A novel 3T3-L1 preadipocyte variant that expresses PPARγ2 and RXRα but does not undergo differentiation

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Abstract **This report describes a novel adipocyte-like cell line termed 3T3-L1/RB1 that was derived from preadipocyte cell line, 3T3-L1. The 3T3-L1/RB1 cells continued to divide after reaching confluence, formed foci, and constitutively expressed a low level of adipose fatty acid binding protein (A-FABP) mRNA. However, 3T3L-1/RB cells did not undergo terminal differentiation as indicated by the failure of insulin and thiazolidendiones to induce the expression of A-FABP, lipoprotein lipase, and fatty acid synthase. We hypothesized that the 3T3-L1/RB1 variant did not respond to differentiation stimuli because it did not express either peroxisomal proliferator activated receptor γ2 (PPARγ2) or its heterodimer partner, retinoid X receptor α (RXRα). Surprisingly, Western blots revealed that 3T3-L1/RB1 cells contained both PPARγ2 and RXRα proteins at levels equal to or greater than that of the parent cell line. However, gel retardation assays using the adipose response element from A-FABP and nuclear protein extracts from 3T3-L1/RB1 cells treated with insulin or pioglitazone revealed that nuclear protein extracts from 3T3-L1/RB1 cells had very little ability to bind the PPARγ2 recognition sequence of the A-FABP gene. These data suggest that the 3T3-L1/RB1 variant contains a mutation that may prevent ligand activation of PPARγ2, and the subsequent conversion of 3T3-L1/RB1 cells to mature fat cells.—**Baillie, R. A., X. Sha, P. Thuillier, and S. D. Clarke. **A novel 3T3-L1 preadipocyte variant that expresses PPARγ2 and RXRα but does not undergo differentiation. *J. Lipid Res.* 1998.** 39: **2048–2053.**

Supplementary key words fatty acid binding protein • adipocytes • differentiation • 3T3-L1 cells • PPAR

The conversion of preadipocytes to mature fat cells is a coordinated response to a collection of external stimuli including signals from cAMP, glucocorticoids, IGF-1, and arachidonate (1–6). These signals initiate a cascade of transcriptional changes which lead to the down-regulation of differentiation suppressors (7), and the up-regulation of differentiation inducers (1). Two transcription factors that are essential to terminal fat cell differentiation and the expression of fat specific genes are PPARγ2 and RXRα (8–11). PPARγ2 is a ligand-activated transcription factor that stimulates the transcription of fat specific genes (e.g., A-FABP) by forming a heterodimer with RXRα and subsequently binding to its DNA recognition site, DR-1 (direct repeat, one base pair spacer) (4, 10). The endogenous ligand activator for PPARγ2 may be the prostanoid 15-deoxy-d12,14-prostaglandin J2 (4). However, the ligand domain of PPARγ2 can bind a number of different activating compounds including fibrates, ETYA, and the novel antidiabetic drugs, thiazolidenediones (12, 13). If PPARγ2, RXRα, or the appropriate PPARγ2 ligand are not present, then the expression of fat specific genes and terminal fat cell differentiation does not occur (6, 11). As an example, the ectopic expression of PPARγ2 in 3T3 fibroblasts did not initiate fat cell differentiation until a ligand activator for PPARγ2 was introduced (14). Similarly, 3T3-L1 preadipocytes do not express A-FABP and terminally differentiate until RXRα expression has been induced by glucocorticoids (9, 11). Thus, it is generally accepted that the events of terminal fat cell differentiation and the expression of fat specific genes such as A-FABP require the presence of PPARγ2, a PPARγ2 ligand activator, and RXRα.

In this report we describe the isolation and characterization of a monoclonal cell line termed 3T3-L1/RB1 that spontaneously arose from the 3T3-L1 adipocyte cell line. Because the 3T3-L1/RB1 variant does not withdraw from the cell cycle and does not undergo fat cell differentiation in response to insulin and the PPARγ2 activator pioglitazone, we have examined the hypothesis that the variant line does not express either PPARγ2 or its heterodimer partner RXRα, and/or that the variant does not produce the ligand activator that is essential for terminal fat cell differentiation and the induction of the fat specific gene, A-FABP.

Abbreviations: PPARγ2, peroxisomal proliferator-activated receptor γ; RXRα, retinoid X receptor α; RXRγ, retinoid X receptor γ; A-FABP, adipose fatty acid binding protein; IGF-1, insulin-like growth factor 1; PREF-1, preadipocyte factor 1; CUP, C/EBP undifferentiated protein; DR-1, direct repeat 1; C/EBPα, ccaat enhancer binding protein; GAPDH, glycceraldehyde phosphate dehydrogenase; ETYA, eicosatetraynoic acid; LPL, lipoprotein lipase.

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MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium, fetal and newborn calf serum, trypsin/EDTA, pennicillin/streptomycin, glyceraldehyde phosphate dehydrogenase cDNA, and the random prime and PCR labeling kits were purchased from Gibco BRL/Life Technologies (Gaithersburg, MD). PPARγ2 antibody was from Affinity Bioreagents, Inc. (Golden, CO). RXRα antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The cDNAs for A-FABP, lipoprotein lipase, RXRα, rat fatty acid synthase (pRFAS-17), and PPARγ2 were provided by D. Bernlohr, (St. Paul, MN), R. Eckel (Denver, CO), T. Neuman (Fort Collins, CO), M. Magnuson, (Nashville, TN), B. Speigelman (Boston, MA), respectively. The [α-^{32}P]dCTP was purchased from DuPont/NEN (Boston, MA). The Enhanced Chemiluminesence kit was purchased from Amersham (Arlington Heights, IL). All other reagents were from Sigma Chemical (St. Louis, MO).

Cell culture

3T3-L1/RB1 cells are a monoclonal cell line derived from a spontaneous aberration found in a single culture of 3T3-L1 cells. A group of putative 3T3-L1/RB1 cells were removed from the culture dish using trypsin/EDTA, an aliquot was counted, and the cells were diluted and plated at a density of 0.8 cells per plate. One culture was selected from this plating and was used for all further experiments. 3T3-L1 preadipocytes (ATCC #F8979) and 3T3-L1/RB1 cells were grown in Dulbecco's modified Eagle's medium that contained 5% fetal calf serum. When the cells reached confluence, they were staged to differentiate by changing the medium to one containing 5% newborn calf serum plus 0.5 mm isobutyl-methylxanthine (IBMX) and 1 μm dexamethasone. IBMX and dexamethasone were removed after 48 h (designated as time 0), and the medium was changed to one containing no hormones, 1 μm insulin, or insulin plus 20 μm pioglitazone. Cell protein was determined by the bicinchoninic acid method (Pierce, Rockford, IL).

RNA isolation and Northern blotting

The relative abundance of mRNAs encoding A-FABP, lipoprotein lipase, fatty acid synthase, glyceraldehyde phosphate dehydrogenase, RXRα, and PPARγ2 was determined by Northern analysis using total RNA isolated by the guanidinium isothiocyanate–phenol method (15). RNA (20 μg) was size fractionated by electrophoresis in a 1.3% agarose gel containing 6.6% formaldehyde, 20 mm MOPS, 1 mm EDTA, and 1 mm sodium acetate. After electrophoresis, the RNA was electroblotted onto Zetaprobe GT® membrane (Bio-Rad, Richmond, CA) and fixed to the membrane by UV crosslinking and baking. The cDNA probes for the Northern blotting analysis were labeled using random prime or PCR labeling. Membranes were hybridized and washed as previously described (16). The membranes were imaged and quantified using an Instantimager (Packard Instrument Company, Meriden, CT) or by exposure to Kodak X-Omat AR film (Rochester, NY) followed by visual imagizing using the AMBIS visualizing system.

Western analysis

Total cellular protein extracts were prepared at the various times cited in the figures. Cells on 60-mm dishes were rinsed twice with PBS and then scraped from the plates using 0.3 ml of RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 5 μg/ml aprotinin, 1 mm sodium orthovanadate). The lysates were passed through a syringe fitted with a 21-gauge needle, transferred to a centrifuge tube and centrifuged at 10, 000 *g* for 20 min at 4°C. The supernatant containing the total cell lysate was stored at −80°C until use. Nuclear protein extracts were prepared using the procedure from Dignam, Lebovitz, and Roeder (17). Proteins (25 μg) were separated by SDS-PAGE on a 10% polyacrylamide gel, transferred, and immobilized on a nitrocellulose membrane. The membrane was blocked by incubation with 5% nonfat dry milk in phosphate-buffered saline at 4°C overnight. The membrane was then washed briefly in Tris-buffered saline, and hybridized with rabbit antibody raised against A-FABP diluted 1:1000, PPARγ2 diluted 1:15000 or RXRα diluted 1:1000 in Tris-buffered saline containing 0.05% Tween 20. Incubation with antibodies and detection of the antigen–antibody complex were performed using the enhanced chemiluminescence.

Gel retardation assay

Nuclear protein extracts (5 μg) isolated using the procedure from Dignam et al. (17) were mixed with 4× Superdex buffer (100 mm KCL, 0.1% NP40, 10% glycerol, 10 mm HEPES, 5 mm $MgCl_2$, 2.5 μm $ZnSO_4$, 0.8 mm DTT), 1 μg of Poly (dA-dT) in the presence of 20, 000 cpm (1 ng) of γ-^{32}P-ATP labeled DNA probe in a total volume of 20 μl and incubated on ice for 20 min. Samples were then loaded on a 4.5% polyacrylamide gel containing 0.1% NP40 and run at 200 volts for 2 h in 40 mm Tris, 380 mm glycine, pH 8.3, buffer containing 0.1% NP40. Gels were dried and quantified by phosphoimagizing. The double-stranded oligonucleotide used for gel shift assays was the adipose response element for PPARγ2/RXRα previously identified for the A-FABP gene (8). The sequence of this response element is: 5′-CAGA AATGCACATTTCACCCAGAGAGAAGGG-3′.

RESULTS

During our studies of the mechanisms governing the conversion of 3T3-L1 fibroblasts to mature fat cells we noted that a small number of the cells no longer stopped dividing when confluence was attained. This sub-group of 3T3-L1 cells was named 3T3-L1/RB1, and they displayed a number of characteristics distinctly different from the parent 3T3-L1 cells. Specifically, the doubling time of the 3T3-L1/RB1 cells was 4–6 h compared to 12–18 h for the 3T3-L1 parent. In addition, unlike the 3T3-L1 cells which upon reaching confluence become rounded, develop a phenotype characteristic of mature fat cells, and subsequently fill with lipid, the 3T3-L1/RB1 cells maintain their fibroblast-like shape and do not develop a gene expression pattern that is characteristic of adipocytes (**Fig. 1** and **Fig. 2**). As an example, 3T3-L1 fibroblasts do not express the adipocyte specific gene, A-FABP, until they have reached confluence and have been induced to differentiate by treatment with IBMX, glucocorticoid, and insulin (Figs. 1 and 2). However, the 3T3-L1/RB1 cells express a low, constitutive level of A-FABP (i.e., A-FABP mRNA and protein were <30% of fully differentiated 3T3-L1 fat cells), and neither A-FABP mRNA nor protein increased in response to the differentiating stimuli of IBMX, dexamethasone, and insulin (Figs. 1, 2 and **Fig. 3**). Similarly, differentiating 3T3-L1 cells form large lipid droplets, and greatly accelerate the expression of adipocyte genes such as lipoprotein lipase and fatty acid synthase (Fig. 2). In constrast, lipid droplets did not form in 3T3-L1/RB1 cells that were treated with IBMX/dexamethasone/insulin. In addition, Northern analysis of total RNA extracts from

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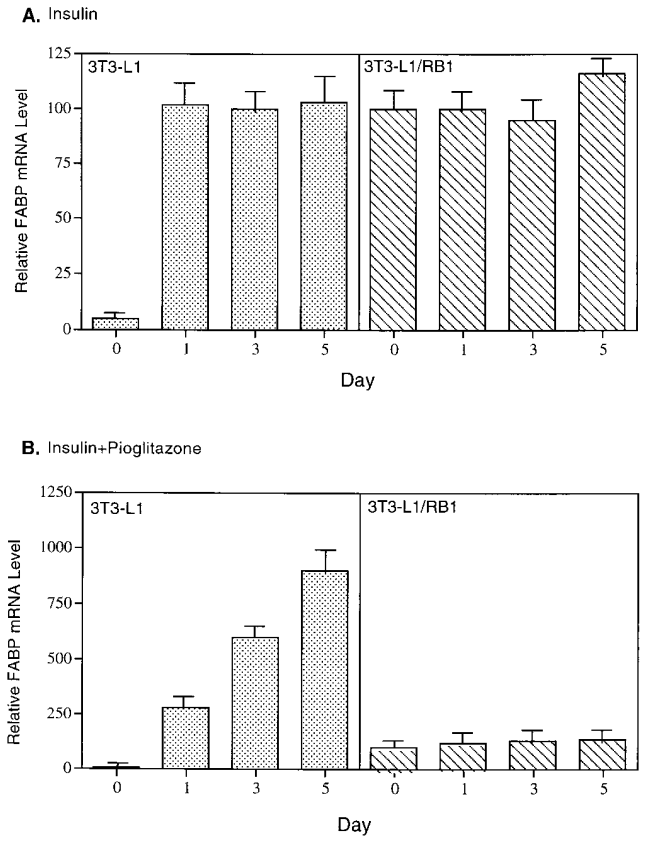

Fig. 1. The influence of insulin and pioglitazone on the abundance of A-FABP mRNA in 3T3-L1 and 3T3-L1/RB1 cells. The 3T3-L1 and 3T3-L1/RB1 preadipocytes were staged to differentiate by treating them with IBMX and dexamethasone for 48 h (Methods). After the 48-h staging period, IBMX and dexamethasone were removed from the media and the cells were cultured for an additional 1, 3, and 5 days with insulin or pioglitazone plus insulin. Transcript abundance was determined by Northern analysis of total RNA. Panel A shows cells treated with insulin. Panel B shows cells treated with insulin and pioglitazone. Note the 10-fold difference in scale between panels A and B. A sample Northern analysis is depicted in Fig. 2. Results shown as means ± SEM (n = 4).

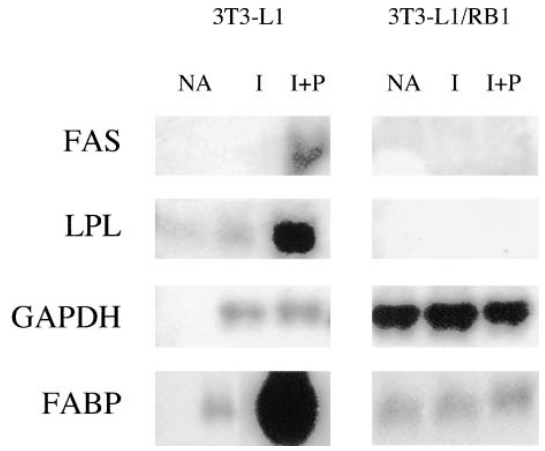

Fig. 2. The influence of insulin and pioglitazone on the expression of lipogenic genes in 3T3-L1 and 3T3-L1/RB1 cells. The 3T3-L1 and 3T3-L1/RB1 preadipocytes were staged to differentiate by treating them with IBMX and dexamethasone for 48 h (Methods). After the 48-h staging period, IBMX and dexamethasone were removed from the media and the cells were cultured for an additional 3 days with no hormones (NA), insulin (I), or pioglitazone plus insulin (I + P). Transcript abundance was determined by Northern analysis using total RNA. Resulting blots depicted are from a single Northern membrane that was sequentially hybridized with cDNAs noted in the figure. The depicted experiment is representative of eight experiments; FAS, fatty acid synthase; LPL, lipoprotein lipase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FABP, adipose fatty acid binding protein.

3T3-L1/RB1 cells (before and after treatment with IBMX/dexamethasone/insulin) failed to detect the presence of either LPL mRNA or fatty acid synthase mRNA (Fig. 2).

Like A-FABP and LPL, the abundance of GAPDH mRNA was low in non-differentiated 3T3/L1 cells, but the level of GAPDH mRNA markedly increased when the 3T3-L1 preadipocytes were induced to differentiate (Fig. 2) (18). In contrast, the level of GAPDH mRNA in 3T3-L1/RB1 cells was approximately 10-fold higher than in 3T3/L1 preadipocytes and, unlike the 3T3-L1 cells, the level of GAPDH mRNA did not change when the the 3T3-L1/RB1 variant was treated with insulin (Fig. 2).

One explanation for the gene expression differences between 3T3-L1 preadipocytes and the 3T3-L1/RB1 variant might be that the 3T3-L1/RB1 cells respond more slowly to differentiation stimuli. To address this hypothesis, the 3T3-L1/RB1 cells were treated with the insulin/IGF-1 sensitizing agent pioglitazone (19, 20). Pioglitazone is a member of the thiazolidenedione family of antidiabetic compounds which functions as a ligand activator for the adipocyte transcription factor, PPARγ2. The consequence of this ligand activation is an acceleration and amplification of adipocyte gene expression, e.g. A-FABP (5, 6, 10, 20). As expected, pioglitazone increased the abundance of A-FABP and LPL mRNA in differentiating 3T3-L1 cells by 10- to 15-fold (Fig. 2). However, pioglitazone treatment of 3T3-L1/RB1 cells did not increase the abundance of either A-FABP mRNA or A-FABP protein (Figs. 1–3). Similarly, the abundance of LPL mRNA and fatty acid synthase mRNA remained undetectable in 3T3-L1/RB1 cells treated with pioglitazone and insulin (Fig. 2).

Because the 3T3-L1/RB1 cells did not respond to pioglitazone, we hypothesized that the PPARγ2 receptor for pioglitazone, and/or its heterodimer partner, RXRα, may not be expressed in the 3T3-L1/RB1 variant. Consistent with our hypothesis, the 3T3-L1/RB1 variant was found by Northern analysis to contain undetectable levels of mRNA for PPARγ2 and RXRα. However, it is possible that low levels of PPARγ2 and/or RXRα may be available for synthesis of the respective proteins. As can seen in **Fig. 4**, the nuclei of both 3T3-L1 and 3T3-L1/RB1 contained comparable levels of PPARγ2 and RXRα; and the amount of PPARγ2 and RXRα protein was not increased by either insulin or pioglitazone (Figs. 3 and 4). Interestingly, the 3T3-L1/RB1 cells contained 2- to 3-fold more RXRα than the parent 3T3-L1, but this was not reflected in the mRNA abundance for RXRα. The regulatory significance of the elevation in RXRα remains unclear.

As both PPARγ2 and RXRα proteins were present in the

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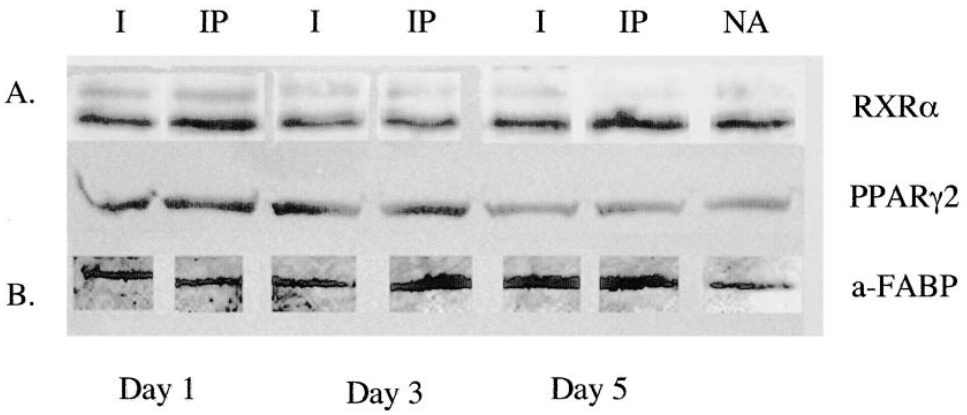

Fig. 3. Insulin and pioglitazone treatment of 3T3-L1/RB1 cells does not increase the total cellular protein level of PPARγ2, RXRα, and A-FABP. The 3T3-L1/RB1 preadipocytes were staged to differentiate by treating them with IBMX and dexamethasone for 48 h (Methods). After the 48-h staging period, IBMX and dexamethasone were removed from the media and the cells were cultured for an additional 1, 3, and 5 days with insulin (I), pioglitazone plus insulin (IP), or no hormones (NA). Total protein was extracted and utilized for Western analysis. The blots were probed with antibodies for PPARγ2 or RXRα or antisera against A-FABP. The depicted results are from a single cell preparation and are representative of three experiments.

3T3-L1/RB1 cells at levels equal to or greater than the amounts found in their 3T3-L1 parent line, we hypothesized that the inability of the 3T3-L1/RB1 cells to express fat specific genes and to terminally differentiate into mature fat cells was perhaps due to a failure of PPARγ2 and RXRα to form a heterodimer and subsequently bind to the PPARγ2 response element. To address this hypothesis, electromobility shift assays were performed using the adipose response element from the A-FABP gene, which is a well-established DNA binding site for the PPARγ2/RXRα heterodimer (4, 8, 9). As expected, nuclear extracts from differentiating 3T3-L1 cells displayed a pronounced gel shift with the adipose response element (**Fig. 5**), while nuclear protein extracts from undifferentiated 3T3-L1 cells (i.e., no addition) had little ability to bind to the adipose response element of the A-FABP gene. On the other hand, nuclear proteins extracted from the 3T3-L1/RB1 variant treated with either insulin or pioglitazone displayed very little ability to interact with the DNA sequence for the adipose response element (Fig. 5). Thus, even though the 3T3-L1/RB1 variant contains ample quantities of PPARγ2 and RXRα protein, the PPARγ2 ligand pioglitazone was unable to induce a PPARγ2 interaction with its *cis*-acting element.

DISCUSSION

The conversion of preadipocytes to mature fat cells is a coordinated response to a collection of external inducers (1, 6, 7). At least three external factors are essential to the differentiation process: *a*) cAMP (22); *b*) glucocorticoids (23); and IGF-1 (2, 3). In addition, long chain fatty acids may function as differentiation inducers in some preadi-

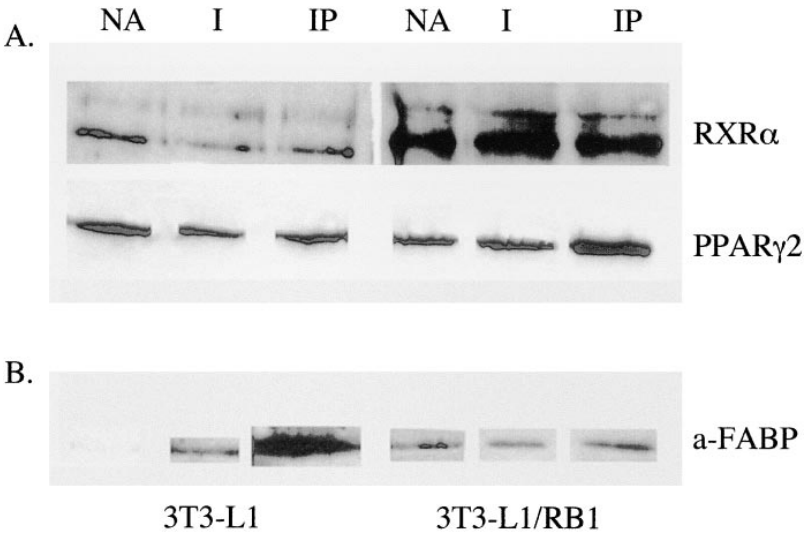

Fig. 4. Nuclear content of PPARγ2 and RXRα in 3T3-L1 and 3T3-L1/RB1 cells. The 3T3-L1 and 3T3-L1/RB1 preadipocytes were staged to differentiate by treating them with IBMX and dexamethasone for 48 h (Methods). After the 48-h staging period, IBMX and dexamethasone were removed from the media and the cells were cultured for additional 3 days with no addition (NA), insulin (I), or pioglitazone plus insulin (IP). Western analysis was used to quantify the abundance of PPARγ2 and RXRα in nuclear protein extracts (17) and to quantify the abundance of A-FABP in total cellular protein extracts. The depicted results are from a single cell preparation and are representative of three experiments.

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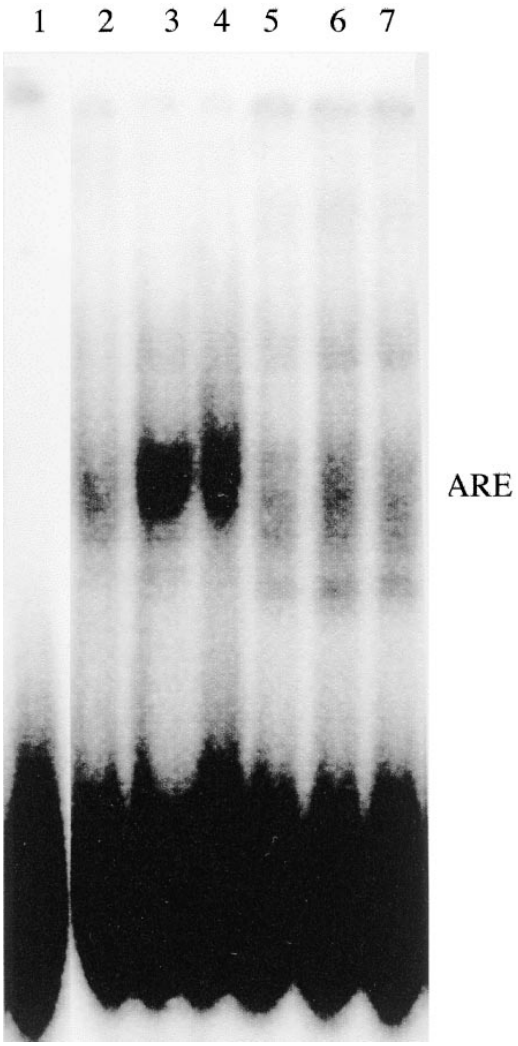

Fig. 5. Gel retardation assay. The 3T3-L1 and 3T3-L1/RB1 preadipocytes were staged to differentiate by treating them with IBMX and dexamethasone for 48 h (Methods). After the 48-h staging period, IBMX and dexamethasone were removed from the media and the cells were cultured for additional 3 days with no hormone, insulin, or pioglitazone plus insulin. Nuclear protein extracts were prepared (17) and utilized in a gel retardation assay with ^{32}P-labeled ARE. Lane 1, free probe; lanes 2–4, 3T3-L1 cells; lanes 5–7, 3T3-L1/RB1 cells. Lanes 2 and 5, no hormone; lanes 3 and 6, insulin; lanes 4 and 7, pioglitazone plus insulin.

pocyte models (23). The function of these inducing agents is to down-regulate repressors of differentiation such as PREF-1 (preadipocyte factor 1), CUP (C/EBP undifferentiated protein), and RARγ (retinoic acid receptor gamma) (1, 7, 9), while concomitantly increasing the expression of transcription factors essential for the transcription of genes characteristic of a terminally differentiated fat cell, e.g. C/EBP, RXRα, and PPARγ2 (5–11, 24). PPARγ2 and RXRα are one set of transcription factors whose synthesis is critical for the expression of fat specific genes and terminal fat cell differentiation (14). These two transcription factors stimulate the transcription of adipose specific genes by forming a heterodimer and subsequently binding to the DNA recognition site, DR-1 (direct repeat sequence with one nucleotide spacer) (8, 9). Like other members of the steroid receptor super-family, PPARγ2 contains a ligand binding domain, and binding of factors such as fatty acids, fibrates, certain prostanoids, and thiazolidenediones to the ligand domain greatly enhances the interaction of PPARγ2 with its DNA recognition site (4, 5, 9, 14), which in turn accelerates the transcription of adipose specific genes such as A-FABP (19, 25).

In our studies of how pioglitazone regulates the conversion of 3T3-L1 preadipocytes to mature fat cells, we discovered a phenotypic change in some of the 3T3-L1 cells, e.g., failure to arrest growth and failure to round up after treatment with insulin. This variant (i.e., 3T3-L1/RB1) constituitively expressed adipocyte specific A-FABP. However, the levels of A-FABP protein and mRNA were not induced by differentiation stimuli such as insulin and pioglitazone (Figs. 1 and 2). The reasons for the low constituitive expression of A-FABP in the 3T3-L1/RB1 cells are unclear. We did not determine whether or not the expression of A-FABP in 3T3-L1/RB1 cells was determined by transcriptional or post-transcriptional mechanisms. However, we hypothesized that the failure of A-FABP to induce its expression in response to insulin and pioglitazone may indicate that the 3T3-L1/RB1 variant did not express PPARγ2 and/or its heterodimer partner RXRα. Surprisingly, Western blot analysis indicated that the total cellular and nuclear content of both PPARγ2 and RXRα in the 3T3-L1/RB1 variant were equal to or greater than the amounts found in differentiated 3T3-L1 parent cells (Figs. 3 and 4). In light of the fact that the 3T3-L1/RB1 variant clearly contained PPARγ2 and RXRα, we evaluated the alternative hypothesis that the variant line may not produce the appropriate ligand needed to activate PPARγ2 and enhance its interaction with the DR-1. To address this alternative, the 3T3-L1/RB1 cells were treated with the high affinity ligand activator, pioglitazone. Pioglitazone is a novel antidiabetic agent that is a member of the thiazolidenedione family, and has the effect of increasing fat cell sensitivity to insulin and IGF-1 several fold (19, 20). In spite of the fact that the 3T3-L1/RB1 variant contained high levels of PPARγ2 and RXRα protein, pioglitazone did not enhance PPARγ2/RXRα interaction with its DR-1 recognition sequence from the A-FABP gene (Fig. 5). Consistent with this observation, A-FABP mRNA abundance was not increased in 3T3-L1/RB1 cells treated with pioglitazone (Figs. 1 and 2). This outcome was very surprising because ectopic expression of PPARγ2 in NIH 3T3 fibroblasts and subsequent treatment of the fibroblasts with pioglitazone led to near 95% conversion of the fibroblasts to mature fat cells (14).

The reasons for why 3T3-L1/RB1 cells do not terminally differentiate can only be speculated. One possibility is that these cells do not express the C/EBP family of proteins that may be needed to achieve full PPARγ2 responsivity. In this regard, ectopic expression in G8 myoblasts of either C/EBPα alone or PPARγ2 (plus activator) alone did not give rise to a fat cell phenotype, but co-expression of the two transcription factors led to 70% conversion of the cells to adipocytes (14). A second possibility is that the

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3T3-L1/RB1 cells contain a differentiation silencer or suppressor protein, e.g., high levels of PREF-1 or CHOP/gadd153 that could block the anti-mitogenic action of C/EBPα and lead to a failure of the cells to growth arrest (5–7). However, the difficulty with both of these possibilities is that neither of them provides a good explanation for why PPARγ2/RXRα do not bind to the DR-1 recognition sequence derived from the A-FABP gene (Fig. 5).

The failure of PPARγ2 to interact with its DNA recognition sequence even in the presence of a high affinity ligand such as pioglitazone has several possible explanations. One obvious possibility is that PPARγ2 of the 3T3-L1/RB1 variant has undergone a mutation in either the ligand or DNA binding domains. Alternatively, the RXRα may contain an amino acid mutation preventing it from forming a heterodimer with PPARγ2. It is also possible that simple ligand binding is not sufficient for PPARγ2 to bind to its recognition sequence. In this respect, pioglitazone treatment of 3T3-L1 cells does not induce fat cell differentiation unless the media contain insulin or IGF-1 (S. D. Clarke and X. Sha, unpublished data). Moreover, the phosphorylation status of PPARγ2, which appears to be regulated by insulin, has been found to govern the ability of PPARγ2 to interact with its DR-1 recognition sequence (8, 9). Thus, it is feasible that the 3T3-L1/RB1 variant has a defect in the insulin signaling cascade. Finally, it is also possible that the 3T3-L1/RB1 variant may over-express an anti-adipogenic signal (e.g., PREF-1) that is blocking PPARγ2 activation. Clearly, all of these possibilities are only speculations, but as we elucidate the cause for the RB1 variant of 3T3-L1 cells, we will gain a better understanding of the mechanism by which PPARγ2 regulates fat cell differentiation.

The work described in this manuscript was supported by NIH grant DK 46363 (SDC) and the American Diabetes Association (SDC). Rebecca A. Baillie was the recipient of postdoctoal traineeship (NIH DK 07658). The authors also wish to thank the sponsors of the M.M. Love Chair in Nutritional, Cellular, and Molecular Sciences (SDC) for their support.

Manuscript received 11 December 1997 and in revised form 16 June 1998.

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