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Retinoic Acid Blocks Adipogenesis by Inhibiting C/EBPβ-Mediated Transcription

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Adipocyte differentiation is thought to involve sequential induction of the transcription factors C/EBPβ, peroxisome proliferator-activated receptor γ (PPARγ), and C/EBPα. C/EBPα expression is both necessary and sufficient for adipocyte differentiation. Here we report that ectopic expression of either C/EBPα or C/EBPβ induces PPARγ expression and adipogenesis and that retinoic acid (RA) completely inhibits adipogenesis by either form of C/EBP. In studies of normal preadipocytes, RA does not prevent C/EBPβ induction but blocks induction of PPARγ, C/EBPα, and adipogenesis. In transient transfection studies, liganded RA receptor (RAR) specifically blocks transcriptional activation by either C/EBPα or C/EBPβ. These results strongly suggest that C/EBPα substitutes for C/EBPβ to induce adipocyte differentiation and that liganded RAR inhibits adipogenesis by blocking C/EBPβ-mediated induction of downstream genes.

Adipocyte differentiation is a complex process involving a cascade of molecular events triggered by an adipogenic stimulus (45). Two classes of transcription factors play important roles as positive mediators of adipogenic signals: the bZip C/EBP proteins (23, 43, 67, 70) and peroxisome proliferator-activated receptor γ (PPARγ) (60). C/EBPα is induced during adipocyte differentiation (7, 15), and there is much evidence that it is both necessary and sufficient for progression to the mature adipocyte phenotype. C/EBPα binds to and transcriptionally activates a number of adipocyte-specific genes (14). Forced expression of C/EBPα in NIH 3T3 cells results in adipose conversion (23, 43), and prevention of C/EBPα induction in 3T3-L1 cells inhibits adipocyte differentiation (42). In addition, mice which do not express C/EBPα due to genetic alterations have a reduced mass of abnormal adipose tissue (65). C/EBPα binds DNA as a homodimer as well as a heterodimer with other bZip proteins (7) and contains at least three distinct transcriptional activation domains (47, 48).

Although C/EBPα is clearly crucial for adipocyte differentiation, it is not induced until 2 to 3 days following adipogenic stimulation of preadipocyte cell lines (7, 15). Thus, it is widely held that adipogenic stimuli induce a cascade of events which precede and cause induction of C/EBPα and, after 5 to 7 days, full adipocyte differentiation (45, 70). One of the earliest events in adipogenesis is a transitory increase in C/EBPβ (15, 70), whose DNA binding properties are similar to those of C/EBPα (7, 50). C/EBPβ is expressed at low levels in the preadipocyte, but forced expression of higher levels of C/EBPβ is sufficient to induce adipocyte differentiation of 3T3-L1 cells (67, 70). C/EBPβ induction may be causally related to the induction of PPARγ (67), whose gene promoter contains a potential C/EBP binding site (73).

Unlike the C/EBP proteins, PPARγ is expressed almost exclusively in adipocytes (12, 62). Both PPARγ mRNA (12, 62) and protein isoforms γ1 and γ2 (69) accumulate within 24 h after adipogenic stimulation of 3T3-L1 cells. A number of lines of evidence indicate that PPARγ is adipogenic. Various PPAR activators induce adipocyte differentiation of 3T3-L1 cells (11, 71). PPARγ is a specific receptor for thiazolidinediones (22, 39), which also induce adipocyte differentiation (31, 56). Furthermore, forced expression of PPARγ in NIH 3T3 cells leads to adipose conversion in the presence of thiazolidinediones or the prostaglandin ligand 15-deoxy, Δ12, 14-prostaglandin J2 (22, 32).

Retinoic acid (RA) receptor (RAR), another member of the nuclear hormone receptor superfamily, mediates inhibition of adipocyte differentiation by RA (11, 35, 59, 69). Preadipocytes express predominantly RARα and RARγ, which are functionally redundant in their abilities to mediate inhibition of adipocyte differentiation by RA (69). RA is effective only during the first 24 to 48 h after exposure of 3T3-L1 cells to adipogenic stimuli (59, 69), but the actual step at which liganded RAR interferes with the adipocyte differentiation cascade is unknown, as is the underlying molecular mechanism.

We were surprised to find that RA could prevent adipocyte differentiation due to ectopic expression of C/EBPα, which is normally induced after cells are no longer responsive to RA. C/EBPα also induced PPARγ expression, suggesting that C/EBPα might mimic the effects of C/EBPβ. Consistent with this possibility, RA also prevented C/EBPβ-induced adipocyte differentiation and PPARγ induction. In addition, RA prevented normal PPARγ induction by differentiation medium (DM) but did not prevent C/EBPβ induction, indicating that it blocked a step distal to C/EBP induction. Since RA blocked the effects of ectopic C/EBP, we studied the ability of liganded RAR to interfere with C/EBP-mediated transcription. Indeed, liganded RAR inhibited C/EBPα- and β-dependent transactivation in transcription assays. An RAR mutant which inhibits RA signaling from hormone-responsive elements did not interfere with RA inhibition of C/EBP-mediated transcription or adipocyte differentiation, suggesting that liganded RAR inhibited adipocyte differentiation by an alternative mechanism which did not involve the retinoid X receptor (RXR) or direct binding to target genes. These results strongly suggest that liganded RAR blocks the transcriptional activity of C/EBP proteins and that this mechanism underlies the inhibitory effects of RA on adipocyte differentiation.

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MATERIALS AND METHODS

Cell culture and differentiation. 3T3-L1 cells were obtained from the American Type Culture Collection (Rockville, Md.). Cells were cultured in growth medium (GM) containing 10% bovine calf serum in Dulbecco's modified Eagle's medium (DMEM). For adipocyte differentiation, cells were placed in DM containing 10% fetal bovine serum, 10 μg of insulin per ml, 1 μM dexamethasone, and 0.5 mM isobutylmethylxanthine 2 days after cell confluency (day 0). Differentiating cells were maintained in post-DM containing 10% fetal bovine serum and 10 μg of insulin per ml. Cells were switched to post-DM 2 days after initiation of differentiation, and the medium was changed every 3 days. RA dissolved in ethanol was used at a concentration of 10 μM in experiments with 3T3-L1 cells. For the RARαΔ403 experiments, RA was added in ethanol along with DM, and the same concentration of RA was maintained when the medium was replaced. 3T3-L1 cells infected with a control retrovirus and RARαΔ403 were grown to confluence in GM. Two days postconfluency, cells were treated for 24 h with 10 μM RA in GM. Cells were then harvested for RNA.

Gene transduction and selection of 3T3-L1 cells ectopically expressing C/EBPα, C/EBPβ, and RARαΔ403. The C/EBP retroviral expression vectors were constructed by ligating the 1.26-kb *Eco*RI-*Bam*HI fragment of pBS-C/EBPα and the 1.5-kb *Eco*RI-*Bam*HI fragment of MSV-CEBPβ into the *Eco*RI and *Bam*HI sites of pLXSN (46). The RARαΔ403 construct described below was ligated into the *Bam*HI site of LXSN. Ectopically expressing cells were made by using retroviral infection as previously described (69). 3T3-L1 cells were split 1:5 at 24 h after infection and selected for 10 days in DMEM containing 10% calf serum and 400 μg of Geneticin G418 (Gibco BRL) per ml to eliminate uninfected cells. For C/EBP experiments, cells were selected in the presence or absence of 10 μM RA. Independent selection of control cells as well as C/EBPα-, C/EBPβ-, RARαΔ403-expressing cells was performed in at least four separate experiments for each construct. In experiments to show the reversibility of the RA inhibition, cells were selected for 10 days in the presence of RA, then split into dishes containing GM, and grown for 15 days either in the absence or presence of RA or in the presence of RA for 5 days followed by removal of RA for 10 days.

Oil red O staining. 3T3-L1 cells were washed twice with phosphate-buffered saline (PBS), fixed in 10% formalin, dissolved in PBS for 1 h, and washed again once with PBS. Cells were then stained with 60% of a filtered oil red O stock solution (0.5 g of oil red O [Sigma] in 100 ml of 99% isopropanol) for 15 min. Cells were washed twice with water for 15 min each time and visualized.

Northern analysis. Isolation of total RNA and Northern analysis were performed as described previously (10). The cDNA probes for aP2, RARα, RARγ2, and actin were labeled with ^{32}P by using random hexamers.

Western (immunoblot) analysis. For experiments shown in Fig. 1B and 2B, 3T3-L1 cells were lysed in buffered sodium dodecyl sulfate (SDS), and lysate was boiled immediately. One hundred to 200 μg of protein was subjected to SDS-polyacrylamide gel electrophoresis. For experiments shown in Fig. 4, cells were lysed in radioimmunoprecipitation assay buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) in the presence of 1 mg of both leupeptin and aprotinin per ml and sheared through a 21-gauge syringe. Lysates were incubated at 4°C for 30 min, and phenylmethylsulfonyl fluoride was added at 10 μM. Lysates were centrifuged, and protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, Calif.). Then 100 μg of protein was subjected to electrophoresis. In all experiments, proteins were electrophoretically transferred to nitrocellulose membranes. After transfer, Ponceau S staining was performed to ensure equal loading of each sample. Membranes were incubated in TTN (40 mM Tris-HCl, 10 mM Tris base, 150 mM NaCl, 0.2% Nonidet P-40) containing 5% bovine serum albumin (BSA; Fisher) for 2 h to block nonspecific binding. The blots were then incubated with primary antibody, polyclonal anti-C/EBPα or anti-C/EBPβ antiserum (1:200 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.), or anti-PPARγ antiserum (69) (1:1,000 dilution) in TTN containing 3% BSA for 2 h. Membranes were then washed five times with TTN and incubated with a secondary antibody, horseradish peroxidase-conjugated donkey anti-rabbit (1:5,000 for C/EBP antibodies and 1:15,000 for PPARγ antibody; Amersham Life Sciences, Arlington Heights, Ill.), in TTN containing 3% BSA for 1 h. After five washes with TTN, the blots were developed by chemiluminescence (Amersham Life Sciences) and visualized by exposure to autoradiography film.

Reporter plasmids. The C/EBPRE×3 (C/EBP response element × 3)-TK (thymidine kinase)-luciferase reporter was constructed by ligating the oligonucleotide GATCAGATTGTGCAATGT (underlined is the C/EBP binding site in the interleukin-6 gene [1]) into the *Bam*HI-*Bgl*II sites of pTK-luciferase (kindly provided by D. Moore). Insertion of three copies of the oligonucleotide was verified by sequencing. The C/EBPα promoter-luciferase construct (kindly provided by M. D. Lane) contained bp −1500 to +125 of the C/EBPα gene promoter (64) in the *Bam*HI-*Nco*I sites of pGL3-Basic (Stratagene). The RARE (RA response element)-TK-luciferase reporter plasmid contains two copies of the modified rat growth hormone RARE (35BA) and has been previously described (4, 18).

Expression plasmids. Murine RARα (mRARα) and mRARγ1 in pSG5 (kindly provided by P. Chambon) were used as RAR expression vectors. cDNAs encoding C/EBPα and C/EBPβ (kindly provided by S. McKnight) were ligated to the *Eco*RI and *Bam*HI sites of pSG5. The cDNA encoding the LAP protein of C/EBPβ (kindly provided by S. Freytag) was subcloned into the *Bam*HI site of pSG5. RARαΔ403 was constructed by PCR to insert an in-frame stop at codon 404, using the following primers: 5′ ACATGCTGCAAGAGC 3′ (P1 from RAR), 5′ TGGCATGGATCATGGGATCTC 3′ (P2 from RAR), 5′ ATGGA GATCCCATGAGGCTCCATG 3′ (P3 from RAR), and 5′ TAATAAGATCT GGATCC 3′ (P4 from pSG5). PCR was applied to mRARα by using P1 and P2 to produce a 172-bp fragment and using P3 and P4 on mRARα to produce a 200-bp fragment. Overlap PCR was performed by using P1 and P4 on these two fragments. The 370-bp fragment was produced and digested with *Mro*I and *Bam*HI to release a 301-bp fragment. Template pSG5-mRARα was cut with *Bam*HI and *Mro*I to produce a 1,088-bp fragment which was ligated in conjunction with the 301-bp PCR product to the *Bam*HI site of pSG5 to generate the desired RARα dominant negative mutant with a stop codon at amino acid 404. The construct was sequenced to verify the presence of the region produced by PCR, including the in-frame stop codon at 404.

Transient transfection transcription assays. JEG-3 cells were obtained from the American Type Culture Collection and cultured in low-glucose DMEM containing 10% fetal bovine serum and then switched to low-glucose DMEM with 10% charcoal-stripped fetal calf serum just prior to transfection. All transfections were normalized for total DNA and promoter concentration. Two micromolar all-*trans* RA (Sigma) or 2 mM ethyl *P*-[(*E*)2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl]benzoic acid (TTNPB) (kindly provided by J. Grippo) in ethanol or ethanol alone was added 16 h after transfection, and cells were harvested 24 h later. Sixty-millimeter-diameter dishes were transfected via the calcium phosphate precipitation method, using 2 to 5 μg of receptor expression vectors and C/EBP expression vectors as indicated in figure legends, 2 μg of luciferase reporter, and 0.5 μg of β-galactosidase (β-Gal) expression vector. Cells transfected were lysed in Triton X-100, and cell lysates were subject to luciferase and β-Gal assays as described previously (2). Results were normalized to β-Gal activity, and fold activation was calculated.

RESULTS

RA inhibits C/EBPα-mediated adipocyte differentiation and induction of PPARγ. One effect of RA is to prevent C/EBPα induction by adipogenic stimuli. However, this cannot be a direct effect of RA since the period of RA effectiveness occurs prior to the induction of C/EBPα (69). Since C/EBPα is necessary and sufficient for adipocyte differentiation (23, 43, 70), we predicted that induction of adipogenesis by ectopic C/EBPα expression should be insensitive to RA. A retroviral system that we have previously used to ectopically express RARγ1 and RARα1 in 3T3-L1 cells (69) was used to express C/EBPα. Figure 1A shows microscopic examination of the cells 10 days after infection, followed by oil red O staining. Expression of ectopic C/EBPα indeed resulted in adipocyte differentiation of 3T3-L1 cells in the absence of any adipogenic stimuli such as DM or PPAR activators. The differentiation occurred in normal GM approximately 7 to 10 days after G418 selection. Remarkably, treatment with RA during the selection process prevented C/EBPα-induced adipogenesis.

To ensure that the antiadipogenic effect of RA was not due to the selection of a population of cells that was incapable of differentiation, C/EBPα-expressing cells that were initially selected in the presence of RA for 10 days to prevent differentiation as in Fig. 1A were split into separate dishes and monitored for an additional 15 days in three different culture conditions: the absence of RA, the continued presence of RA, and 5 days of RA followed by 10 days of culture in the absence of RA. Figure 1B shows the macroscopic oil red O staining of these cells. After removal of RA, adipogenesis was observed (Fig. 1B, left) to an extent similar to that seen in cells which were not treated with RA. In contrast culture in the continued presence of RA prevented differentiation (Fig. 1B, middle). However, even after culture in RA for 5 additional days, removal of RA for 10 days resulted in spontaneous differentiation of the C/EBPα-expressing cells (Fig. 1B, right) to an extent similar to that for cells which were never treated with RA. Thus, the inhibitory effects of RA on C/EBP-mediated adipogenesis were reversible and not related to selection of cells which lost the inherent capability to differentiate into adipocytes.

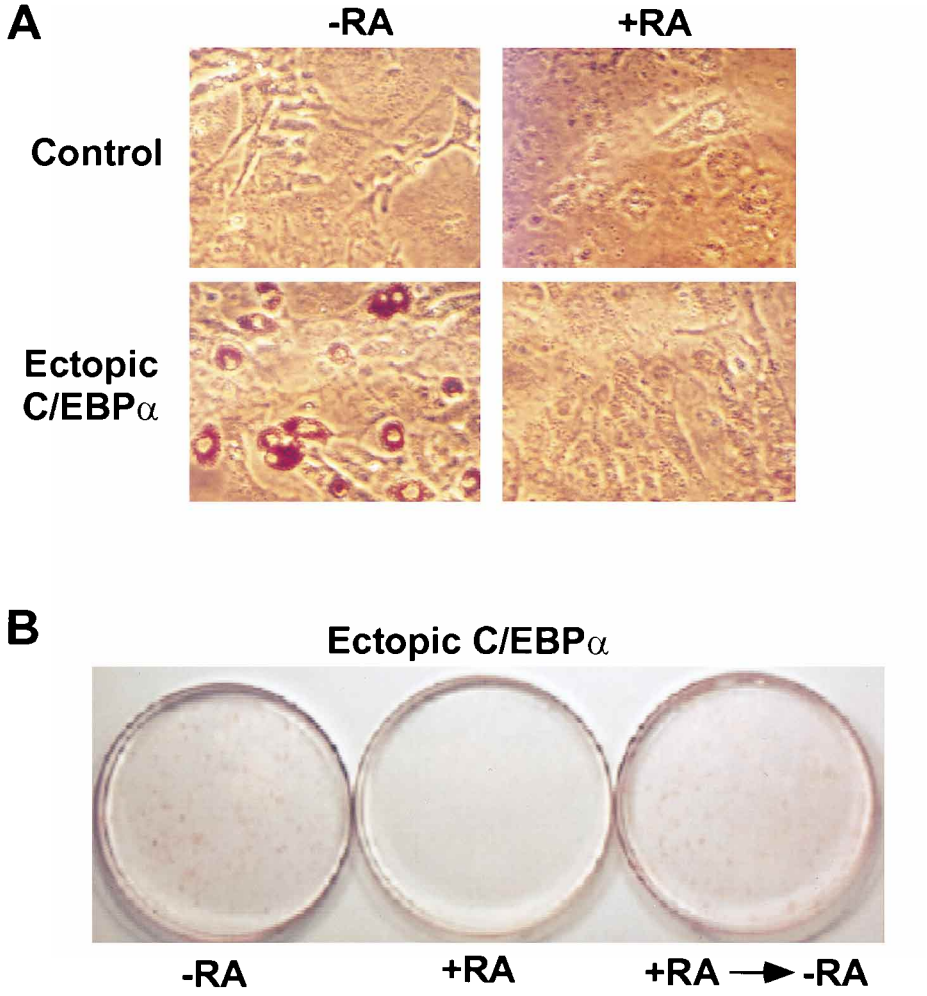

FIG. 1. RA inhibits adipogenesis due to ectopic expression of C/EBPα. (A) Morphology, determined by microscopic analysis of cells stained with oil red O 10 days after infection with control virus or virus directing expression of C/EBPα, in the absence and presence of RA. (B) Macroscopic analysis of cells stained with oil red O. All cells were initially selected in the presence of RA for 10 days and then were split and cultured for 15 days either in the absence (−RA) or presence (+RA) of RA or for 5 days in the presence of RA followed by 10 days in the absence of RA (+RA→−RA). (C) Western analysis of control and ectopic C/EBPα-expressing 3T3-L1 cells for expression of C/EBPα and PPARγ. Pre-ad (lane 1), confluent control 3T3-L1 preadipocytes; Ad (lane 2), 3T3-L1 adipocytes on day 7 of a standard differentiation protocol; lane 3, 3T3-L1 cells ectopically expressing C/EBPα; lane 4, cells ectopically expressing C/EBPα but treated with RA as described in the text. Note that expression of the endogenous C/EBPα (as reflected by the 29-kDa species) and of PPARγ was inhibited by RA, whereas RA did not prevent retrovirally mediated expression of the 43-kDa form of C/EBPα. *, a nonspecific band seen in all lanes.

Western analyses shown in Fig. 1C indicate that ectopic C/EBPα expression also induced the expression of endogenous C/EBPα as well as PPARγ proteins. Two endogenous C/EBPα proteins, 43 and 29 kDa, are induced during adipocyte differentiation (lane 2 and references 42 and 51), whereas the retroviral transgene expressed exclusively the 43-kDa form. Lane 3 shows that the ectopic C/EBPα induced the expression of the 29-kDa protein from the endogenous C/EBPα gene. Consistent with its overall inhibition of adipocyte differentiation, RA blocked the expression of the endogenous C/EBPα gene, as indicated by nearly complete absence of the 29-kDa protein (lane 4). Although this form of C/EBPα itself is not sufficient for adipocyte differentiation (44), prevention of its expression could contribute to the antiadipogenic effects of RA. In contrast, although the total amount of 43-kDa C/EBPα protein was reduced in the presence of RA because of inhibition of endogenous C/EBP synthesis (compare lanes 3 and 4), the adipogenic C/EBPα protein was still present at high levels in the RA-treated cells (lane 4). Thus, RA did not prevent constitutive expression of the ectopic C/EBPα from the retroviral long terminal repeat, indicating that the ability of RA to prevent C/EBPα-induced adipocyte differentiation was not due to reduced expression of the transgenic C/EBPα protein.

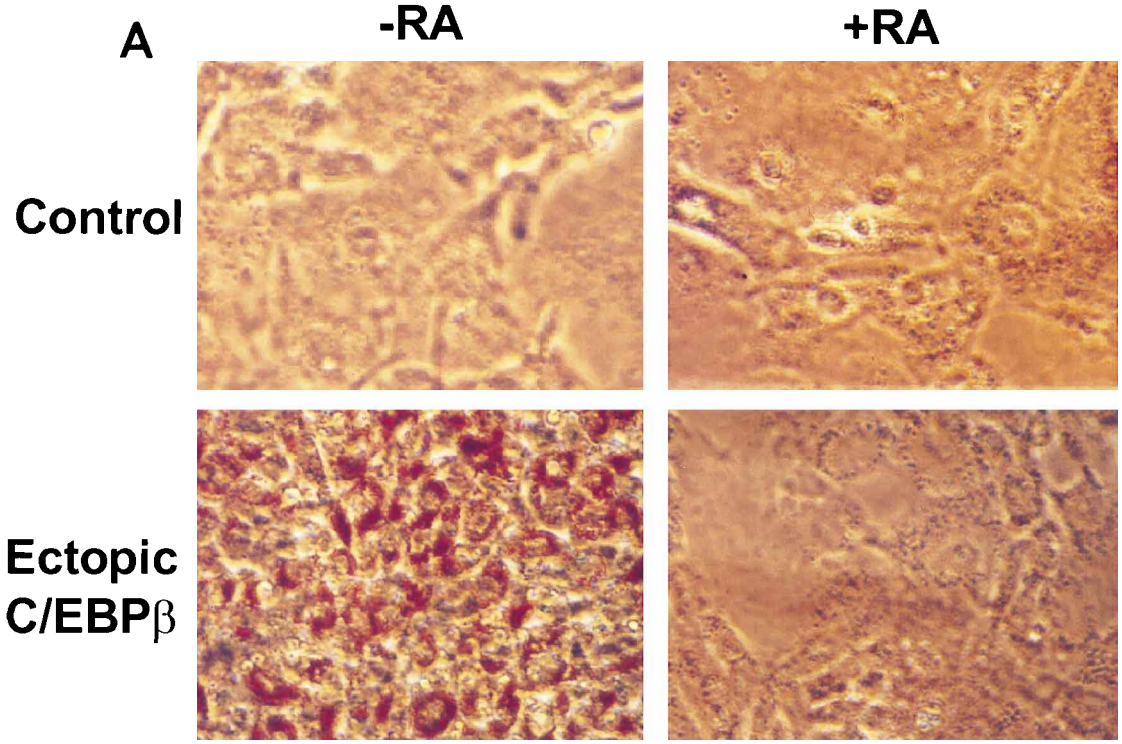

FIG. 2. RA inhibits adipogenesis due to ectopic expression of C/EBPβ. (A) Morphology, determined by oil red O staining of cells 10 days after infection of 3T3-L1 cells with control virus or virus directing expression of C/EBPβ, in the absence (−RA) and presence (+RA) of 10 μM RA. (B) Western analysis of control and ectopic C/EBPβ-expressing 3T3-L1 cells for PPARγ protein expression in the presence and absence of RA.

Ectopic C/EBPα also induced expression of both PPARγ1 and PPARγ2 proteins (Fig. 1C, lane 3). Like adipocyte differentiation in general, the induction of PPARγ was also prevented by RA (Fig. 1C, lane 4). The ability of C/EBPα expression to induce PPARγ is interesting because during normal adipocyte differentiation, C/EBPα is ordinarily induced after PPARγ and is therefore unlikely to be the usual initial inducer of PPARγ (61, 69). Perhaps the physiological role of C/EBPα induction of PPARγ is to maintain the high-level expression of PPARγ in the mature adipocyte.

RA inhibits C/EBPβ-mediated adipocyte differentiation and induction of PPARγ. The effects of C/EBPα on PPARγ expression may be explained by the presence of a consensus C/EBP binding site in the promoter of the PPARγ gene (73). In wild-type cells, C/EBPα may contribute to expression of PPARγ in the mature adipocyte, but this is unlikely to explain the induction of PPARγ early in differentiation because PPARγ is normally expressed before C/EBPα. However, C/EBPβ is induced prior to PPARγ (7) and has DNA binding (3, 50) and transactivation (47, 66) properties which are similar to those of C/EBPα. Indeed, C/EBPβ has been shown to induce the expression of the PPARγ gene and is itself adipogenic (67, 70). We therefore hypothesized that ectopic C/EBPα was mimicking the effects of C/EBPβ, in which case RA would also prevent C/EBPβ-induced adipocyte differentiation. To test this possibility, C/EBPβ was expressed in the same retroviral system as used above, using a cDNA which has previously been shown to promote adipocyte differentiation (67, 70). Figure 2A shows that ectopic expression of C/EBPβ, like C/EBPα, induced adipocyte differentiation. Furthermore, RA completely prevented C/EBPβ-induced adipogenesis. Similarly, PPARγ proteins were expressed in the C/EBPβ-induced adipocytes, and this induction was blocked by RA (Fig. 2B). These results were consistent with previous results showing that RA not only prevents PPARγ induction but, in circumstances where RA inhibits adipocyte differentiation of cells which are already expressing PPARγ, also reverses the induction of PPARγ protein and mRNA (69). Thus, RA prevents adipocyte differentiation by interfering with the actions of C/EBPβ as well as C/EBPα.

RA does not inhibit C/EBPβ induction by adipogenic stimulation. For the reasons described above, the likely step at which liganded RAR interferes with normal adipogenesis is transactivation of genes involved in the differentiation cascade, including PPARγ, by C/EBPβ. This hypothesis predicts that RA will have little effect on the transient induction of C/EBPβ that occurs prior to PPARγ induction. Figure 3 shows that wild-type 3T3-L1 cells express two C/EBPβ-proteins due to translational initiation from different start sites. The larger

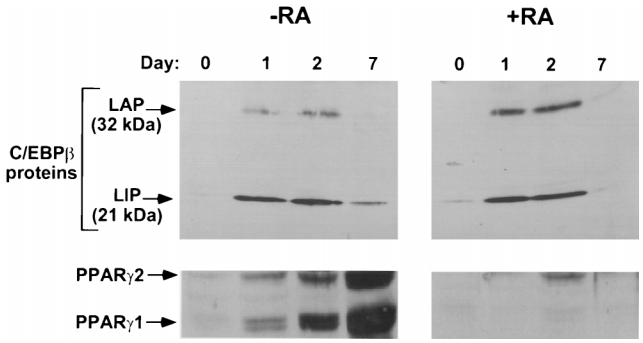

FIG. 3. RA inhibits the normal induction of PPARγ but not C/EBPβ by DM. Shown is Western analysis of C/EBPβ and PPARγ proteins in wild-type 3T3-L1 cells treated with DM for the indicated times in the absence (−RA) and presence (+RA) of RA.

form is a transcriptional activator referred to as LAP, whereas the smaller form, called LIP, lacks the transcriptional activation domains but is still able to bind DNA and may function as an inhibitor (19). Both LIP and LAP are transiently induced early in adipocyte differentiation of wild-type 3T3-L1 cells, as shown in Fig. 3. Consistent with the work of others, some C/EBPβ forms are present in the preadipocyte, and all are maximally induced by day 1 (70). Importantly, Fig. 3 shows that RA treatment did not prevent C/EBPβ induction. This normal regulation of C/EBPβ expression in the presence of RA stands in contrast to the regulation of PPARγ, as shown in Fig. 3, as well as numerous other genes, including those encoding C/EBPα, Rev-ErbAα, lysyl oxidase, and aP2, whose regulated expression during adipocyte differentiation is completely prevented by RA (10, 20, 69). Since RA blocked the induction of PPARγ but not C/EBPβ, the primary mechanism of RA inhibition in normal adipogenesis appears to be at the level of C/EBP-mediated transcription, which would be consistent with the ability of RA to prevent both adipocyte differentiation and PPARγ expression in the cells expressing ectopic C/EBPα or -β.

Liganded RAR inhibits C/EBP-mediated transcription. The ability of RA to block adipocyte differentiation induced by both C/EBPα and C/EBPβ suggested that liganded RAR might directly prevent transcriptional activation by C/EBP proteins. This was tested in transient cotransfection transcription assays in which C/EBP proteins were expressed in the presence of RAR and the effects of RAR ligands were ascertained. JEG-3 human choriocarcinoma cells were used for these experiments because low transfection efficiency of 3T3-L1 cells prevented their use. Figure 4A shows that C/EBPα activated a luciferase reporter containing three copies of the C/EBP-binding site from the interleukin-6 gene (1) and that this was greatly inhibited by RARα in the presence of RA. Figure 4B shows that the RAR-specific ligand TTNPB had a similar effect. Figure 4C shows that RARγ also had the ability to inhibit transactivation due to C/EBPα in the presence of specific ligand. We considered the possibility that liganded RAR could block C/EBP-dependent transactivation by decreasing C/EBPα protein levels. However, the inset to Fig. 4A shows that the level of C/EBPα protein after transfection into JEG-3 cells was minimally affected by coexpression of RAR and treatment with RA.

We also tested the effects of liganded RAR on C/EBPβ-induced transactivation. For these experiments we used the natural C/EBPα promoter, which contains a C/EBPRE (64). Figure 4D shows that C/EBPβ activated transcription from the

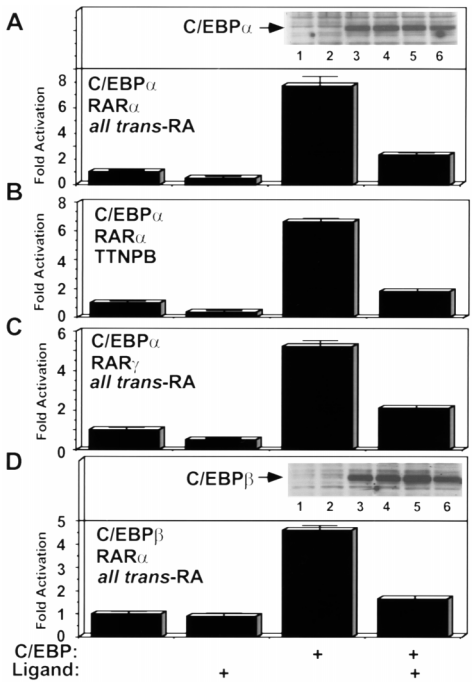

FIG. 4. Liganded RAR inhibits transcription mediated by C/EBPα and β. JEG-3 cells were transfected with RARα or RARγ expression vector in the presence or absence of retinoid ligand or C/EBPα or -β expression vector as indicated. (A) RARα, C/EBPα, and all-*trans* RA with (C/EBPRE×3)-TK-luciferase reporter. Inset, Western analysis of the 43-kDa C/EBPα protein. Lanes 1 and 2, control transfections, without and with RA, respectively; lanes 3 and 4, transfection of C/EBPα, without and with RA, respectively; lanes 5 and 6, transfection of C/EBPα and RARα, without and with RA, respectively. No 29-kDa protein was observed. (B) RARα, C/EBPα, and TTNPB with (C/EBPRE×3)-TK-luciferase reporter. (C) RARγ, C/EBPα, and all-*trans* RA with (C/EBPRE×3)-TK-luciferase reporter. (D) RARα, C/EBPβ, and all-*trans* RA with C/EBPα promoter-luciferase reporter (64). The C/EBPβ expression vector (kindly provided by S. Freytag) was engineered to allow only LAP (and not LIP) expression. Inset, Western analysis of the 32-kD C/EBPβ (LAP) protein. Lanes 1 and 2, control transfections, without and with RA, respectively; lanes 3 and 4, transfection of C/EBPα, without and with RA, respectively; lanes 5 and 6, transfection of C/EBPα and RARα, without and with RA, respectively. No LIP was observed. Unless otherwise indicated, 2 μg of expression vector was used, and the ligand concentration was 2 μM. In each case, the results of a representative experiment are shown as the mean and range of duplicate samples. Each experiment was repeated four to eight times, with similar results.

C/EBPα promoter and that liganded RARα was also able to abolish or greatly reduce transactivation by C/EBPβ. This was not due to a significant decrease in C/EBPβ expression in the transfected cells (inset). Thus, just as endogenous liganded RAR can inhibit C/EBPα- or C/EBPβ-induced adipogenesis in vivo, transfected liganded RAR can inhibit C/EBPα- or C/EBPβ-mediated transactivation in the context of two different promoters and C/EBP binding sites. Since the C/EBPs are thought to induce adipogenesis by directly transactivating adipocyte-specific genes (45), these effects of liganded RAR would be consistent with its role in inhibiting adipocyte differentiation in vivo.

Liganded RAR specifically inhibits the transcriptional activation function of C/EBP. To better understand the mechanism by which liganded RAR inhibited C/EBP function, we tested the effects of liganded RAR on a Gal4-C/EBPα fusion protein which lacks the bZip region but contains the transactivation domains of C/EBPα (amino acids 1 to 199) (47). Figure 5 shows that liganded RAR inhibited transactivation from Gal4 binding sites mediated by the Gal4-C/EBP fusion protein. Both all-*trans* RA (Fig. 5A) and the RAR-specific TTNPB

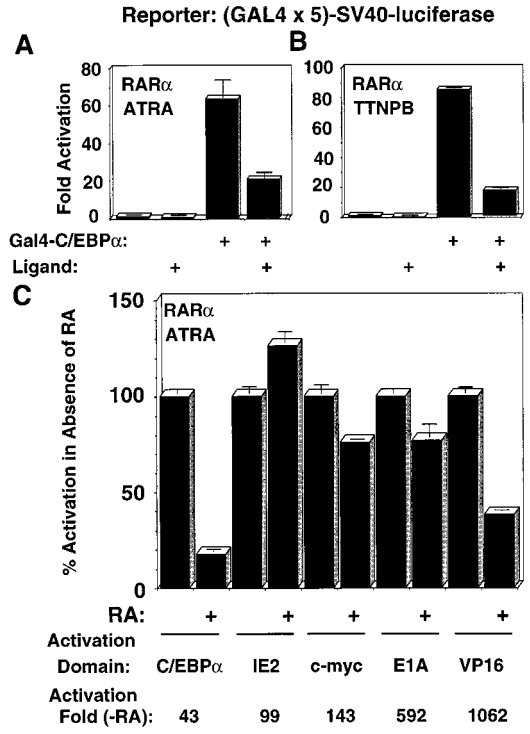

FIG. 5. Liganded RAR inhibits the activity of the C/EBPα transactivation domain fused to a heterologous DNA binding domain. (A) Transfection of JEG-3 cells with RARα expression vector in the presence or absence of all-*trans* RA (ATRA) and the presence or absence of Gal4-C/EBPα expression vector. (B) Same as panel A except that the ligand was TTNPB. (C) Same as panel A except that Gal4(1-147, in pSG424) was also fused to activation domains from c-Myc (amino acids 1 to 262), cytomegalovirus IE2 protein (amino acids 1 to 85), adenovirus E1A protein (amino acids 121 to 223), and VP16 (amino acids 413 to 490) as indicated. For each activation domain, the results are normalized to those in the absence of 2 μM all-*trans* RA. The actual fold activation with each construct in the absence of RA is provided. In each case, 2 μM ligand was used, and 1 μg of each Gal4 activation domain construct was transfected except for Gal4-VP16, in which 10 ng of expression plasmid was transfected because of the relative strength of this activation domain.

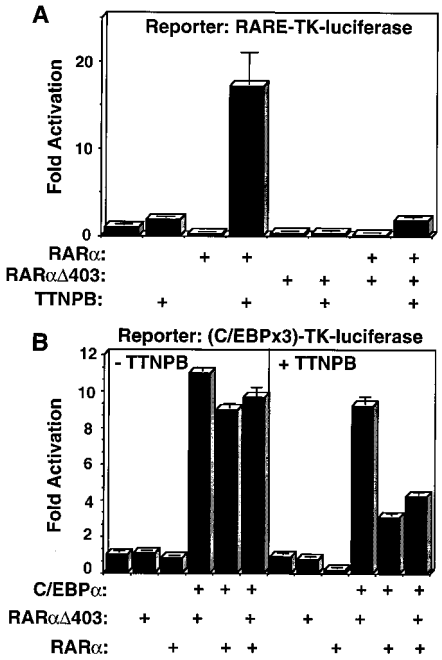

FIG. 6. Inhibition of C/EBP-mediated transcription by liganded RAR is not blocked by a dominant negative RAR. (A) RARαΔ403 dominantly inhibits ligand-dependent transactivation from an RARE by RAR. JEG-3 cells were cotransfected with RARα and RARαΔ403 expression vectors using (rat growth hormone RARE 35BA)-TK-luciferase (4, 18) as reporters; 2 μg of expression vector and 2 μM TTNPB were used. (B) RARαΔ403 has no effect on RA-dependent inhibition of C/EBP-mediated transcription by RAR. JEG-3 cells were cotransfected with combinations of C/EBPα (5 μg), RARα (2 μg), and RARαΔ403 (2 μg) expression vectors in the absence (left) or presence (right) of TTNPB (2 μM).

(Fig. 5B) allowed RAR to function as an inhibitor of Gal4-C/EBPα. Thus, liganded RAR inhibited transactivation by the C/EBPα activation domain in a manner which was independent of DNA binding as well as the bZip region of C/EBP. Consistent with this conclusion, RAR did not displace C/EBPα from binding to a canonical C/EBP binding site (data not shown).

We next examined the specificity of the effects of liganded RAR on C/EBP-mediated transcription. This was tested by comparing the effects of liganded RAR on a variety of activation domains fused to GAL4. Figure 5C shows that a variety of transcriptional activation domains, including those from c-Myc (amino acids 1 to 262 [30]), cytomegalovirus IE2 protein (amino acids 1 to 85 [53]), and adenovirus E1A protein (amino acids 121 to 223 [41]) were essentially unaffected by cotransfection of RAR and treatment with RA under the same conditions in which Gal4-C/EBPα, as well as C/EBP itself, were dramatically inhibited. Interestingly, the potent VP16 transcriptional activation domain was inhibited by liganded RAR, although to a lesser extent than was Gal4-C/EBP. This result is consistent with the recent finding that ligand-dependent transactivation by nuclear receptors involves a protein, SRC-1, which also functions as a coactivator for VP16 (49). Thus, one explanation for the findings is that ligand-bound RAR, C/EBP, and VP16 utilize a related, limiting coactivator which is less important or uninvolved with the transactivation domains of c-Myc, IE2, and E1A.

An RAR mutant which acts in a dominant negative manner on RA-responsive genes does not prevent C/EBP-mediated transcription or inhibition of adipocyte differentiation by RA. The role of the AF2 activation domain of RAR in the inhibition of C/EBP-mediated transcription was explored by using RARαΔ403, a dominant negative RAR mutant which lacks the AF2 activation domain (17, 21) and functions as a constitutive repressor of RARE-containing genes by binding as a heterodimer with RXR (13, 16). We examined whether RARαΔ403 could inhibit C/EBP-mediated transactivation on its own or whether it could reverse the inhibitory effects of liganded RAR on this process. Figure 6A confirms that RARαΔ403 did not activate transcription from an RARE, but due to its preserved ability to interact with RAREs as a heterodimer with RXR, this AF2-defective RAR was able to dominantly inhibit retinoid stimulation of RARE-dependent transcription (compare lanes 4 and 8). Figure 6B shows that RARαΔ403 had no significant effect on C/EBP-activated transcription. Moreover, RARαΔ403 had little or no ability to reverse the inhibitory effect of liganded RAR on transactivation by C/EBP (Fig. 6B; compare lanes 11 and 12). Thus, consistent with its inability to prevent the inhibitory effects of RA on adipogenesis, the AF2-deleted RAR mutant lacks the ability to reverse inhibition of C/EBP-mediated transcription by liganded RAR.

The data thus far suggest that liganded RAR inhibits adipocyte differentiation by inhibiting C/EBP-mediated transcrip-

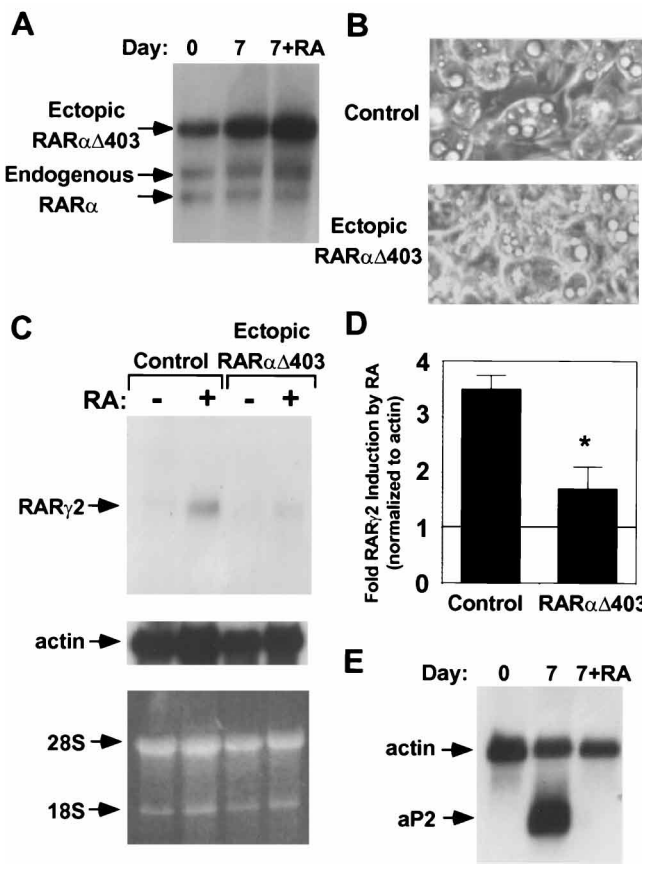

FIG. 7. Dominant negative RAR does not prevent the inhibitory effects of RA on adipocyte differentiation. (A) Expression of RARαΔ403 in 3T3-L1 cells by using a retrovirus. Northern analysis using an RARα-specific probe shows endogenous and ectopic RARα before (day 0) and after treatment with DM in the absence and presence of RA (day 7 and 7+RA, respectively). The ectopically expressed RARαΔ403 transcript is larger than the two endogenous transcripts, as expected. (B) 3T3-L1 cells expressing RARαΔ403 differentiate normally. Cells are shown 7 days after treatment with DM 2 days postconfluency. (C) Ectopic expression of RARαΔ403 inhibits RA induction of the RARγ2 gene. Northern analyses using probes specific for RARγ2 and actin are shown. Also shown are β-actin hybridization and ethidium bromide fluorescence of rRNA on the gel used for the representative RARγ2 Northern blot shown. (D) Quantitation of the RARγ2 expression in three separate experiments. Results were quantified by densitometry and phosphorimaging and were normalized to β-actin levels and to the RARγ2 expression in the absence of RA. Shown are the mean results of three experiments and the standard deviations of the measurements. *, $P < 0.05$ compared with fold activation in the absence of RA. (E) Ectopic expression of RARαΔ403 does not block RA inhibition of adipocyte differentiation. The Northern blot shown in Fig. 1A was reprobed for the adipocyte-specific aP2.

tion rather than by directly regulating RARE-containing genes. This was tested by constitutively expressing RARαΔ403 in 3T3-L1 cells by using the retroviral expression system used to express C/EBPα and -β in the earlier experiments. Figure 7A shows that the transgene was expressed at levels equal or greater than that of endogenous RARα, in a manner which was independent of RA. These expression levels were similar to those observed by other groups using this mutant to interrupt RA-dependent hematopoiesis (63) as well as skin development (26). Nevertheless, the cells differentiated into adipocytes, as shown morphologically in Fig. 7B and by aP2 expression in Fig. 7E. In some experiments, there was less than 100% adipose conversion, perhaps reflecting levels high enough to compete with PPAR for RXR, as was previously

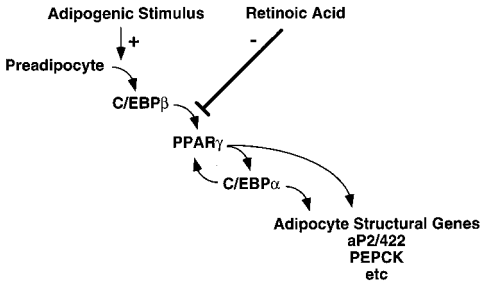

FIG. 8. Cascade model of adipocyte differentiation. Time proceeds from left to right. The ability of C/EBPβ expression to induce PPARγ (67) was confirmed in this study. The ability of C/EBPα expression to induce PPARγ was shown in this study for the first time. PPARγ and C/EBPα both induce a number of adipocyte-specific genes, as indicated. In addition, C/EBPα promoter is regulated by C/EBPα (64) and probably C/EBPβ (Fig. 4D). This study shows that RA blocks adipocyte differentiation by ectopic C/EBPα or C/EBPβ and that the likely mechanism of RA inhibition of wild-type adipocyte differentiation is interference with C/EBPβ function, as shown. PEPCK, phosphoenolpyruvate carboxykinase.

observed when full-length RAR was ectopically expressed in 3T3-L1 cells (69).

The effectiveness of the transgene at inhibiting RA-dependent transcription was tested by studying the ability of RA to induce the expression of RARγ2, which is expressed in 3T3-L1 cells (69). RARγ2 mRNA has been previously shown to be RA inducible in preadipocytes, and the RARγ2 gene promoter contains a well-characterized RARE (28, 40). Figure 7C shows that RARγ2 gene expression was indeed induced by RA treatment of cells which had been infected with control retrovirus. In contrast, the effect of RA on RARγ2 expression in the RARαΔ403-expressing cells was greatly attenuated. Quantitation of the results, shown in Fig. 7D, indicated that the RA-dependent increase was reduced from ~250 to 70%. This result was consistent with the inhibitory function of the dominant negative form of RAR on the RARE-containing gene, indicating that the RARαΔ403 protein was functional. Remarkably, however, despite the expression of functional dominant negative RAR, RA was completely effective in preventing adipocyte differentiation of the RARαΔ403-expressing cells, as shown by expression of the adipocyte-specific marker aP2 (Fig. 7E) as well as by cell morphology (not shown). These results strongly support the model that endogenous liganded RAR inhibits adipocyte differentiation by a nonclassical mechanism which does not involve interaction with RAREs, i.e., by inhibiting C/EBP-mediated transcription.

DISCUSSION

We have shown that liganded RAR inhibits C/EBP-mediated adipocyte differentiation, most likely by inhibiting C/EBP-dependent gene transcription. The interpretation of our results in the context of the cascade model of adipocyte differentiation is shown in Fig. 8. This model is consistent with the findings that strongly suggests that RA inhibits adipocyte differentiation by interfering with the function of C/EBPβ: (i) RA did not block the induction of endogenous C/EBPβ early in adipogenesis, (ii) C/EBPβ is sufficient to induce adipogenesis, and (iii) RA blocked C/EBPβ-induced adipocyte differentiation.

We considered the possibility that RAR inhibits adipocyte differentiation by interfering with PPARγ signaling. RAR and PPAR both require RXR for high-affinity DNA binding (5, 33, 72), and the RAR-RXR heterodimer inhibits transcription from DR1 sites whereas PPAR-RXR activates from similar sites (34, 36). Thus, RAR could potentially inhibit PPAR func-

tion by sequestration of RXR or by competing for with PPAR-RXR heterodimers for DR1 PPAR response elements. However, the present results show that inhibition of PPARγ function cannot be the primary mechanism of RA inhibition of adipocyte differentiation because RA prevents the induction of PPARγ. This finding indicates that the liganded RAR affects a step in the adipocyte differentiation cascade prior to expression of PPARγ. RA also has the ability to reverse induction of PPARγ in cells treated with RA after exposure to adipogenic stimulation but before commitment to differentiation (reference 69 and data not shown). By contrast, RA did not block C/EBPβ induction during adipogenesis. Expression of C/EBPβ induces PPARγ, as shown in this report and by others (67, 68). Thus, it is likely that RA prevents the induction of PPARγ by blocking this step, i.e., direct transcriptional activation of PPARγ expression by C/EBPβ.

RA also inhibited C/EBPα-induced adipocyte differentiation. This is unlikely to be the mechanism by which RA prevents normal adipocyte differentiation because RA is effective only at times prior to the induction of C/EBPα. Our observation that C/EBPα induces PPARγ, which is ordinarily induced prior to C/EBPα, strongly suggests that C/EBPα induces adipocyte differentiation by substituting for C/EBPβ. This would be consistent with the extensive similarities between the transactivation domains and DNA binding specificities of C/EBPα and C/EBPβ (66). Nevertheless, C/EBP induction is necessary for normal adipocyte differentiation (42), and C/EBPα regulates numerous adipocyte structural genes at times when C/EBPβ expression has returned to levels comparable to those in the preadipocyte (reference 45 and references therein). Many of these genes are also targets of PPARγ, which may synergize with C/EBPα and prevent RA from inhibiting this process at later times when expression of both PPARγ and C/EBPα is high. Alternatively, loss of responsiveness to RA may reflect changes in cellular coactivators during differentiation.

The inability of dominant negative RAR to prevent inhibition of adipogenesis by RA suggests that RA inhibits C/EBP function by a mechanism which does not involve RAR binding to RAREs. Similar cross talk between RAR, as well as other nuclear receptors, and AP1 signaling has been described (reviewed in references 52 and 55). In addition, the glucocorticoid receptor can inhibit NF-κB (6, 54, 57), while the estrogen receptor was recently shown to interfere with NF-κB and C/EBPβ (58). The ability of liganded RAR to inhibit transcriptional stimulation by Gal4-C/EBP indicates that this inhibition does not require specific DNA binding by the C/EBP bZip region but does involve the activation domain of C/EBP. Furthermore, the lack of effectiveness of RARαΔ403 suggests that the C-terminal activation domain (AF2) of RAR is required for interference with C/EBP function. This could occur at the level of a shared coactivator whose binding requires AF2 such as SRC-1 (49), RIP-140 (8), Trip1 (38), TIF-1 (37), and estrogen receptor-associated proteins (24) or a more downstream target analogous to RA inhibition of AP1 signaling by converging on CBP/p300 (9, 25, 29). However, to date these cofactors have not been particularly receptor specific. In contrast, the biological phenomenon of RA inhibition of adipogenesis is clearly receptor specific since RXR-specific ligands do not interfere with adipocyte differentiation (69) despite the fact that 3T3-L1 cells express ample amounts of RXR (11). The ability of liganded RAR to inhibit C/EBP transcription was specific, in that a number of activation domains from other transcription factors were minimally affected. Another activation domain, from VP16, was modestly inhibited by liganded RAR, which is perhaps not surprising given the known functional interaction of VP16 with SRC-1 (49) as well as the likely involvement of some coactivators in mediating transcription by different families of transcription factors (27). Such issues of specificity will need to be further explored.

In sum, we have identified C/EBP-mediated transcription as the target for RA inhibition of adipogenesis. It is important to emphasize that this mechanism pertains to RA interruption of normal adipocyte differentiation and, moreover, involves endogenous RAR. Thus, in addition to demonstrating the novel convergence between RAR and C/EBP, this work provides a rare example of the physiological relevance of nuclear hormone receptor cross talk with other signaling pathways.

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The first two authors contributed equally to this work.

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