Cytosolic and nuclear distribution of PPARγ2 in differentiating 3T3-L1 preadipocytes

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Abstract **In light of the pivotal role that PPARγ2 plays in the expression of fat specific genes (e.g., A-FABP), we have examined the hypothesis that a rise in PPARγ2 protein is required for the expression of A-FABP, and that the acceleration of fat cell differentiation by the thiazolidinedione agent, pioglitazone (PIOG), reflects an increase in the abundance of PPARγ2 mRNA and protein. Western analyses surprisingly revealed that undifferentiated 3T3-L1 fibroblasts contained significant levels of PPARγ2 protein; that the amount of total cellular PPARγ2 only increased 2-fold during differentiation; and that the levels of PPARγ2 protein and mRNA were not increased by PIOG even though fat cell differentiation was accelerated by PIOG as revealed by a 20-fold increase in A-FABP expression. Cell fractionation studies revealed that PPARγ2 was evenly distributed between the cytosolic and nuclear compartments in both undifferentiated and differentiating 3T3-L1 cells. Immunocytochemical studies with a PPARγ2-specific antibody indicated that PPARγ2 was diffusely distributed throughout the cytosol of undifferentiated 3T3-L1 cells, but as the differentiation progressed, the PPARγ2 became focused around the developing lipid droplets. In contrast to PPARγ2, undifferentiated 3T3-L1 cells contained no measurable quantities of RXRα, but once fat cell differentiation was initiated by treatment with IBMX and dexamethasone, the cellular content of RXRα increased several fold. The rise in RXRα content paralleled the induction of A-FABP, but the expression of RXRα was not enhanced by PIOG. Although the amount of PPARγ2 and RXRα was unaffected by PIOG, gel shift assays revealed that PIOG stimulated PPARγ2/RXRα binding to the adipose response element of A-FABP by 5-fold in less than 12 h. Apparently, RXRα rather than PPARγ2 is the pivotal trans-factor essential for the initiation of terminal fat cell differentiation. However, the high cytsolic content of PPARγ2 and its association with the lipid droplet of differentiating 3T3-L1 cells suggests PPARγ2 may possess a cytosolic function in the developing fat cell.—**Thuillier, P., R. Baillie, X. Sha, and S. D. Clarke. **Cytosolic and nuclear distribution of PPARγ2 in differentiating 3T3-L1 preadipocytes.** ***J. Lipid Res.*** **1998.** 39: **2329–2338.**

Supplementary key words insulin • fatty acid binding protein • gene expression • pioglitazone • PPARγ2 • RXRα

Adipocytes first appear late in fetal development in preparation for postnatal life when a substantial energy reserve is needed to survive periods of fasting. However, excessive development of adipose tissue affects over 30% of all adults in the United States and represents a significant risk factor for non-insulin-dependent diabetes mellitus (NIDDM), coronary artery disease, and hypertension. Considerable progress has been made during the past few years in our understanding of the molecular control of adipogenesis and adipocyte-specific gene expression. Using the adipocyte-specific fatty acid binding protein (A-FABP) gene as a model, several *cis*-acting elements and *trans*-acting factors have been identified that act cooperatively to trigger the terminal differentiation program of adipocytes (1–5). Particular attention has focused on the fat-specific region of the A-FABP gene which is located from −5400 to −4900 bp. This region of the gene contains the adipose response elements (AREs) that instill adipose tissue-specific expression of A-FABP. The AREs are similar to the direct repeat (DR-1) response elements that bind members of the peroxisome proliferator-activated receptor (PPAR) family of transcription factors (5–14). The identification of the ARE (DR-1) element led to the cloning and identification of a new member of the PPAR family, PPARγ2, which is found in high abundance in adipose tissue and appears to be responsible for regulating the expression of adipose specific genes such as A-FABP (5, 15).

The induction of fat-specific genes such as A-FABP by PPARγ2 requires that PPARγ2 bind to the ARE (DR-1) sequence as a heterodimer complex with the retinoid X receptor alpha (RXRα) (5, 6). Moreover, interaction of the PPARγ2 with the ARE (DR-1) appears to be dependent upon its ability to bind a collection of ligand activators in-

Abbreviations: A-FABP, adipocyte fatty acid binding protein; ARE, adipocyte response element; DEX, dexamethasone; GRE, glucocorticoid response element; IBMX, isobutylmethylxanthine; IGF-1, insulin-like growth factor-1; PIOG, pioglitazone; PPAR, peroxisomal proliferator-activated receptor; PPRE, PPAR response element; RXR, retinoid X receptor.

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cluding eicosanoids (11–20), fatty acids, and insulin-sensitizing drugs such as pioglitazone (21–24). Although these observations demonstrate the importance of PPARγ2 in fat cell differentiation, it is unknown whether the abundance of PPARγ2 protein is the limiting determinant for terminal fat cell differentiation. Thus, we have hypothesized that an increase in PPARγ2 protein is required for the conversion of preadipocytes to mature fat cells, and that activating ligands of PPARγ2 such as the thiazolidinedione, pioglitazone, accelerate fat cell differentiation by increasing the abundance of PPARγ2 protein. In fulfilling our objective we have made two interesting discoveries: *a*) nearly 50% of the total cellular content of PPARγ2 is located in the cytosol compartment of both preadipocytes and mature fat cells; and *b*) early events of fat cell differentiation appear to be dependent upon the induction of RXRα expression rather than upon the expression of PPARγ2.

EXPERIMENTAL PROCEDURES

Cell culture

3T3-L1 preadipocytes (ATCC #F8979) were grown in Dulbecco's modified Eagle's medium (Gibco BRL) that contained 5% fetal calf serum. When the cells were 70% confluent they were staged to differentiate by changing the medium to one containing 5% newborn calf serum plus 10 μm IBMX and 1 μm dexamethasone. The IBMX and dexamethasone were removed after 48 h (designated as time 0 h), and the medium was changed to one containing either no hormones, 1 μm insulin, or insulin plus 10 μm pioglitazone.

Anti-PPARγ2 antibody preparation

To address our hypothesis that an increase in PPARγ2 is a crucial step in terminal fat cell differentiation, antibodies to two domains of PPARγ2 were prepared: *a*) amino acid residues 2–16 (p2–16) which are specific for PPARγ2; and *b*) amino acid residues 32–54 (p32–56) which is a domain shared by both PPARγ2 (56 kDa) and PPARγ1 (52 kDa). The anti-serum to mPPARγ2 was raised in rabbits injected with synthetic peptide antigen corresponding to either p2–16 or p32–56 of PPARγ2. Antisera were harvested every 2 weeks and their antigenicity against the respective peptides was determined. Antiserum collected at day 70 was the most antigenic and was subsequently harvested for antibody purification. Affinity-purified anti-PPARγ2 was prepared from serum using the respective peptides linked to sulfo-link affinity columns (Pierce). As can be seen in **Fig. 1**, Western blot analysis of total protein extracts from undifferentiated and differentiated 3T3-L1 cells revealed that the PPARγ2 specific antibody (p2–16) detected a single band at 56 kDa (lanes 3 and 4); and that this band co-migrated with a protein produced from an in vitro transcription–translation reaction programmed with a vector containing the PPARγ2 open-reading frame (Fig. 1, lane 5). As expected, antibody prepared to the p32–54 domain of PPARγ2 detected both the larger PPARγ2 (56 kDa) and the smaller PPARγ1 (52 kDa) (Fig. 1, lanes 1 and 2). Clearly, the dominant protein in both undifferentiated and differentiated 3T3-L1 cells was the PPARγ2 isoform.

RNA isolation and Northern analysis

The abundance of A-FABP, PPARγ2, and RXRα mRNA was determined by Northern analysis at the times indicated in the various figures. RNA isolation and Northern blotting were performed as described previously (25). Twenty μg of total RNA was loaded on a 1% formaldehyde–agarose gel as described (26) and transferred to a Zeta probe membrane (Bio-Rad). The membranes were hybridized with cDNA probes labeled with α-^{32}P-dCTP (DuPont NEN). The full-length cDNA for A-FABP was provided by D. Bernlohr, University of Minnesota (27). PPARγ2 cDNA was a generous gift from B. M. Spiegelman, Dana-Farber Cancer Institute, and RXRα cDNA was provided by T. Neuman, Colorado State University. Autoradiographs were digitally captured and quantified using the Optical Integrator and Image software from Ambis.

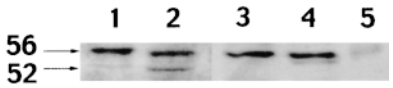

Fig. 1. PPARγ protein expression in undifferentiated and differentiated 3T3-L1 cells. Western analysis was conducted utilizing total cellular protein extracts (50 μg per lane) from undifferentiated (lanes 1 and 3) and fully differentiated (lanes 2 and 4) 3T3-L1 cells. After separating the proteins by polyacrylamide gel electrophoresis, lanes 1 and 2 were hybridized with anti-PPARγ$_{32–54}$ which is a peptide domain located in both PPARγ1 (52 kDa) and PPARγ2 (56 kDa); and lanes 3–5 were hybridized with anti-PPARγ$_{4–26}$ which is a peptide domain located in only PPARγ2 (56 kDa). Lane 5, which was hybridized with anti-PPARγ$_{4–26}$, is an aliquot of an in vitro transcribed and translated reaction that used PPARγ2 cDNA as a template. Note that the predominant form of PPARγ in both undifferentiated and differentiated 3T3-L1 cells is PPARγ2, and that the in vitro translated PPARγ2 co-migrates with the 56 kDa protein.

Cellular protein extraction and Western analysis

Total cellular protein extracts were prepared from undifferentiated and differentiated 3T3-L1 cells grown in 100-mm petri dishes. The plates were first washed twice with PBS, and the cells were subsequently scraped from the plates using 0.6 ml of RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.25 mm PMSF, 5 mg/ml aprotinin, 1 mm Na orthovanadate). A cell lysate was prepared by passing the cells through a syringe fitted with a 21-gauge needle; the lysate was transferred to a microfuge tube, incubated on ice for 60 min, and subsequently was microfuged at 10,000 *g* for 20 min at 4°C. The supernatant containing the total cell lysate was saved and stored at −80°C until use.

Cytosolic and nuclear protein extracts were prepared according to the method of Sanchez et al. (28). Briefly, 3T3-L1 cells grown in a 100-mm dish were rinsed with ice-cold PBS and scraped into a microfuge tube with 1.0 ml PBS. The cells were pelleted by centrifugation (1000 *g*, 10 min). The resulting cell pellet was resuspended in 250 ml of 10 mm HEPES, 1 mm EDTA, 0.5 mm DTT, 0.25 mm PMSF, 50 mm NaF, 2 mm Na metavanadate, and 5 mg/ml leupeptin and pepstatin (buffer A), and homogenized in a dounce homogenizer with 20 strokes. This homogenate was centrifuged at 1000 *g* for 10 min. The resulting low speed nuclear pellet was utilized for protein extraction as described below, and the supernatant (S1) was processed as follows: 4 m NaCl was added to the S1 fraction to make a final concentration of 0.5 m NaCl and the mixture was incubated on ice for 1 h with occasional vortexing. The S1 fraction was centrifuged at 100,000 *g* for 1 h and the supernatant was saved as cytosolic extract. Extracts were aliquoted and stored in liquid nitrogen until use. The low speed nuclear pellet was washed twice by resuspension in 250 ml buffer A containing 250 mm sucrose and pelleted at 1000 *g* for 10 min. The

pellet was then resuspended in 100 ml buffer A and 100 ml 1 m NaCl was added to make a final concentration of 0.5 m NaCl. The pellet was there incubated on ice for 1 h with occasional vortexing. After salt extraction the nuclear pellet was centrifuged at 8000 *g* for 30 min; the supernatant was saved as nuclear extract. Extracts were aliquoted and stored in liquid nitrogen until use.

For Western analysis, proteins were separated by SDS-PAGE, transferred, and immobilized on a nitrocellulose membrane at 4°C. The membrane was blocked by incubation with 5% non-fat dry milk in phosphate-buffered saline (PBS) at 4°C overnight. The membrane was then washed briefly in PBS and hybridized with rabbit antibody raised against rat PPARγ2 diluted 1/15,000, or against rat RXRα (Santa Cruz, CA) diluted 1:500 in PBS containing 0.1% Tween 20 (PBS-T). Incubation with antibodies and detection of the antigen–antibody complex were performed using the ECL kit (Amersham) according to the manufacturer's instructions, except that washes after secondary antibody incubation consisted of six changes of buffer over 1 h.

Immunocytochemistry

Undifferentiated and differentiated 3T3-L1 cells were grown in 12-well plates and induced to differentiate as described previously. At each time indicated in the figure, the cells were fixed in formallin for 15 min. Subsequently, each well was rinsed twice with PBS and treated with 0.03% H_2O_2 in methanol. After a 20-min incubation, the cells were washed three times with PBS containing 0.1% bovine serum albumin. Subsequently, the cells were incubated with 10% goat serum for 20 min, and then treated for 45 min with a 1:500 dilution of affinity-purified anti-PPARγ2 (p2–16). After antibody incubation the cells were washed with PBS containing bovine serum albumin. Subsequently, the cells were treated for 30 min with anti-rabbit IgG (Vector Lab, Inc.). After three washes with PBS-albumin, antibody staining was performed using vectastain as described by the manufacturer (Vector Lab, Inc.). Nuclei were visualized by 1 min incubation with Harris's hematoxylin.

Gel mobility shift assay

Five μg of nuclear protein extract (29) was mixed with 4X Superdex buffer (100 mm KCl, 0.1% NP40, 10% glycerol, 10 mm HEPES, 5 mm $MgCl_2$, 2.5 mm $ZnSO_4$, 0.8 mm DTT), 1 μg of poly dA-dT, and 1 ng of γ-^{32}P-ATP-labeled DNA probe (20,000 dpm). The 20 ml reaction was incubated on ice for 20 min. For cold competition, 200 ng of unlabeled ARE, PPRE, or GRE was added to the binding reaction and kept on ice for 20 min. For supershift, 2 ml of anti-PPARγ2 (p32–54) was added to the tubes at the end of the binding reaction, and incubated on ice for 30 min. Samples were then loaded onto a 4.5% acrylamide gel containing 0.1% NP40. Electrophoresis was run at 200 v for 3 h in a 40 mm Tris and 380 mm glycine buffer (pH 8.3) containing 0.1% NP40. Gels were dried and quantified by radioimagizing.

Ablation of the PPARγ2 gel shift by anti-PPARγ2 (p2–16) was achieved by incubating overnight 5 ml of affinity-purified antibody with 5 μg nuclear protein extract in 1X Superdex buffer. After the overnight incubation at 4°C, radiolabeled ARE (40,000 dpm) was added to the reaction mixture and the incubation was continued on ice for an additional 25 min. After incubation the mixture was separated by electrophoresis as described above.

The double-stranded oligonucleotides used were (only one strand is shown):

ARE: 5′-CAGAAATGCACATTTCACCCAGAGAGAAGGG-3′
GRE: 5′-AGTTTTTGGTTACAAACTGTTCTTAAAACG AGG-3′
PPRE: 5′-GATCTGTGACCTTTGTCCTAGTAAG-3′

RESULTS

PPARγ2 expression during 3T3-L1 differentiation

When 3T3-L1 preadipocytes undergo terminal differentiation to mature fat cells, the level of PPARγ2 mRNA increases significantly (9, 10). However, the relationship between the level of PPARγ2 mRNA and PPARγ2 protein is unknown. This is particularly true for the early stages of 3T3-L1 differentiation. Using a two-stage differentiation protocol (see Experimental Procedures), we discovered that total protein extracts from undifferentiated 3T3-L1 cells contained a significant quantity of PPARγ2 protein (see −48 h in **Fig. 2**). In fact, the level of immunoreactive PPARγ2 in preadipocytes was approximately 30% of that found in mature fat cells. Similarly, the 3T3-L1 preadipocytes contained a level of PPARγ2 mRNA that was comparable to the amount of PPARγ2 protein (**Fig. 3**). As expected, the level of PPARγ2 mRNA increased significantly in response to differentiation stimuli. However, by applying our two-step differentiation protocol, we discovered that IBMX and dexamethasone were largely responsible for the increased abundance of PPARγ2 mRNA (Fig. 3). Interestingly, the rise in PPARγ2 mRNA resulting from IBMX and dexamethasone treatment was not accompanied by an increase in PPARγ2 protein (Fig. 2). However, when the medium containing IBMX and dexamethasone

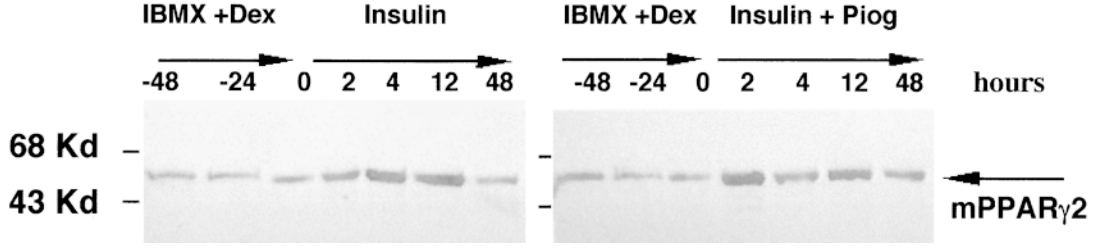

Fig. 2. Increases in the cellular content of PPARγ2 protein during fat cell differentiation. Undifferentiated 3T3-L1 cells were grown to confluence (−48 h) and staged to differentiate as described in Methods. After a 48-h staging period with IBMX and dexamethasone (0 h), the cells were induced to differentiate by changing the media to one containing either insulin (1 μm) or insulin plus pioglitazone (10 μm, PIOG). Total protein extracts (20 μg per lane) were prepared for Western analysis at the hours indicated in the figure. The protein blots were hybridized with anti-PPARγ$_{32–54}$. The 56 kDa PPARγ2 is noted by the arrow.

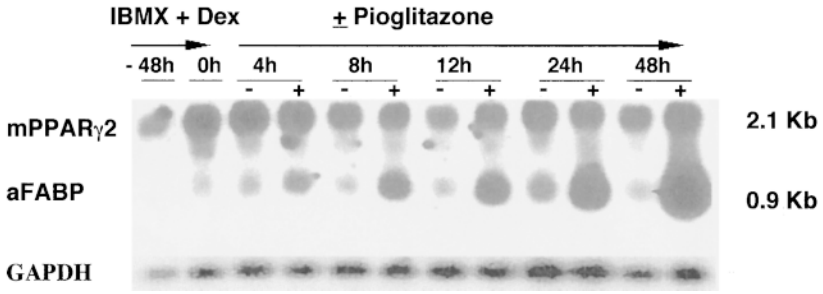

Fig. 3. Increases in PPARγ2 and A-FABP mRNA abundance during the early stages of preadipocyte differentiation. Undifferentiated 3T3-L1 cells were grown to confluence (−48 h) and staged to differentiate as described in Methods. After a 48-h staging period with IBMX and dexamethasone (0 h), the cells were induced to differentiate by changing the media to one containing either 1 μm insulin (designated with − symbol), or insulin plus 10 μm pioglitazone (designated with + symbol). Total RNA was extracted at the hours indicated in the figure, and the Northern blot was sequentially hybridized with cDNAs for mPPARγ2, A-FABP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The molecular sizes of the PPARγ2 and A-FABP transcripts are noted at the right.

was removed and replaced by one that contained insulin, the amount of PPARγ2 protein increased 2- to 3-fold within 2–4 h after insulin exposure (Fig. 2). This increase in PPARγ2 protein was not enhanced by the pioglitazone, a potent enhancer of fat cell differentiation.

Western analysis of cytosolic and nuclear protein extracts from undifferentiated 3T3-L1 cells indicated that PPARγ2 protein was distributed evenly between the cytosol and nucleus (**Fig. 4** and **Fig. 5**). When the undifferentiated 3T3-L1 cells were treated with insulin, the 2- to 3-fold increase in total cellular PPARγ2 protein that was noted previously (Fig. 2) appeared to be restricted to the cytosolic fraction, while the nuclear fraction of PPARγ2 remained relatively constant in undifferentiated and differentiated 3T3-L1 cells (Figs. 4 and 5). Even when the cells were treated with pioglitazone, which is a potent ligand activator for PPARγ2 (8, 19), the amount of immuno-reactive PPARγ2 in the nucleus did not appear to increase (Fig. 4). However, one interesting observation associated with the pioglitazone-treatment of 3T3-L1 cells was that the nuclear protein extracts from pioglitazone-treated cells did not appear to contain the lower molecular weight PPARγ1, while nuclear extracts from cells treated with insulin contained the predominate PPARγ2 protein, and a small quantity of immunoreactive PPARγ1 (Fig. 4, lanes 1 and 3).

The cellular fractionation studies indicated that a significant quantity of PPARγ2 was located in the cytosol. To verify this observation, an antibody specific for the N-terminal peptide domain of PPARγ2 (p2–16) was used in a collection of immunocytochemical studies. These studies supported our conclusion that undifferentiated 3T3-L1 preadipocytes contained both cytosolic and nuclear PPARγ2 (Fig. 5). The staining pattern for immunoreactive PPARγ2 indicated that PPARγ2 protein was scattered diffusely throughout the cytosol (Fig. 5). However, as the preadipoctyes underwent differentiation to mature fat cells, the cytosolic content of PPARγ2 increased, and the cytosolic PPARγ2 appeared to be concentrated around the lipid vacuoles of the maturing fat cell (Fig. 5). The concentrating of the PPARγ2 around the lipid vacuoles is particularly noticeable at day 5 and day 10 (Fig. 5).

Expression of RXRα during fat cell differentiation

Because undifferentiated 3T3-L1 fibroblasts expressed significant levels of PPARγ2 mRNA and protein (i.e., 30% of fully differentiated cells) (Figs. 2–4), we have con-

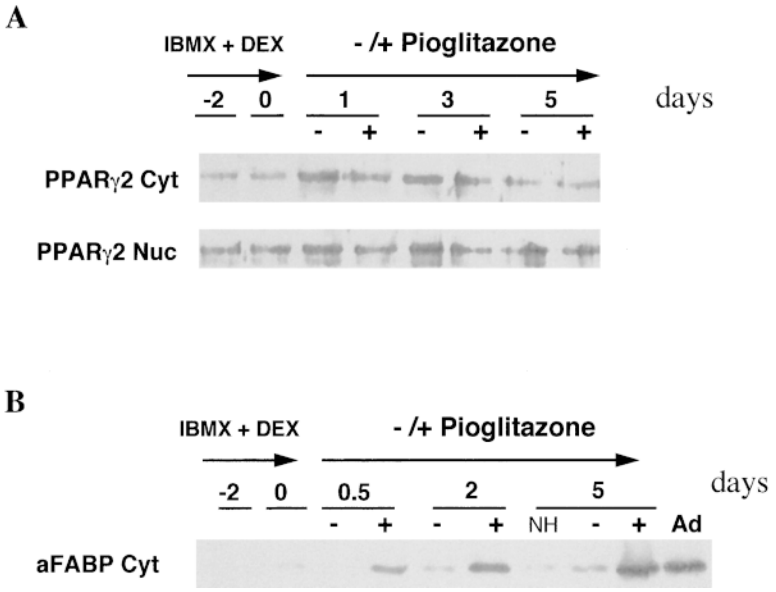

Fig. 4. Changes in nuclear and cytosolic content of PPARγ2 protein during the conversion of 3T3-L1 preadipocytes to mature fat cells. Undifferentiated 3T3-L1 cells were grown to confluence (−2 days) and staged to differentiate as described in Methods. After a 48-h staging period with IBMX and dexamethasone (0 days), the cells were induced to differentiate by changing the media to one containing either 1 μm insulin (designated with the − symbol), or insulin plus 10 μm pioglitazone (designated with the + symbol). (A) At the days indicated in the figure, cytosolic (Cyt) and nuclear (Nuc) protein extracts (10 μg per lane) were prepared for Western blot analysis of PPARγ2 using anti-PPARγ$_{32–54}$. (B) A-FABP protein abundance was determined at the days indicated. Lane NH shows the level of A-FABP in 3T3-L1 cells after they have undergone 2 days of IBMX and dexamethasone treatment followed by 5 days of no hormone or pioglitazone (NH) treatment. The abundance of A-FABP in rat adipose tissue protein extract is depicted in the "Ad" lane (10 μg).

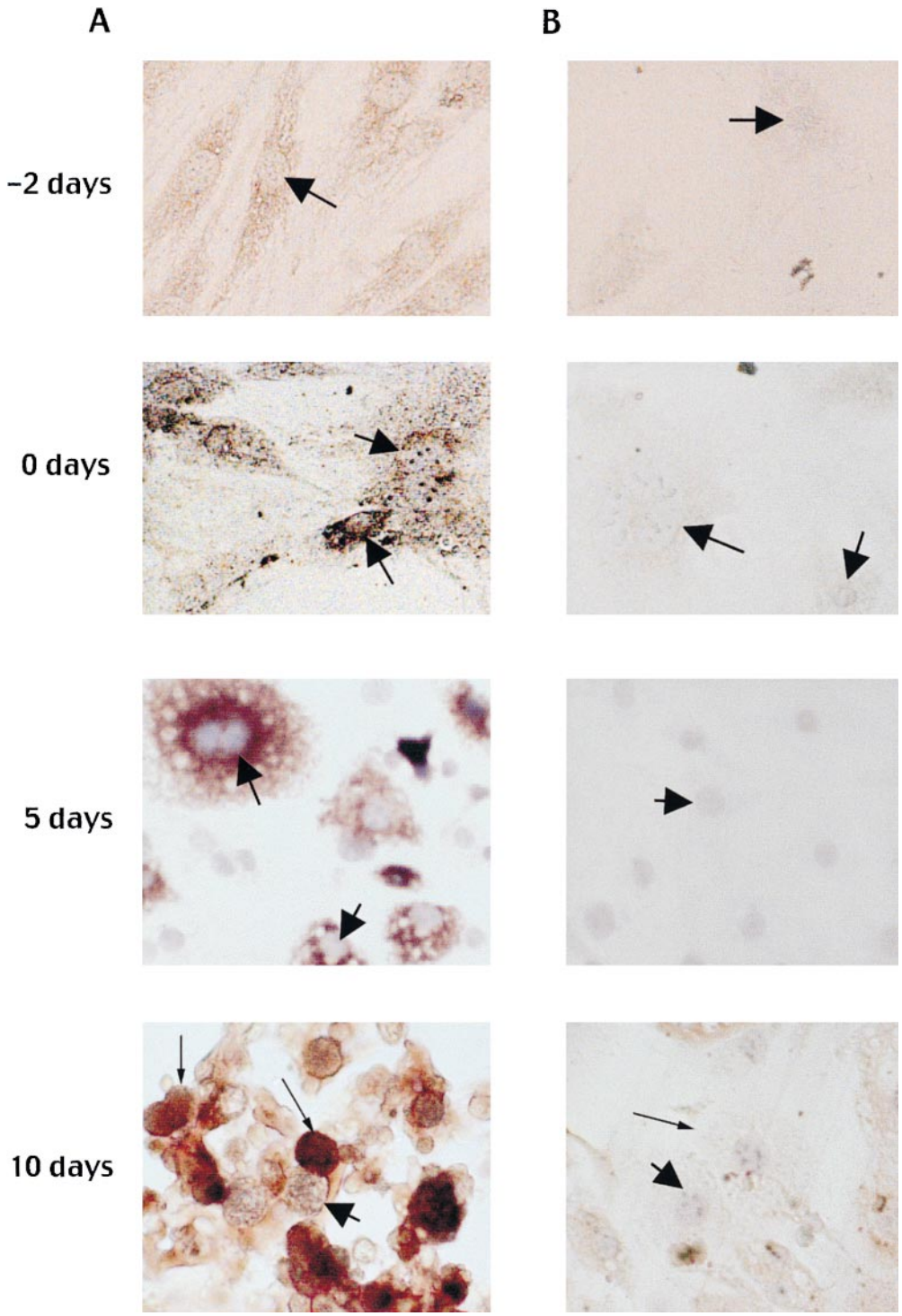

Fig. 5. Intracellular distribution and abundance of PPARγ2 protein in undifferentiated and differentiated 3T3-L1 cells. Undifferentiated 3T3-L1 cells were grown to confluence (−2 days) and staged to differentiate as described in Methods. After a 48-h staging period with IBMX and dexamethasone (0 days), the cells were induced to differentiate by changing the media to one containing 1 μm insulin plus 10 μm pioglitazone. At −2, 0, 5, and 10 days of treatment, the cellular localization of PPARγ2 protein (red) was determined by immunocytochemical staining using PPARγ2 specific anti-PPARγ2$_{4-26}$ (A). Preincubating the anti-PPARγ2 with its specific peptide completely blocked PPARγ2 protein staining (B). Nuclei were detected by hematoxylin staining (pale blue) and are marked by the large arrows. Lipid vacuoles were detected by oil red O staining and are noted by the thin arrows.

cluded that the expression of PPARγ2 is not the limiting factor for the initiation of terminal fat cell differentiation. However, PPARγ2 induces the transcription of fat-specific genes (e.g., A-FABP) by binding to the ARE (DR-1) DNA recognition sequence as a heterodimer with the *trans*-factor RXRα. In light of the fact that PPARγ2 requires RXRα as a partner, we hypothesized that the stimulus for terminal fat cell differentiation may be the induction of

RXRα expression, and that the acceleration of fat cell differentiation by pioglitazone may reflect an amplification in RXRα expression. Consistent with this hypothesis, preadipocytes did not contain measurable quantities of RXRα or A-FABP protein (**Fig. 6**), but treatment of undifferentiated 3T3-L1 cells with IBMX and dexamethasone for 48 h greatly increased the cellular content of RXRα. Cell fractionation studies revealed that unlike PPARγ2, virtually all of the RXRα was located in the nuclear compartment. Moreover, the nuclear content of RXRα increased 6-fold during the subsequent 5 days differentiation (Fig. 6). This rise in nuclear RXRα content was paralleled by an increase in the cellular content of A-FABP mRNA and protein (Figs. 3, 4, and 6). Although it appears that RXRα is required for A-FABP expression and fat cell differentiation, pioglitazone did not enhance or accelerate the expression of RXRα (Fig. 6 and **Fig. 7**).

One surprising finding was that Northern blot analysis of total RNA revealed that abundance of RXRα mRNA was high in undifferentiated 3T3-L1 preadipocytes, but RXRα mRNA was not translated into RXRα protein. RXRα protein was only expressed after the preadipocytes were stimulated to differentiate by treating them with IBMX and dexamethasone (Fig. 6). It would appear that the translation of RXRα mRNA is suppressed in the undifferentiated 3T3-L1 cell.

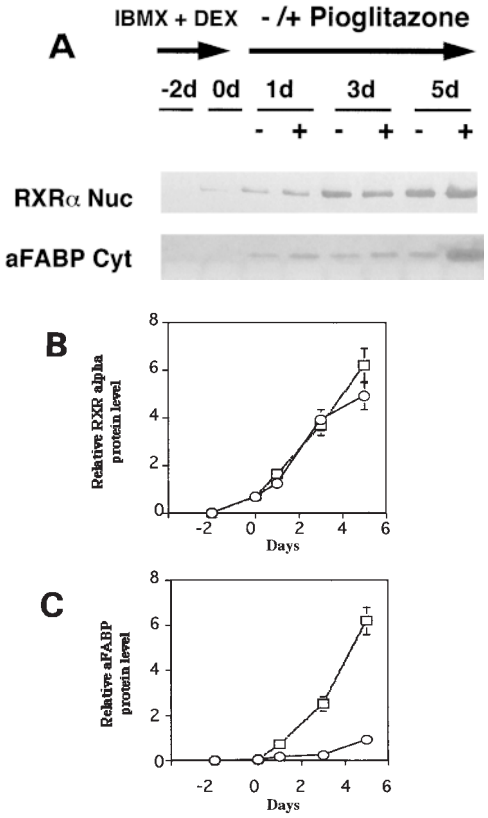

Fig. 6. Pattern of RXRα and A-FABP protein expression as undifferentiated 3T3-L1 cells undergo conversion to mature fat cells. Undifferentiated 3T3-L1 cells were grown to confluence (−2 days) and staged to differentiate as described in Methods. After a 48-h staging period with IBMX and dexamethasone (0 days), the cells were induced to differentiate by changing the media to one containing either 1 μm insulin (designated by the − symbol) or insulin plus 10 μm pioglitazone (designated by the + symbol). (A) Nuclear (Nuc) and cytosolic (Cyt) protein extracts (10 μg per lane) were prepared and utilized for Western blot analysis. Frames (B) and (C) depict the relative changes in RXRα and A-FABP protein as undifferentiated 3T3-L1 preadipocytes undergo differentiation. Values are means ± SD for n = 2–4 cell preparations; circles, insulin and squares, insulin plus pioglitazone.

Pioglitazone enhances the PPARγ2/RXRα interaction with ARE

The previously described data indicate that pioglitazone does not induce PPARγ2 or RXRα gene expression, nor does it increase the cellular content of PPARγ2 or RXRα proteins. Nevertheless, pioglitazone did accelerate the differentiation process as revealed by the marked and rapid induction of A-FABP (Figs. 3, 4, and 6). As pioglitazone is a well-established high affinity, ligand activator of PPARγ2, we examined the ability of pioglitazone to stimulate PPARγ2 binding to its ARE (DR-1) recognition sequence (**Fig. 8**). As expected, nuclear proteins extracted from pioglitazone-treated 3T3-L1 preadipocytes readily bound to the ARE (DR-1) recognition sequence of A-FABP (Fig. 8). This increase in protein binding appeared to involve enhanced PPARγ2 binding because: *a*) cold DR-1 competed for binding; *b*) anti-PPARγ2/γ1 treatment yielded a supershift; and *c*) prolonged incubation of the nuclear extract with PPARγ2-specific antibody abolished protein binding to the ARE (DR-1).

Relative to cells treated with insulin alone, pioglitazone increased PPARγ2 binding to the ARE (DR-1) of A-FABP by 5-fold within 12 h (Fig. 8, lanes 3 and 4). However, after 5 days of insulin treatment, PPARγ2 binding to the ARE (DR-1) was comparable to that of pioglitazone (Fig. 8, lanes 7 and 8). Apparently, sufficient endogenous ligand was produced after 5 days of insulin stimulation to fully activate PPARγ2. Also as expected, the binding of PPARγ2 to its ARE (DR-1) recognition sequence was paralleled by an increase in A-FABP mRNA and an increase in A-FABP protein (Figs. 3, 4, and 6). Finally, the ability of pioglitazone to stimulate PPARγ2 binding to its ARE (DR-1) recognition sequence was very rapid. As an example, nuclear extracts from 3T3-L1 preadipocytes staged to differentiate by treatment with IBMX and dexamethasone showed virtually no PPARγ2 binding to the ARE (DR-1), but PPARγ2 binding to the ARE oligo was detectable within 2–4 h of adding pioglitazone to the media, and near maximal PPARγ2 binding occurred after 12 h of pioglitazone treatment (data not shown).

DISCUSSION

Several years ago we reported that the insulin or IGF-1 induction of fat cell differentiation could be greatly accelerated by the insulin sensitizing agent, pioglitazone (21, 23). One of the most pronounced responses to this thiazolidinedione derivative was the greatly amplified level of expression for the adipocyte-specific gene, A-FABP (23). A

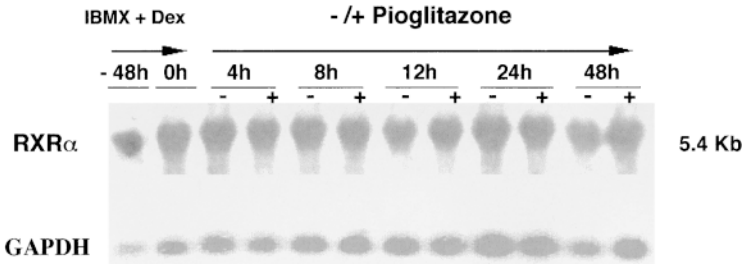

Fig. 7. Changes in RXRα mRNA abundance during the early phases of 3T3-L1 preadipocyte differentiation. Undifferentiated 3T3-L1 cells were grown to confluence (−48 h) and staged to differentiate as described in Methods. After a 48-h staging period with IBMX and dexamethasone (0 h), the cells were induced to differentiate by changing the media to one containing either 1 μm insulin (designated with − symbol), or insulin plus 10 μm pioglitazone (designated with + symbol). Total RNA was extracted at the hours indicated in the figure, and the Northern blot was sequentially hybridized with cDNAs for RXRα and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The molecular size of the RXRα transcript is noted at the right.

collection of studies since this initial observation indicate that thiazolidinediones, as well as free fatty acids, enhance fat cell differentiation by functioning as ligand activators for a group of transcription factors termed PPARs. PPARs, notably PPARγ2, induce the transcription of adipose-specific genes by binding to a DR-1 motif in the 5′-flanking region of the genes (11, 22, 24). The importance of PPARs in fat cell differentiation has been clearly demonstrated by ectopic expression of the PPARγ2 and PPARδ in CHO cells and muscle cells respectively (30, 31). In both of these cases, the cells began to express A-FABP and accumulate lipid in response to ligand activation of the PPARs (30, 31). In light of the pivotal role that PPARs play in fat cell differentiation and adipose-specific gene expression, we hypothesized that insulin stimulated terminal fat cell differentiation by inducing the synthesis of PPARγ2, and that the thiazolidinedione, pioglitazone, accelerated fat cell differentiation by inducing the expression of PPARγ2 and/or its heterodimer partner RXRα. In addition, we hypothesized that PPARγ2, like other steroid-type receptors, existed in both the cytosolic and nuclear compartments, and that ligand activation by pioglitazone would enrich the nuclear content of PPARγ2.

The experimental approach to these hypotheses differed from that of most other investigators in that a two-step differentiation protocol was used. Most prior reports induce the conversion of 3T3-L1 preadipocytes to mature fat cells by first treating the preadipocytes with a combination of insulin, IBMX, and dexamethasone; and then subsequently removing the IBMX and dexamethasone after 48 h. In order to separate the effects of IMBX/dexamethasone from those of insulin, we first treated the 3T3-L1 preadipocytes with IBMX/dexamethasone for 48 h, and then changed the media to one containing insulin. In applying this two-step differentiation approach, we discovered that the marked induction in PPARγ2 mRNA associ-

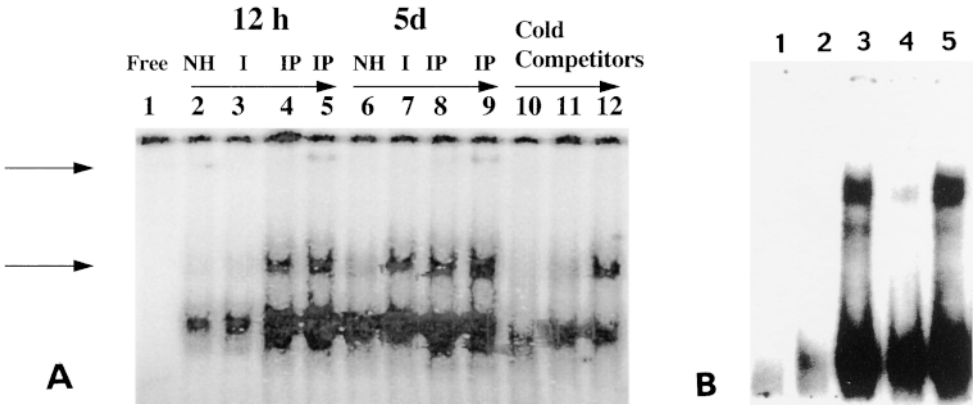

Fig. 8. Pioglitazone enhances PPARγ2 binding to the ARE (DR-1) of the adipose response element from the A-FABP gene. Undifferentiated 3T3-L1 cells were grown to confluence and staged to differentiate as described in Methods. (A) After a 48-h staging period with IBMX and dexamethasone, the cells were induced to differentiate by changing the media to one containing no hormones (NH), 1 μm insulin (I), or insulin plus 10 μm pioglitazone (IP). After 12 h (12 h) and 5 days (5 d) nuclear protein extracts were prepared and utilized in a gel retardation assay using ^{32}P-labeled ARE (DR-1) from the adipose response element of the A-FABP gene. Nuclear protein extracts from cells treated with insulin plus pioglitazone were used in competition assays (lanes 10–12). Lane 10 is a 100-fold molar excess of unlabeled DR-1 oligo from the adipose response element, and lane 11 is a 100-fold molar excess of DR-1 oligo from the peroxisome proliferator response element. Lane 12 is a 100-fold molar excess of a glucocorticoid response element. PPARγ2 binding to the DR-1 oligo is depicted by the lower arrow. Treatment with anti-PPARγ$_{32–54}$ resulted in a supershifted band (upper arrow). (B) Nuclear protein extracts were prepared from confluent untreated, undifferentiated 3T3-L1 cells (lane 2), and fully differentiated (10 days of pioglitazone and insulin treatment) 3T3-L1 cells (lanes 3–5). PPARγ2 interaction with the ARE (DR-1) of the adipose response element is depicted in lane 3; treating the nuclear extract with anti-PPARγ2$_{4–26}$ nearly eliminated the shifted band (lane 4); but treating the nuclear extract with anti-PPARδ had no effect on the PPARγ2 shift. Lane 1 depicts the free probe.

ated with fat cell differentiation (5, 9, 13) was not in response to insulin or pioglitazone, but rather the rise in PPARγ2 mRNA that occurred during fat cell differentiation appeared to be an early phase response to IBMX (presumably resulting from an increase in cAMP) and/or dexamethasone. Interestingly, the increase in PPARγ2 mRNA induced by IBMX/dexamethasone was not associated with an increase in PPARγ2 protein (Figs. 2 and 4). However, when the IBMX/dexamethasone mixture was removed and insulin was added to the media, total cellular PPARγ2 protein increased 2- to 3-fold within 2 h (Fig. 2). These data suggest that during the early stages of fat cell differentiation, the amount of cellular PPARγ2 is, in part, governed by translation control mechanisms, and that these mechanisms may be insulin dependent.

Even though pioglitazone treatment of differentiating 3T3-L1 cells greatly enhanced the expression of A-FABP (Fig. 2), as well as increased the expression of lipoprotein lipase and fatty acid synthase (data not shown), pioglitazone had no effect on the abundance of PPARγ2 mRNA or on the total cellular content of PPARγ2 protein of differentiating 3T3-L1 cells (Figs. 2 and 3). However, pioglitazone treatment of differentiating 3T3-L1 preadipocytes greatly stimulated PPARγ2 binding to its DR-1 recognition sequence of the A-FABP gene. Thus, the pioglitazone-dependent acceleration of fat cell differentiation appears to be largely due to its function as a ligand activator of PPARγ2, rather than as an inducer of PPARγ2 expression.

Because PPARγ2 has been considered the pivotal transcription factor for terminal fat cell differentiation (6, 9–14), we were surprised to discover that 3T3-L1 preadipocytes contained significant quantities (i.e., 30–50% of fully differentiated cells) of PPARγ2 mRNA and protein. Equally surprising was the discovery that PPARγ2 existed in both the cytosolic and nuclear compartments of non-differentiating and differentiating 3T3-L1 cells, and that the insulin-dependent increase in total cellular PPARγ2 occurred largely in the cytosolic compartment. Moreover, the PPARγ2 ligand activator, pioglitazone, did not increase the fraction of PPARγ2 located in the nuclear compartment. The cytosolic component of PPARγ2 appeared to be diffusely distributed throughout the cytosol of the undifferentiated 3T3-L1 cells, but as the preadipocytes underwent differentiation and accumulated lipid, the PPARγ2 became associated with the lipid droplets (Fig. 5). The significance of PPARγ2's association with the lipid droplets is unclear, but PPARγ2 is a receptor for free fatty acids and certain prostanoids. Thus, PPARγ2's association with lipid droplets may simply place it in closer proximity to its fatty acid ligand. Similarly, we can only speculate as to the function of cytosolic PPARγ2, but the relatively high cytosolic content of PPARγ2 in preadipoctyes combined with the observation that the 2- to 3-fold increase in total cellular PPARγ2 occurring during early differentiation is largely restricted to the cytosolic compartment strongly suggest that PPARγ2 may have a cytosolic function. In this regard, it is interesting to note that the 3′-untranslated region of the fatty acid synthase gene contains a consensus DR-1 recognition sequence for PPARγ2/RXRα. Because the rise in fatty acid synthase mRNA of differentiating 3T3-L1 cells is predominately the product of an increased mRNA stability, one might speculate that PPARγ2 binding to the DR-1 of the 3′-untranslated region of the fatty acid synthase transcript could regulate the cellular abundance of fatty acid synthase mRNA by altering its stability. Whatever the function may be, the discovery that significant quantities of PPARγ2 exist in the cytosol of both preadipocytes and mature adipocytes suggests a new cellular role for PPARγ2.

While our data demonstrated that 3T3-L1 preadipocytes contained significant quantities of PPARγ2 protein, nuclear proteins extracted from undifferentiated preadipocytes were not able to bind to the DR-1 recognition sequence (Fig. 8), nor did pioglitazone treatment of preadipocytes stimulate A-FABP expression. If preadipocytes contain ample levels of PPARγ2, what then is the determinant for the expression of fat specific genes such as A-FABP? The answer to this question may lie in the temporal pattern of RXRα expression and/or the production of the ligand activator for RXRα (i.e., 9-*cis* retinoic acid). Transcriptional activation of genes involved in the terminal stages of fat cell differentiation (e.g., A-FABP) requires that PPARγ2 bind to its response element (e.g., TGCACATTTCACC) as a heterodimer complex with RXRα (32, 33). However, even though preadipocytes contain a significant level of RXRα mRNA, they contain no detectable amount of RXRα protein. The reason why the RXRα transcript is translationally repressed in undifferentiated 3T3-L1 cells is unknown, but the nature of this mechanism would appear to play a pivotal role in the terminal differentiation of 3T3-L1 preadipocytes. Regardless of the mechanism, it is only when the preadipocytes are treated with IBMX and dexamethasone that the cellular content of RXRα begins to increase. As the preadipocytes lack RXRα protein, PPARγ2 cannot form a heterodimer with RXRα and consequently it cannot interact with the DR-1 recognition sequence. Thus, the transcription of fat specific genes (i.e., A-FABP) is not initiated, and terminal fat cell differentiation does not occur. In addition to being dependent upon the amount of RXRα, PPARγ2/RXRα interaction with the DR-1 recognition sequence may be enhanced by the binding of 9-*cis* retinoic acid to the ligand binding domain of RXRα. If production of 9-*cis* retinoic acid were low in the differentiating 3T3-L1 preadipocytes, then the rate of differentiation would be dependent upon the rate of 9-*cis* retinoic acid synthesis. Such a possibility is suggested by the observation that RXRα and PPARγ2 proteins are both present in 3T3-L1 preadipocytes after 48 h of IBMX/dexamethasone treatment (i.e., time 0), but detectable levels of A-FABP mRNA and protein are not observed until 4–12 h after the addition of insulin and pioglitazone (Figs. 3 and 4). However, if ligand for RXRα were limiting for A-FABP transcription, then PPARγ2/RXRα binding to the DR-1 (ARE) recognition sequence would be expected to be low in cells treated with and without the PPARγ2 ligand, pioglitazone. In other words, activation of PPARγ2/RXRα binding would be dependent upon the availability of RXRα ligand, and would not be enhanced by the PPARγ2 ligand. This was not the

case. Specifically, treating differentiating 3T3-L1 preadipocytes with pioglitazone enhanced PPARγ2/RXRα binding to the DR-1 recognition sequence several fold within 12 h (Fig. 8). As this increase in DNA binding occurred without a change in RXRα protein concentration, the determining factor for PPARγ2/RXRα binding to the DR-1 (ARE) recognition sequence of the A-FABP gene would appear to be the availability of PPARγ2 ligand, and not the availability of RXRα ligand. This conclusion is supported by the observations that ectopic expression of PPARγ2 has no effect of lipogenic gene expression until PPARγ2 is activated by its ligand (9).

While it is clear that the synthesis of RXRα is essential for the expression of A-FABP and for the terminal differentiation of fat cells, the ability of thiazolidinediones such as pioglitazone to accelerate fat cell differentiation and increase insulin/IGF-1 sensitivity appears to be independent of RXRα expression and the nuclear content of RXRα protein (Fig. 6). Given that pioglitazone does not increase the expression of either PPARγ2 or RXRα during the early phase of terminal fat cell differentiation, the question remains as to how pioglitazone accelerates the differentiation of 3T3-L1 preadipocytes and induces the expression of A-FABP. The answer to this question appears to lie in the observation that pioglitazone is a high affinity ligand for PPARγ2 (11, 22) and that ligand binding to PPARγ2 stimulates PPARγ2/RXRα interaction with its ARE (DR-1) DNA response element (Fig. 8), which in turn induces A-FABP transcription (23, 24). While this mechanism may explain how thiazolidinediones accelerate the transcription of genes involved in terminal fat cell differentiation, it does not explain how pioglitazone shifts the dose–response curve for IGF-1 to the left (i.e., increased IGF-1 sensitivity) (21, 23). The explanation for the enhanced IGF-1 responsivity remains to be elucidated.

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