 48 h. During this period the only gene that was induced was PPARγ. After the initial 2-d exposure to these inducers, the cells were switched to maintenance medium containing FBS, insulin, and the PPARγ ligand ciglitazone, which resulted in sequential activation of the adipogenic mRNAs. aP2 and adipsin mRNAs reached maximum levels of expression by day 4 of the differentiation process, whereas GPD and GLUT4 continued to accumulate and finally reached a plateau between 8 and 10 d. As observed in Fig. 2, C/EBPα mRNA was not activated at any time during the conversion of the β/δ39 cells into adipocytes. The presence of tetracycline in the differentiation medium for the entire 10-d period completely inhibited expression of the adipocyte mRNAs. To assess the extent of conversion of the β/δ39 fibroblasts into fat-laden adipocytes, cells were induced to differentiate for 8 d, and were then stained with Oil Red O. Approximately 100% of the cells contained fat droplets when

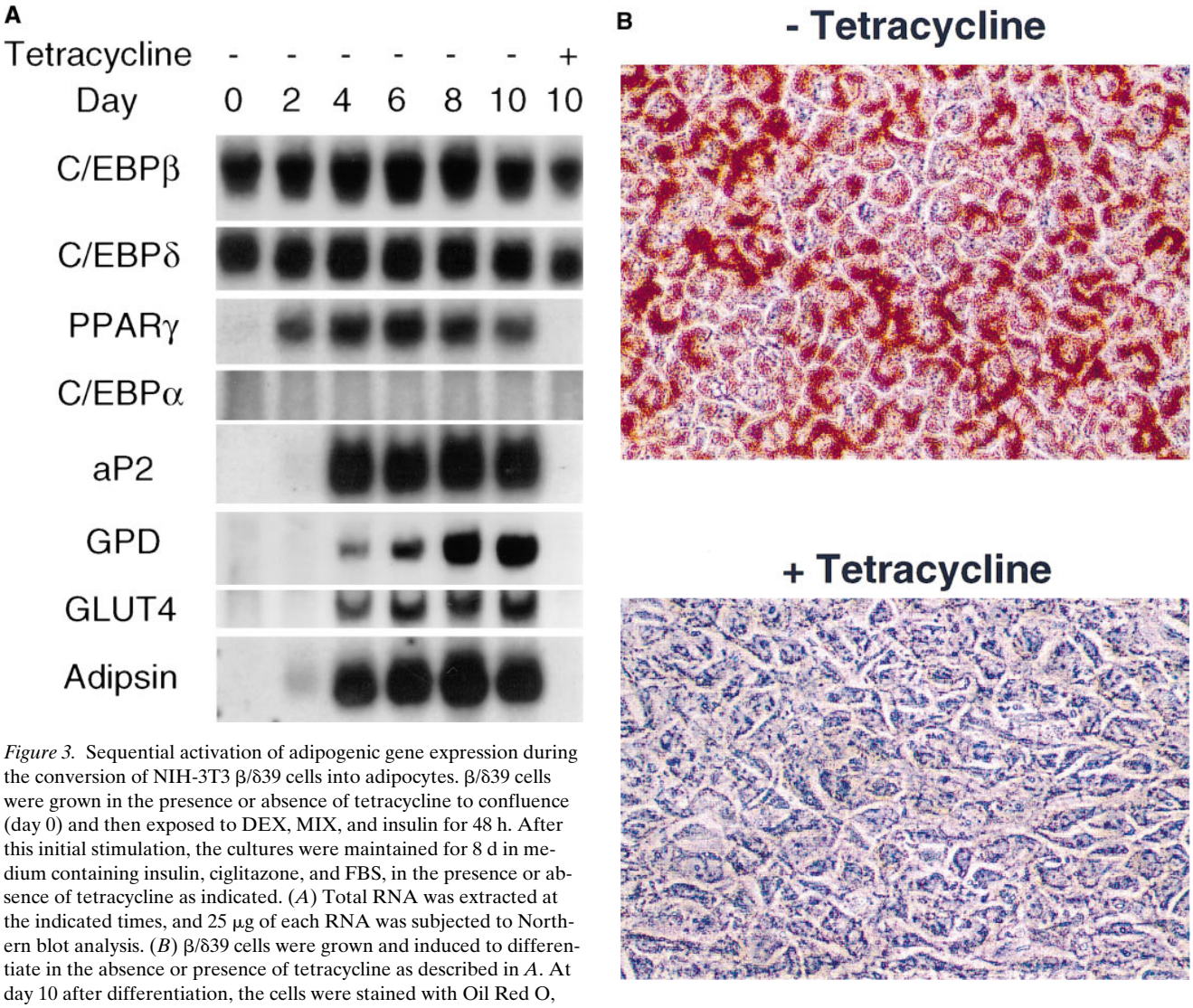

Figure 3. Sequential activation of adipogenic gene expression during the conversion of NIH-3T3 β/δ39 cells into adipocytes. β/δ39 cells were grown in the presence or absence of tetracycline to confluence (day 0) and then exposed to DEX, MIX, and insulin for 48 h. After this initial stimulation, the cultures were maintained for 8 d in medium containing insulin, ciglitazone, and FBS, in the presence or absence of tetracycline as indicated. (*A*) Total RNA was extracted at the indicated times, and 25 μg of each RNA was subjected to Northern blot analysis. (*B*) β/δ39 cells were grown and induced to differentiate in the absence or presence of tetracycline as described in *A*. At day 10 after differentiation, the cells were stained with Oil Red O, and then counterstained with giemsa before being photographed.

cultured in the absence of tetracycline, whereas cells exposed to the antibiotic for the entire differentiation period exhibited a typical 3T3 fibroblast morphology (Fig. 3 *B*). One of the similarities of the sequential gene activation during differentiation of both 3T3-L1 and the β/δ39 cells is the activation of PPARγ before the induction of other adipogenic genes.

Induction of GLUT4 gene expression in the β/δ39 cells requires exposure to the adipogenic inducers that also stimulate PPARγ production. To identify the effectors responsible for activating GLUT4 expression and to correlate this process with the expression of PPARγ, we cultured the β/δ39 cells in tetracycline-free medium, thereby enhancing C/EBPβ and C/EBPδ expression, and then exposed them to various combinations of insulin, MIX, and DEX in the presence of the PPARγ ligand ciglitazone. The Northern blot in Fig. 4 shows that culture of these cells in medium containing only insulin activates PPARγ to a minor extent, and is incapable of inducing GLUT4 expression (lane *1*). Exposure of the cells to MIX in the absence or presence of insulin (lanes *2* and *4*) stimulates PPARγ expression somewhat further than insulin, but only modestly enhances GLUT4 expression. As observed previously (30), the most potent inducer of PPARγ mRNA production is DEX, which also activates GLUT4 as well as aP2 and adipsin mRNAs, whether the cells are cultured in DEX alone (lane *3*) or DEX in combination with the other effectors (lanes *5*, *6*, and *7*). The level of expression of these adipogenic genes (PPARγ, GLUT4, aP2, and adipsin) in these fibroblasts (β/δ39 cells) is

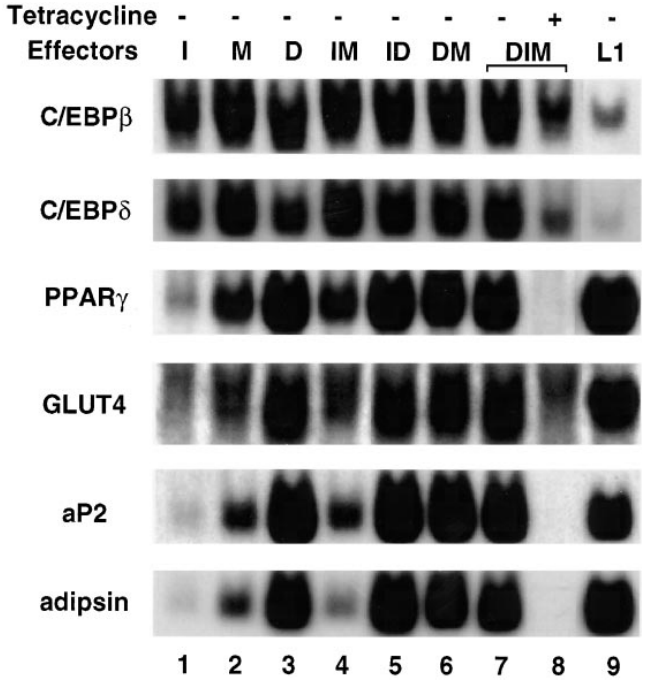

Figure 4. Effects of the adipogenic inducers on activation of PPARγ and GLUT4 mRNA in the β/δ39 cells. β/δ39 cells were grown to confluence in the absence or presence of tetracycline. They were then exposed to various combinations of the effectors DEX, MIX, and insulin for 48 h. They were subsequently maintained in medium containing FBS, insulin, and ciglitazone for an additional 4 d. Tetracycline (1 μg/ml) was present in the control culture as indicated. Total RNA was extracted at day 6 from each culture, and 20 μg of each was subjected to Northern blot analysis. *I*, insulin (5 μg/ml); *D*, DEX (1 μM); *M*, MIX (0.5 mM). L1, total RNA isolated from 3T3-L1 adipocytes cultured for 8 d as described in Fig. 1.

almost equivalent to that expressed in fully differentiated 3T3-L1 adipocytes (lane *9*). The β/δ39 cells maintained in tetracycline throughout the culture period exhibit normal fibroblast levels of C/EBPβ and C/EBPδ, and do not express either PPARγ or GLUT4 mRNAs, even though they have been exposed to DEX, MIX, and insulin (lane *8*).

Induction of GLUT4 expression is dependent on exposure of β/δ39 cells to a PPARγ ligand. In order for PPARγ to act as a transcription factor it needs to associate with an appropriate ligand such as the thiazolidine ciglitazone, or the prostaglandin PGJ2. Thus, to assess the role of PPARγ in regulating GLUT 4 gene expression, we analyzed the effect of PPAR ligands on GLUT4 mRNA production during the conversion of β/δ39 cells into adipocytes. In the experiment shown in Fig. 5, β/δ39 cells were primed to differentiate by culture in tetracycline-free medium containing DEX, MIX, insulin, and 10% FBS for 48 h, which activated PPARγ expression. The cells were then exposed to fresh medium containing insulin, 10% FBS, and the PPARγ ligands ciglitazone or PGJ2, as well as ETYA (an activator of PPAR) for an additional 4 d, at which time total RNA was isolated and subjected to Northern blot analysis. Fig. 5 *A* shows that the adipogenic inducers DEX, MIX, insulin, and FBS are potent inducers of PPARγ, aP2, and adipsin mRNAs, but they are incapable of activating GLUT4 mRNA production in the β/δ39 cells (lane *1*). In contrast, exposure of these cells to ciglitazone, ETYA, or PGJ2 after stimulation with the adipogenic inducers results in activation of GLUT4 mRNA expression to varying extents (lanes *2–4*). Fig. 5 *B* shows that the dose of ciglitazone required to induce GLUT4 mRNA expression in the presence of elevated levels of C/EBPβ, C/EBPδ, and PPARγ is 10 μM. As seen in Fig. 5 *A*, production of aP2 mRNA occurs in the absence of ciglitazone, although its level of expression is enhanced severalfold with increasing concentrations of the thiazolidinedione. To determine the kinetics of induction of GLUT4 gene expression, β/δ39 cells were treated with FBS, DEX, MIX, and insulin for 48 h, followed by maintenance in medium containing FBS and insulin for an additional 2 d to allow PPARγ expression to be fully induced ("primed cells"; Fig. 5 *C*, lane *1*). The abundance of GLUT4 mRNA was measured by Northern blot analysis at different times after exposure of these cells to 10 μM of ciglitazone. Fig. 5 *C* shows that expression of GLUT4 mRNA is not detectable until 12–24 h after addition of ciglitazone. Furthermore, it takes 48 h for its expression to reach a maximum level. This is in contrast to the rapid activation of aP2 mRNA production, which occurs during the initial 4 h of drug treatment.

The ciglitazone-dependent induction of GLUT4 mRNA expression in the β/δ39 cells is accompanied by synthesis of GLUT4 protein and an increase in glucose uptake in response to insulin. One of the major functions of fully mature adipocytes is to increase glucose uptake in response to elevated levels of insulin, which is facilitated by a transport system that involves GLUT4 protein. The results shown above demonstrate that enhanced expression of an activated form of PPARγ induces the expression of abundant amounts of GLUT4 mRNA in NIH-3T3 fibroblasts. Previous investigations have shown, however, that overexpression of GLUT4 mRNA in nonadipogenic cells that have insulin receptors and normal intracellular organelles does not recapitulate the insulin-regulatable glucose transport system (54–56). To assess the extent to which the β/δ39 cells express the mature adipogenic phenotype, we

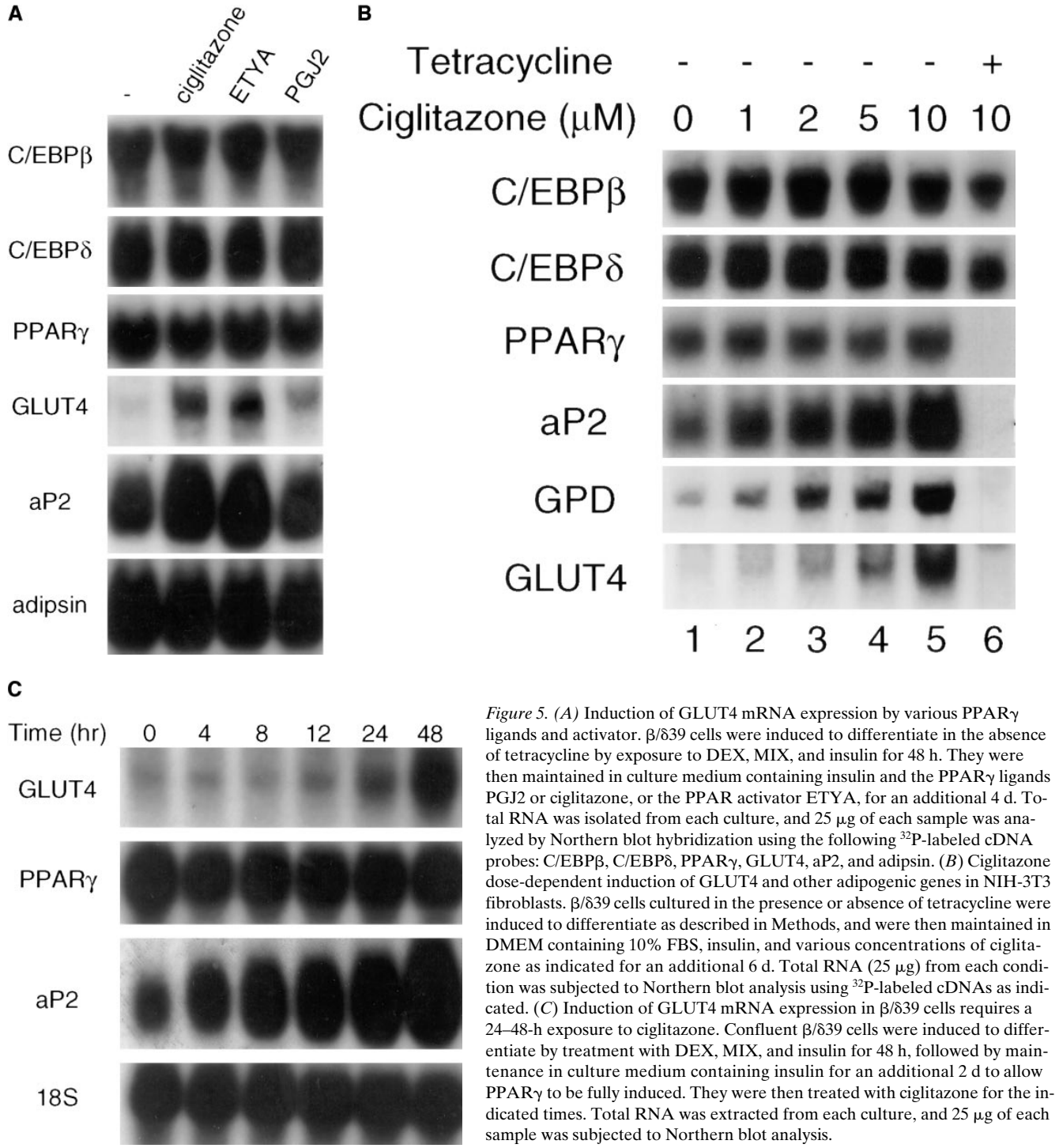

Figure 5. (A) Induction of GLUT4 mRNA expression by various PPARγ ligands and activator. β/δ39 cells were induced to differentiate in the absence of tetracycline by exposure to DEX, MIX, and insulin for 48 h. They were then maintained in culture medium containing insulin and the PPARγ ligands PGJ2 or ciglitazone, or the PPAR activator ETYA, for an additional 4 d. Total RNA was isolated from each culture, and 25 μg of each sample was analyzed by Northern blot hybridization using the following ^{32}P-labeled cDNA probes: C/EBPβ, C/EBPδ, PPARγ, GLUT4, aP2, and adipsin. (*B*) Ciglitazone dose-dependent induction of GLUT4 and other adipogenic genes in NIH-3T3 fibroblasts. β/δ39 cells cultured in the presence or absence of tetracycline were induced to differentiate as described in Methods, and were then maintained in DMEM containing 10% FBS, insulin, and various concentrations of ciglitazone as indicated for an additional 6 d. Total RNA (25 μg) from each condition was subjected to Northern blot analysis using ^{32}P-labeled cDNAs as indicated. (*C*) Induction of GLUT4 mRNA expression in β/δ39 cells requires a 24–48-h exposure to ciglitazone. Confluent β/δ39 cells were induced to differentiate by treatment with DEX, MIX, and insulin for 48 h, followed by maintenance in culture medium containing insulin for an additional 2 d to allow PPARγ to be fully induced. They were then treated with ciglitazone for the indicated times. Total RNA was extracted from each culture, and 25 μg of each sample was subjected to Northern blot analysis.

analyzed whether expression of GLUT4 mRNA in these fibroblasts gives rise to the corresponding protein, and whether induction of PPARγ can also turn on the insulin-responsive glucose transporter system. In the experiment shown in Fig. 6 and Table I, the β/δ39 cells were cultured in the presence of different extracellular inducers, resulting in varying levels of PPARγ and GLUT4 mRNA expression. The Western blot analysis shown in Fig. 6 demonstrates that abundant quantities of GLUT4 protein are synthesized only in the β/δ cells that also express an activated form of PPARγ. In other words, these cells are cultured in tetracycline-free medium to enhance C/EBPβ and C/EBPδ, and are then exposed to DEX and ciglitazone, which effectively induces PPARγ and ensures the presence of a potent ligand. In fact, if cells are cultured in medium either containing tetracycline or in the absence of ciglitazone where differentiation either does not occur or progresses poorly, the amount of GLUT4 protein is negligible. The data showing the results of the glucose uptake studies are presented in Table I. In 3T3-L1 adipocytes, glucose uptake is increased about fivefold in response to insulin stimulation. In the β/δ39 cells, glu-

Table I. Insulin-dependent Glucose Uptake
2-Deoxyglucose Uptake (pmol/mg/min)

Cell line	β/δ39			L1
Tetracycline	−	+	−	−
Ciglitazone	+	−	−	−
+ Insulin	726±53	593±41	582±31	735±67
− Insulin	353±27	607±36	539±23	145±25
Fold	2.1	1.0	1.1	5.1

β/δ39 cells were grown and induced to differentiate under the conditions as described in Fig. 6. Cells were subjected to [^{3}H]2-deoxyglucose uptake assay as described in Methods at day 8 after the initiation of differentiation. 3T3-L1 adipocytes were included in the assay as a control.

cose uptake is increased about twofold under optimal differentiation conditions. In the control cells, however, where the cells are cultured either in the presence of tetracycline or in the absence of ciglitazone, not only is GLUT4 protein minimally expressed, correlating with the expression of the mRNA, but the cells are not responsive to stimulation with insulin measured by glucose uptake. These data demonstrate that PPARγ activates the fully differentiated adipogenic program that includes both the induction of GLUT4 gene expression and insulin-responsive glucose uptake.

Discussion

Adipocytes are highly specialized cells that play a major role in energy homeostasis in the organism. Their primary function is to synthesize and store triglycerides at times of caloric excess and to mobilize these stores when caloric intake is low. Insulin plays an important role in regulating lipid metabolism in adipose tissue by stimulating glucose uptake and lipogenesis, as well as inhibiting lipolysis. To facilitate the response to insulin, adipocytes express a host of proteins and enzymes that function in many different metabolic processes to maintain the balance of energy. Transcription of several of the genes that code for these proteins is activated during the differentiation of preadipocytes into adipocytes (57). The present study provides evidence demonstrating that induction of the insulin-dependent glucose transport system during conversion of mesenchymal stem cells into adipocytes is regulated in part by PPARγ. Specifically, the data show that ectopic expression of C/EBPβ and C/EBPδ in NIH-3T3 cells exposed to glucocorticoids induces PPARγ expression, and in the presence of a PPARγ ligand (ciglitazone) these cells are stimulated to convert into fat-laden adipocytes that express both GLUT4 mRNA and protein (Figs. 3 and 6). In addition, expression of a ligand-activated form of PPARγ in NIH-3T3 fibroblasts also activates the insulin-responsive glucose transport system since exposure of the β/δ39 adipocytes to insulin stimulates 2-deoxyglucose uptake (Table I). Activation of the adipogenic program in these fibroblasts occurs in the absence of C/EBPα. We can conclude, therefore, that the induction of PPARγ in nonadipogenic fibroblasts exposed to a PPARγ ligand is sufficient to promote their differentiation into mature adipocytes that express the insulin-dependent glucose transporter GLUT4. Based on the results acquired in this investigation and our previous studies (30, 36), we outline a potential network of regulatory pathways involving PPARγ and C/EBPs that regulate adipogenesis (Fig. 7).

Although the molecular mechanisms that regulate expression of the GLUT4 gene during adipogenesis are unknown,

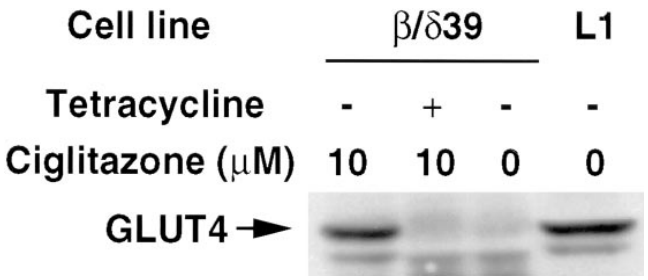

Figure 6. Induction of GLUT4 protein during adipogenic conversion of the β/δ39 cells. β/δ39 cells were grown to confluence and then induced to differentiate by exposure to DEX, MIX, and insulin for 48 h in the presence or absence of tetracycline. After initiation of differentiation, the cells were maintained in DMEM and 10% FBS in the presence or absence of tetracycline, with or without ciglitazone as indicated for an additional 6 d. Total membrane protein was isolated from each culture at day 8, and 300 μg of each was subjected to Western blot analysis using anti-GLUT4 and anti–mouse polyvalent immunoglobulins (IgA, IgM, IgG) as primary and secondary antibodies, respectively. Total membrane protein (150 μg) from 3T3-L1 adipocytes at day 10 of differentiation was included as a positive control.

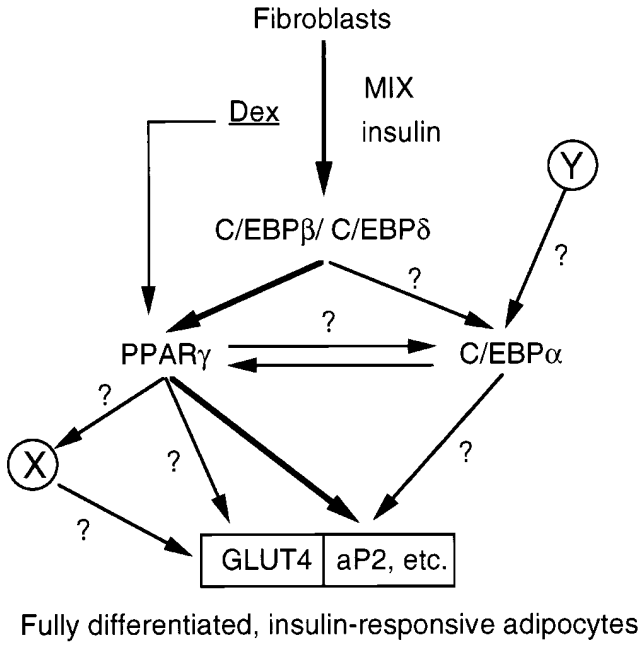

Figure 7. Schematic model of the pathways controlling differentiation of fibroblasts into adipocytes. Overexpression of C/EBPβ and C/EBPδ in NIH 3T3 fibroblasts activates PPARγ expression in the presence of adipogenic inducers DEX or MIX and insulin. PPARγ induces the expression of aP2, GLUT4, and other adipocyte differentiation-dependent genes, and converts these fibroblasts into fully differentiated insulin-dependent adipocytes. Expression of GLUT4 in these fibroblasts is activated by PPARγ, which likely involves other unknown factor(s) (*X*). C/EBPα, however, is absent in these cells despite the fact that C/EBPβ and C/EBPδ as well as PPARγ are expressed. This fact suggests that regulation of C/EBPα requires additional unknown factor(s) (*Y*).

the observations presented in this study allow us to speculate on the role of at least two families of transcription factors: the C/EBPs and the PPARs. These data strongly suggest that PPARγ plays a significant role in activating GLUT4 transcription during the terminal stages of adipogenesis in NIH-3T3 cells. In addition, induction of GLUT4 expression by PPARγ requires 24–48 h of exposure to ciglitazone, and appears to be dependent on protein synthesis (data not shown). These data suggest that PPARγ activates the expression of additional transcription factors (*X*, see Fig. 7) that may cooperate with PPARγ to initiate GLUT4 transcription. The genes encoding aP2 and PEPCK have a similar temporal pattern of expression during adipogenesis as the GLUT4 gene; the promoters for these genes have been shown to contain PPAR binding sites that can facilitate their respective activation by PPARγ in in vitro transfection assays (17, 52). Thus, it is also possible that there is a direct interaction between PPARγ and the corresponding nuclear hormone response elements in the promoter/enhancer of the GLUT4 gene. This possibility needs to be further investigated in the future when more sequences of the GLUT4 gene become available.

The role of the C/EBPs in activating GLUT4 gene expression is unclear. A previous study identified a C/EBP binding site in the promoter of the mouse GLUT4 gene (31). The data presented in our study, however, demonstrate that overexpression of C/EBPβ and C/EBPδ are not sufficient to induce GLUT4 transcription (Fig. 3 *A*, lane *1*; and Fig. 4, lane *1*). It appears that the major role of C/EBPβ and C/EBPδ along with glucocorticoids is to activate PPARγ expression, which is responsible for the induction of GLUT4 transcription. In addition, C/EBPα is not required for activation of GLUT4 and the other adipocyte genes since it is absent in the fully differentiated cell lines (including β2, β/δ39, and Pγ2) that express GLUT4 as well as other major markers of the mature adipocyte phenotype. It is possible, however, that the artificially high level of C/EBPβ in some NIH-3T3 cell lines (β2 and β/δ39) may be capable of substituting for C/EBPα by synergizing with PPARγ to activate GLUT4 expression during the terminal stages of the differentiation process.

The absence of C/EBPα in the differentiated NIH-3T3 cell lines (β2, β/δ39, and Pγ2) suggests that PPARγ alone or in combination with C/EBPβ and C/EBPδ is not sufficient to activate C/EBPα expression in nonadipogenic fibroblasts. One possibility is that the C/EBPα gene is maintained in a repressed state by suppressor proteins, which may need to be inactivated before the required transcription factors can induce its expression.

GLUT4 plays an important role in maintaining glucose homeostasis in mammals. Down regulation of its expression in adipocytes is a universal feature of insulin-resistant states. A number of transgenic studies show that overexpression of GLUT4 in either muscle (58, 59) or adipose tissue (60) alone or together (61, 62) enhances glucose tolerance and insulin-dependent glucose uptake in vivo. Specifically, elevating the level of GLUT4 expression in these tissues prevents insulin resistance in normal mice (62) and ameliorates diabetes in mice with metabolic pertubations (58, 60). The molecular mechanisms regulating this important transporter, however, are essentially unknown. The results presented here shed light on the transcriptional regulation of GLUT4 during adipocyte differentiation.

As mentioned at the outset, the thiazolidinediones (TZDs) were originally developed as synthetic drugs that are capable of sensitizing individuals who suffer from non-insulin-dependent diabetes mellitus to insulin and effectively lower blood glucose (63). More recently, investigations have demonstrated that TZDs are synthetic PPARγ ligands and as such are capable of promoting the conversion of some PPARγ-expressing cell lines into adipocytic cells (19–21). It is not known, however, whether the PPARγ ligand activity of the TZDs is involved in processes that lower blood glucose in animals with diabetes. The results presented here not only demonstrate that PPARγ induces GLUT4 expression, they also suggest a mechanism by which the TZDs increase the sensitivity of adipose tissue to insulin. Such a mechanism may include induction of genes that encode other proteins involved in insulin-dependent glucose uptake, since overexpression of the GLUT4 protein in nonadipogenic cells does not recapitulate the insulin-regulatable glucose transport system (54–56). The decrease in blood glucose levels in response to elevated levels of insulin is facilitated by muscle and liver as well as adipose tissue. The mechanisms by which TZDs improve insulin sensitivity in these tissues are also unknown. Studies have shown that pioglitazone can increase insulin sensitivity by activating insulin receptor kinase activity in muscles of obese or high fat–fed rats (64, 65). This TZD was also demonstrated to increase P-I-3-kinase activity upon insulin stimulation in cultured myotubes (66). In addition, it was found to increase insulin sensitivity in diabetic rat liver by regulating the transcription of two key enzymes—glucokinase and PEPCK—in gluconeogenic/glycolytic pathways (23). One possibility is that TZDs function in all insulin-responsive tissues by activating PPARγ, which induces the appropriate genes involved in glucose metabolism. In the case of liver, TZDs may activate PPARγ1, the hepatic isoform of PPARγ, and in doing so may regulate gluconeogenesis/glycolysis. In muscle tissue, PPARγ is expressed at very low levels, but it is possible that its level of expression may be enhanced by TZDs via an autoregulatory mechanism. In this regard, it will be important to determine whether expression of the GLUT4 gene in muscle tissue is similarly regulated by PPARγ. It is also possible that the action of TZDs through PPARγ in adipose tissue might result in production and secretion of molecules that in turn regulate the insulin-signaling pathway in liver and muscle. Further analysis of other targets of PPARγ and their possible involvement in insulin signaling may provide important information to help combat obesity and its related disorders.

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References

1. Pessin, J.E., and G.I. Bell. 1992. Mammalian facilitative glucose transporter family: structure and molecular regulation. *Annu. Rev. Physiol.* 54:911–930.

2. McGowan, K.M., S.D. Long, and P.H. Pekala. 1995. Glucose transporter gene expression: regulation of transcription and mRNA stability. *Pharmacol. Ther.* 66:465–505.

3. Birnbaum, M.J. 1989. Identification of a novel gene encoding an insulin-

responsive glucose transporter protein. *Cell*. 57:305–315.

4. Charron, M.J., F.C.I. Brosius, S.L. Alper, and H.F. Lodish. 1989. A glucose transporter protein expressed predominantly in insulin-responsive tissues. *Proc. Natl. Acad. Sci. USA*. 86:2535–2539.

5. James, D.E., M. Strube, and M. Mueckler. 1989. Molecular cloning and characterization of an insulin-regulatable glucose transporter. *Nature*. 338:83–87.

6. Fukumoto, H., T. Kayano, J.B. Buse, Y. Edwards, P.F. Pilch, G.I. Bell, and S. Seino. 1989. Cloning and characterization of the major insulin-responsive glucose transporter expressed in human skeletal muscle and other insulin-responsive tissues. *J. Biol. Chem.* 264:7776–7779.

7. Kaestner, K.H., R.J. Christy, J.C. McLenithan, L.T. Braiterman, P. Cornelius, P.H. Pekala, and M.D. Lane. 1989. Sequence, tissue distribution, and differential expression of mRNA for a putative insulin-responsive glucose transporter in mouse 3T3-L1 adipocytes. *Proc. Natl. Acad. Sci. USA*. 86:3150–3154.

8. Santalucia, T., M. Camps, A. Castello, P. Munoz, A. Nuel, X. Testar, M. Palacin, and A. Zorazno. 1992. Developmental regulation of GLUT-1 (erythroid/HepG2) and GLUT-4 (muscle/fat) glucose transporter expression in rat heart, skeletal muscle, and brown adipose tissue. *Endocrinology*. 130:837–846.

9. Garcia de Herreros, A., and M.J. Birnbaum. 1989. The acquisition of increased insulin-responsive hexose transport in 3T3-L1 adipocytes correlates with expression of a novel transporter gene. *J. Biol. Chem.* 264:19994–19999.

10. Richardson, J.M., and J.E. Pessin. 1993. Identification of a skeletal muscle-specific regulatory domain in the rat GLUT 4/muscle-fat gene. *J. Biol. Chem.* 268:21021–21027.

11. Liu, M.L., A.L. Olson, N.P. Edgington, W.S. Moye-Rowley, and J.E. Pessin. 1994. Myocyte enhancer factor 2 (MEF2) binding site is essential for C2C12 myotube-specific expression of the rat GLUT4/muscle-adipose facilitative glucose transporter gene. *J. Biol. Chem.* 269:28514–28521.

12. Evans, R.M. 1988. The steroid and thyroid hormone receptor superfamily. *Science*. 240:889–895.

13. Giguere, V. 1994. Retinoic acid receptors and cellular retinoid binding proteins: complex interplay in retinoid signaling. *Endocr. Rev*. 15:61–79.

14. Kliewer, S.A., B.M. Forman, B. Blumberg, E.S. Ong, U. Borgmeyer, D.J. Mangelsdorf, K. Umesono, and R.M. Evans. 1994. Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc. Natl. Acad. Sci. USA*. 91:7355–7359.

15. Keller, H., C. Dreyer, J. Medin, A. Mahfoudi, K. Ozato, and W. Wahli. 1993. Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid × receptor heterodimers. *Proc. Natl. Acad. Sci. USA*. 90:2160–2164.

16. Brun, R.P., P. Tontonoz, B.M. Forman, E. Ramsey, J. Chen, R.M. Evans, and B.M. Spiegelman. 1996. Differential activation of adipogenesis by multiple PPAR isoforms. *Genes Dev*. 10:974–984.

17. Tontonoz, P., E. Hu, R.A. Graves, A.I. Budavari, and B.M. Spiegelman. 1994a. mPPARγ: tissue-specific regulator of an adipocyte enhancer. *Genes Dev*. 8:1224–1234.

18. Tontonoz, P., E. Hu, and B.M. Spiegelman. 1994b. Stimulation of adipogenesis in fibroblasts by PPARγ, a lipid-activated transcription factor. *Cell*. 79: 1147–1156.

19. Lehmann, J.M., L.B. Moore, T.A. Smith-Oliver, W.O. Wilkison, T.M. Willson, and S.A. Kliewer. 1995. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). *J. Biol. Chem.* 270:12953–12956.

20. Forman, B.M., P. Tontonoz, J. Chen, R.P. Brun, B.M. Spiegelman, and R.M. Evans. 1995. 15-deoxy-$\Delta^{12,14}$ -prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. *Cell*. 83:803–812.

21. Kliewer, S.A., J.M. Lenhard, T.M. Wilson, I. Patel, D.C. Morris, and J.M. Lehmann. 1995. A prostaglandin J_2 metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. *Cell*. 83:813–819.

22. Chaiken, R.L., M. Eckert-Norton, R. Pasmantier, G. Boden, I. Ryan, R.A. Gelfand, and H.E. Lebovitz. 1995. Metabolic effects of darglitazone, an insulin sensitizer, in NIDDM. *Diabetologia*. 38:1307–1312.

23. Hofmann, C., K. Lorenz, D. Williams, B.J. Palazuk, and J.R. Colca. 1995. Insulin sensitization in diabetic rat liver by an antihyperglycemic agent. *Metab. Clin. Exp*. 44:384–389.

24. Young, P.W., M.A. Cawthorne, P.J. Coyle, J.C. Holder, G.D. Holman, I.J. Kozka, D.M. Kirkham, C.A. Lister, and S.A. Smith. 1995. Repeat treatment of obese mice with BRL 49653, a new potent insulin sensitizer, enhances insulin action in white adipocytes. Association with increased insulin binding and cell-surface GLUT4 as measured by photoaffinity labeling. *Diabetes*. 44:1087–1092.

25. Cao, Z., R.M. Umek, and S.L. McKnight. 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev*. 5: 1538–1552.

26. Birkenmeier, E.H., B. Gwynn, S. Howard, J. Jerry, J.I. Gordon, W.H. Landschulz, and S.L. McKnight. 1989. Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. *Genes Dev*. 3:1146–1156.

27. Lin, F.-T., and M.D. Lane. 1992. Antisense CCAAT/enhancer-binding protein RNA suppresses coordinate gene expression and triglyceride accumulation during differentiation of 3T3-L1 preadipocytes. *Genes Dev*. 6:533–544.

28. Freytag, S.O., D.L. Paielli, and J.D. Gilbert. 1994. Ectopic expression of the CCAAT/enhancer-binding protein α promotes the adipogenic program in a variety of mouse fibroblastic cells. *Genes Dev*. 8:1654–1663.

29. Yeh, W.-C., Z. Cao, M. Classon, and S.L. McKnight. 1995. Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. *Genes Dev*. 9:168–181.

30. Wu, Z., N.L.R. Bucher, and S.R. Farmer. 1996. Induction of peroxisome proliferator-activated receptor γ during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPβ, C/EBPδ, and glucocorticoids. *Mol. Cell. Biol.* 16:4128–4136.

31. Kaestner, K.H., R.J. Christy, and M.D. Lane. 1990. Mouse insulin-responsive glucose transporter gene: Characterization of the gene and transactivation by the CCAAT/enhancer binding protein. *Proc. Natl. Acad. Sci. USA*. 87:251–255.

32. Kletzien, R.F., S.D. Clarke, and R.G. Ulrich. 1992. Enhancement of adipocyte differentiation by an insulin-sensitizing agent. *Mol. Pharmacol.* 41: 393–398.

33. Sandouk, T., D. Reda, and C. Hofmann. 1993. Antidiabetic agent pioglitazone enhances adipocyte differentiation of 3T3-F442A cells. *Am. J. Physiol.* 264:C1600–C1608.

34. Gossen, M., and H. Bujard. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA*. 89:5547–5551.

35. Rana, B., Y. Xie, N.L.R. Bucher, and S.R. Farmer. 1995. The DNA binding activity of C/EBP transcription factors is regulated in G1 phase of the hepatocyte cell cycle. *J. Biol. Chem.* 270:18123–18132.

36. Wu, Z., Y. Xie, N.L.R. Bucher, and S.R. Farmer. 1995. Conditional ectopic expression of C/EBPβ in NIH-3T3 cells induces PPARγ and stimulates adipogenesis. *Genes Dev*. 9:2350–2363.

37. Student, A.K., R.Y. Hsu, and M.D. Lane. 1980. Induction of fatty acid synthetase synthesis in differentiating preadipocytes. *J. Biol. Chem.* 255: 4745–4750.

38. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*162:156–159.

39. Rana, B., D. Mischoulon, Y. Xie, N.L. Bucher, and S.R. Farmer. 1994. Cell-extracellular matrix interactions can regulate the switch between growth and differentiation in rat hepatocytes: reciprocal expression of C/EBP alpha and immediate-early growth response transcription factors. *Mol. Cell. Biol*. 14: 5858–5869.

40. Spiegelman, B.M., M. Frank, and H. Green. 1983. Molecular cloning of mRNA from 3T3 adipocytes. *J. Biol. Chem.* 258:10083–10089.

41. Bernlohr, D.A., C.W. Angus, M.D. Lane, M.A. Bolanowski, and J.T.J.J. Kelly. 1984. Expression of specific mRNAs during adipose differentiation: identification of an mRNA encoding a homologue of myelin P2 protein. *Proc. Natl. Acad. Sci. USA*. 81:5468–5472.

42. Wilkinson, W.O., H.Y. Min, K.P. Claffey, B.L. Satterberg, and B.M. Spiegelman. 1990. Control of the adipsin gene in adipocyte differentiation. Identification of distinct nuclear factors binding to single- and double-stranded DNA. *J. Biol. Chem.* 265:477–482.

43. Dobson, D.E., D.L. Groves, and B.M. Spiegelman. 1987. Nucleotide sequence and hormonal regulation of mouse glycerophosphate dehydrogenase mRNA during adipocyte and muscle cell differentiation. *J. Biol. Chem.* 262: 1804–1809.

44. Clancy, B.M., and M.P. Czech. 1990. Hexose transport stimulation and membrane redistribution of glucose transporter isoforms in response to cholera toxin, dibutyryl cyclic AMP, and insulin in 3T3-L1 adipocytes. *J. Biol. Chem.* 265:12434–12443.

45. Cornelius, P., M.D. Lee, N. Marlowe, and P.H. Pekala. 1990. The growth factor like effects of tumor necrosis factor-α: stimulation of glucose transport activity and induction of glucose transporter and immediately early gene expression. *J. Biol. Chem.* 265:20506–20516.

46. Thoidis, G., N. Kotliar, and P.F. Pilch. 1993. Immunological analysis of GLUT4-enriched vesicle. *J. Biol. Chem.* 268:11691–11696.

47. Stephens, J.M., and P.H. Pekala. 1991. Transcriptional repression of the GLUT 4 and C/EBP genes in 3T3-L1 adipocytes by tumor necrosis factor-α. *J. Biol. Chem.* 266:21839–21845.

48. Green, H., and O. Kehinde. 1974. Sublines of mouse 3T3 cells that accumulate lipid. *Cell*. 1:113–116.

49. Christy, R.J., V.W. Yang, J.M. Ntambi, D.E. Getman, W.H. Landschulz, A.D. Friedman, Y. Nakabeppu, J.T. Kelly, and M.D. Lane. 1989. Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. *Genes Dev*. 3:1323–1335.

50. McGrane, M.M., J. Yun, W.H. Lamers, G.K. Hendrick, B.M. Arafah, E.A. Park, T.E. Wagner, and R.W. Hanson. 1990. Metabolic effects of developmental, tissue-, and cell-specific expression of a chimeric phosphoenolpyruvate carboxykinase (GTP)/bovine growth hormone gene in transgenic mice. *J. Biol. Chem.* 265:22371–22379.

51. Park, E.A., W.J. Roesler, J. Liu, D.J. Klemm, A.L. Gurney, J.D. Thatcher, J. Schuman, A. Friedman, and R.W. Hanson. 1990. The role of the CAAT/enhancer-binding protein in the transcriptional regulation of the gene for phosphoenolpyruvate carboxykinase (GTP). *Mol. Cell. Biol.* 10:6264–6272.

52. Tontonoz, P., E. Hu, J. Devine, E.G. Beale, and B.M. Spiegelman. 1995.

PPARγ2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene. *Mol. Cell. Biol.* 15:351–357.

53. Hu, E., P. Tontonoz, and B.M. Spiegelman. 1995. Transdifferentiation of myoblasts by the adipogenic transcription factors PPARγ and C/EBPα. *Proc. Natl. Acad. Sci. USA*. 92:9856–9860.

54. Haney, P.M., J.W. Slot, R.C. Piper, D.E. James, and M. Mueckler. 1991. Intracellular targeting of the insulin-regulatable glucose transporter (GLUT4) is isoform specific and independent of cell type. *J. Cell Biol.* 114:689–699.

55. Hudson, A.W., M. Ruiz, and M.J. Birnbaum. 1992. Isoform-specific subcellular targeting of glucose transporters in mouse fibroblasts. *J. Cell Biol*. 116: 785–797.

56. Kotliar, N., and P.F. Pilch. 1992. Expression of the glucose transporter isoform GLUT4 is insufficient to confer insulin-regulatable hexose uptake to cultured muscle cells. *Mol. Endocrinol*. 6:337–345.

57. Cornelius, P., O.A. MacDougald, and M.D. Lane. 1994. Regulation of adipocyte development. *Annu. Rev. Nutr.* 14:99–129.

58. Leturque, A., M. Loizeau, S. Vaulont, M. Salminen, and J. Girard. 1996. Improvement of insulin action in diabetic transgenic mice selectively overexpressing GLUT4 in skeletal muscle. *Diabetes*. 45:23–27.

59. Tsao, T.S., R. Burcelin, E.B. Katz, L. Huang, and M.J. Charron. 1996. Enhanced insulin action due to targeted GLUT4 overexpression exclusively in muscle. *Diabetes*. 45:28–36.

60. Tozzo, E., L. Gnudi, and B.B. Kahn. 1997. Amelioration of insulin resistance in streptozotocin diabetic mice by transgenic overexpression of GLUT4 driven by an adipose-specific promoter. *Endocrinology*. 138:1604–1611.

61. Ikemoto, S., K.S. Thompson, M. Takahashi, H. Itakura, M.D. Lane, and O. Ezaki. 1995. High fat diet-induced hyperglycemia: prevention by low level expression of a glucose transporter (GLUT4) minigene in transgenic mice. *Proc. Natl. Acad. Sci. USA*. 92:3096–3099.

62. Gibbs, E.M., J.L. Stock, S.C. McCoid, H.A. Stukenbrok, J.E. Pessin, R.W. Stevenson, A.J. Milici, and J.D. McNeish. 1995. Glycemic improvement in diabetic db/db mice by overexpression of the human insulin-regulatable glucose transporter (Glut4). *J. Clin. Invest.* 95:1512–1518.

63. Nolan, J.J., B. Ludvik, P. Beerdsen, M. Joyce, and J. Olefsky. 1994. Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone. *N. Engl. J. Med.* 331:1188–1193.

64. Kobayashi, M., M. Iwanishi, K. Egawa, and Y. Shigeta. 1992. Pioglitazone increases insulin sensitivity by activating insulin receptor kinase. *Diabetes*. 41:476–483.

65. Iwanishi, M., and M. Kobayashi. 1993. Effect of pioglitazone on insulin receptors of skeletal muscles from high-fat-fed rats. *Metabolism*. 42:1017–1021.

66. Zhang, B., D. Szalkowski, E. Diaz, N. Hayes, R. Smith, and J. Berger. 1994. Potentiation of insulin stimulation of phosphatidylinositol-3-kinase by thiazolidinedione-derived antidiabetic agents in chinese hamster ovary cells expressing human insulin receptors and L6 myotubes. *J. Biol. Chem.* 269:25735–25741.