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Distinct Stages in Adipogenesis Revealed by Retinoid Inhibition of Differentiation after Induction of PPARγ

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Retinoic acid (RA) inhibits adipocyte differentiation of 3T3-L1 preadipocytes but is effective only early in adipogenesis. RA prevented induction of the adipogenic factors PPARγ and C/EBPα. Using receptor-specific ligands, we determined that the effects of RA were mediated by liganded RA receptors (RARs) rather than retinoid X receptors. Preadipocytes expressed primarily RARα and RARγ; during adipocyte differentiation, RARα gene expression was nearly constant, whereas RARγ1 mRNA and protein levels dramatically decreased. Ectopic expression of RARγ1 extended the period of effectiveness of RA by 24 to 48 h; RARα expression had a similar effect, suggesting functional redundancy of RAR subtypes. Remarkably, RA inhibited differentiation when added after PPARγ1 and PPARγ2 proteins had already been expressed and resulted in the loss of PPARγ proteins from cells. By 72 to 96 h after the induction of differentiation, RA failed to prevent differentiation of even ectopic-RAR-expressing cells. Thus, the unresponsiveness of 3T3-L1 preadipocytes to RA after the induction of differentiation is initially due to the reduction in cellular RAR concentration rather than to the induction of PPARγ. At later times cells continue along the differentiation pathway in a manner which is RA and RAR independent.

Retinoic acid (RA) acts as an inducer of differentiation in a number of biological systems (62). However, RA is an inhibitor of adipocyte differentiation (38, 59). Adipocyte differentiation is a complex process, during which a variety of transcription factors, including c-jun and c-fos (16), C/EBPα, -β, and -δ (5, 12), CHOP (56), ADD1 (68), PPARγ (9, 66), and Rev-ErbAα (7), are induced. C/EBPα and -β are sufficient for the induction of adipocyte differentiation of NIH 3T3 fibroblast cells (20, 49, 72, 74), as is the nuclear receptor PPARγ (65). Together, C/EBPα and PPARγ synergistically induce adipogenesis not only in fibroblasts but also in myoblasts (27). Adipocyte differentiation, induced either by a standard hormone cocktail or by PPAR activators, is prevented by the addition of RA (8, 38, 63) only when the RA is added within 24 to 48 h of the exposure of preadipocytes to differentiating conditions (38, 63).

RA regulates gene expression through two classes of nuclear hormone receptors, RA receptors (RARs) and retinoid X receptors (RXRs) (45, 51). Many of the effects of RA are due to direct binding to RARs, which function best as transcriptional regulators in the form of heterodimers with RXRs. While RXR can regulate gene transcription either as a homodimer or as a liganded heterodimer partner of orphan receptors, such as LXR and NGFI-B (1, 14, 19, 55, 71), it appears that the RXR partner of the RAR-RXR heterodimer often functions as an unliganded, "silent" partner (39). There are three RAR subtypes encoded by different genes, with multiple isoforms generated by alternative promoter usage and alternative splicing (45, 51). One of the most important issues raised by the discovery of multiple RARs concerns the specificity of gene regulation by individual RARs. The results of transient-transfection experiments have been inconclusive because although there are some examples of qualitative isoform-specific gene regulation, most differences in target gene regulation are quantitative, with much overlap (54). Mice which lack a single RAR subtype are nearly normal (47, 50), suggesting functional redundancy. Knockout of endogenous RARα and RARγ in embryonic stem cells has suggested that these subtypes have different functions in vivo (4), but the recent finding that many of the defects in RARγ null cell lines can be compensated for by RARα or RARβ also suggests at least partial functional redundancy (64).

We have investigated the mechanism of RA inhibition of adipocyte differentiation by determining the retinoid receptors which mediate the RA signal, the molecular basis for the early loss of responsiveness to this signal, and the effects of RA on adipogenic factors C/EBPα and PPARγ. Studies with receptor-specific retinoids indicated that the actions of RA were predominantly mediated by RARs. 3T3-L1 preadipocytes were found to express primarily RARα and RARγ. Both RARγ1 mRNA and protein were rapidly down-regulated during adipocyte differentiation. Ectopic expression of RARγ1 extended the period during which after exposure to differentiation medium RA was effective in preventing adipogenesis. During this time, RA inhibited adipocyte differentiation of cells which had already induced PPARγ protein and led to the loss of PPARγ from cells. At later times, cells progressed to another stage marked by the inability of RA to prevent terminal adipocyte differentiation despite ectopic expression of RAR.

MATERIALS AND METHODS

Chemicals. All-*trans* RA was purchased from Sigma (St. Louis, Mo.), 9-*cis* RA and TTNPB were obtained from J. Grippo (Hoffmann-La Roche), and SR11237 were obtained from Jeffrey Winkler (University of Pennsylvania).

Cell culture and differentiation. 3T3-L1 cells were obtained from the American Type Culture Collection (Rockville, Md.). Cells were cultured in growth medium containing 10% bovine calf serum (HyClone) in Dulbecco's modified Eagle's medium. For adipocyte differentiation, cells were switched to differentiation medium (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, referred to as day 0. Differentiating cells were maintained in post-DM containing 10% fetal bovine serum and 10 μg of insulin per ml with a change of post-DM at an interval of 3 days after 2 days of initiation of differ-

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entiation by DM. In some experiments, retinoids were added in ethanol along with DM and the same concentration of retinoid was maintained when the medium was changed. For RA time course experiments, 10 μM all-*trans* RA dissolved in ethanol was added to stable cell lines either at the time of DM (day 0) or after 24, 48, or 72 h (days 1 through 3, respectively) and the concentration was consistently maintained after addition.

Gene transduction and selection of 3T3-L1 cells ectopically expressing RARs. The RARγ1 and RARα viral expression vectors were constructed by ligating the full-length cDNA encoding each receptor into the *Eco*RI site of LXSN (53). 293T cells were seeded on six-well dishes and transfected at 10^6 cells per well by calcium phosphate precipitation with 7.5 μg each of SV-E-MLV-env-, SV-Ψ$^-$-env$^-$-MLV-, and LXSN-derived expression plasmids as previously described (41). Viral supernatants were harvested at 48 h after transfection. Dishes (diameter, 100 mm) of 3T3-L1 preadipocytes were infected at 70% confluency with each recombinant virus. Viral supernatants were added to cells in Dulbecco's modified Eagle's medium containing 10% calf serum and 8 μg of Polybrene per ml in a final volume of 8 ml. Cells were split 1:5 at 24 h after infection and selected for 7 days in Dulbecco's modified Eagle's medium containing 10% calf serum and 400 μg of Geneticin (G418; Gibco BRL) per ml to eliminate uninfected cells. Independent selection of control cells as well as RARα1- and RARγ1-expressing cells was performed at least three times for each virus.

Northern (RNA) analysis. Isolation of total RNA and Northern analysis were performed as described previously (7). The cDNA probes for aP2, RARγ1, PPARγ1, C/EBPα, and actin were labeled with ^{32}P by using random hexamers.

RT-PCR and Southern analysis. First-strand cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (RT; Gibco BRL) on the primer of oligo(dT) from total RNA extracts. For the detection of the signals of RARγ1 and RARγ2 mRNAs and the existence of a dominant negative form, the primers specific for RARγ1 (P4), RARγ2 (P5), and dominant negative form (P3) were used in PCR with *Pfu* polymerase (Stratagene) to amplify specific signals. The sequences of the primers were as follows: P1 (nucleotides 754 to 768 of RARγ2 [22]), 5′-ATT GTG GAG TTT GCG-3′; P2 (nucleotides 1306 to 1323 of RARγ2 [22]), 5′-GCC AGG CTT CGA GGA GTC-3′; P3 (nucleotides 1207 to 1224 of dominant negative RARγ [52]), 5′-CCC GGA TCC GAA TTC GAG AGA GTT GAC TCA GAA-3′; P4 (nucleotides 501 to 518 of RARγ1 [31]), 5′-GGT CGC CAC CAT TTG AGA-3′; and P5 (nucleotides 166 to 183 of RARγ2 [22]), 5′-TGC TTC GCC GGA CTT GAG-3′. To exclude the possibility of genomic-DNA contamination in RNA samples, a control reaction was performed under the same conditions as those of experimental reactions, except for the lack of Moloney murine leukemia virus. RT-PCR products were run on 1% agarose gels and transferred to nylon membranes. Southern blot was performed with ^{32}P-labeled RARγ probe with sequences common to RARγ1 and RARγ2.

Nuclear extraction and electrophoretic gel mobility assay. For nuclear extraction, cells were washed with phosphate-buffered saline (PBS) once, harvested in PBS, and then collected by centrifugation (1,000 × *g*). The cell pellet was resuspended in nuclear harvest buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 8.0], 50 mM NaCl, 0.5 M sucrose, 0.1 mM EDTA, 0.5% Triton X-100, 1 mM dithiothreitol 5 mM $MgCl_2$) with 1 μg of leupeptin per ml, 1 μg of aprotinin per ml, and 0.125 mM phenylmethylsulfonyl fluoride; nuclei were collected by centrifugation (2,000 × *g*) and resuspended in the same buffer; and then spermidine and sodium chloride were added to final concentrations of 5 mM and 0.5 M, respectively. Samples were mixed well, incubated on ice for 30 min, and then centrifuged at 20,000 rpm in an Eppendorf microcentrifuge for 30 min. The supernatants were dialyzed against dialysis buffer (10 mM HEPES [pH 8.0], 1 mM dithiothreitol, 20% glycerol, 50 mM KCl) with 0.125 mM phenylmethylsulfonyl fluoride. Then the protein concentrations of samples were determined with a Bio-Rad protein assay reagent. For gel shift experiments, the RA response element (RARE) from the RARβ gene was labeled with [α-^{32}P]dCTP and used as a probe. Nuclear extracts or in vitro-translated RARα and RXRα were used in a 30-μl reaction mixture [10 mM HEPES (pH 7.9), 80 mM KCl, 5% glycerol, 33.3 μg of poly(dI-dC) per ml, 25 ng of denatured salmon sperm DNA per ml, 10 mM dithiothreitol, 33.3 mg of bromphenol blue per ml, 100,000 cpm of ^{32}P-labeled RARβ RARE], in some cases with the addition of 5 μl RARγ1-specific antiserum or nonimmune serum. The reaction mixtures were incubated at room temperature for 30 min and then run on a 6% acrylamide gel, and the dried gel was subjected to autoradiography.

Antibodies. The peptide EMLSPSFRGLGQPDLPKEC (with the amino acids corresponding to amino acids 40 to 57 within the AB domain of mouse RARγ1 [31] and unique to RARγ1 underlined) was synthesized and coupled to keyhole limpet hemocyanin. This was used to raise antipeptide antiserum in rabbits. The peptide SEKTQLYNRPHEEPSNSC (with the amino acids corresponding to amino acids 87 to 103 of mouse PPARγ1 [10, 35] and 118 to 133 of PPARγ2 [66] underlined) was synthesized and coupled to keyhole limpet hemocyanin. This was used to raise antipeptide antiserum in rabbits.

Western (immunoblot) analysis. Extracts from 3T3-L1 cells were harvested as previously described (69). Fifty micrograms of cell protein from cells harvested at day 0 through day 7 of the differentiation protocol were loaded on a sodium dodecyl sulfate–10% polyacrylamide gel along with cell extracts from 293T cells transfected with PPARγ1 (35) or PPARγ2 (66) expression plasmids. Western blotting and chemiluminescent detection (Amersham) were performed essentially as previously described (32). Rabbit anti-PPARγ immunoglobulin G was purified and used at a dilution of 1:2,000. Horseradish peroxidase-linked donkey anti-rabbit secondary antibody was used at a dilution of 1:30,000.

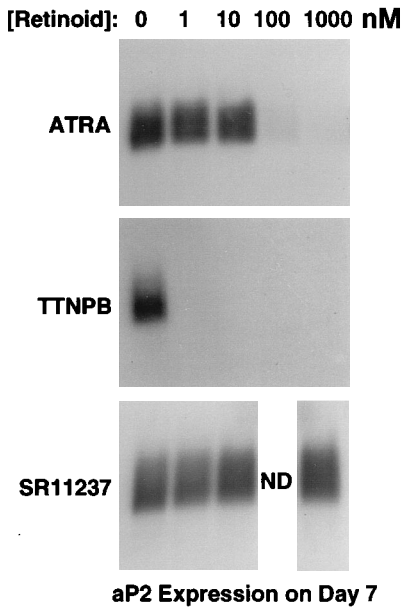

FIG. 1. Inhibition of adipocyte differentiation by retinoids. For Northern analysis, aP2 expression on day 7 after exposure to DM was used as a marker of the adipocyte phenotype. Comparison of all-*trans* RA (ATRA) with RAR-specific TTNPB and RXR-specific SR11237. In all cases, compounds were added simultaneously with DM on day 0. ND, not done.

RESULTS

RA inhibition of adipocyte differentiation is mediated by RARs. The efficacies of various retinoids at inhibiting adipocyte differentiation were assessed by adding retinoids at various doses along with DM to 3T3-L1 preadipocytes. The RAR-specific retinoid TTNPB was much more effective at inhibiting adipocyte differentiation than was all-*trans* RA, as assessed by aP2 expression (Fig. 1) as well as morphology (data not shown). In contrast, the RXR-specific retinoid SR11237 (14, 43) was ineffective at inhibiting adipocyte differentiation. Therefore, the inhibitory effects of RA on adipocyte differentiation were likely mediated by RARs.

3T3-L1 cells express primarily RARα and RARγ, and RARγ1 is selectively down-regulated during adipocyte differentiation. Figure 2A shows that 3T3-L1 preadipocytes were found to express primarily RARα and RARγ, in agreement with the results of earlier reports (29, 46, 58). RARα mRNA levels were essentially unchanged during the course of adipocyte differentiation. Both RARγ1 and RARγ2 were expressed in preadipocytes but differentially regulated during adipogenesis. RARγ1 mRNA was rapidly down-regulated during the first 24 h of adipocyte differentiation, whereas RARγ2 mRNAs levels remained essentially constant. Down-regulation of RARγ1 was prevented by RA just as RA prevents the induction of other genes that are clearly part of the differentiation program, such as PPARγ, C/EBPα, and aP2 (Fig. 2B).

It was recently reported that some cells express relatively high levels of an RARγ variant which lacks the C-terminal RA-dependent transactivation domain (AF2) and therefore functions as an endogenous negative regulator of bona fide RARs (52). A similar mutation of RARα has the ability to inhibit PPAR activation as well (28). The expression of such an

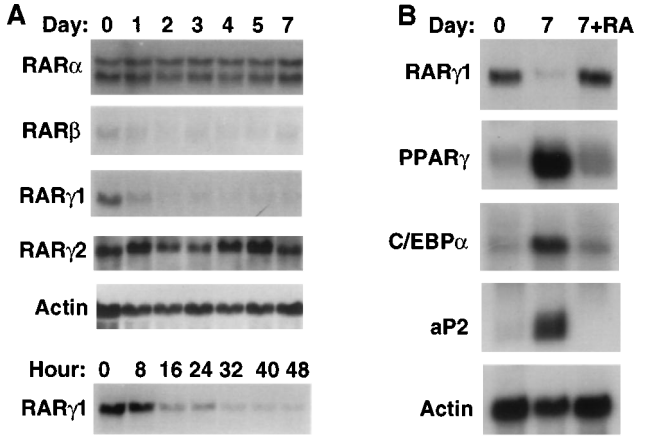

FIG. 2. RAR gene expression during adipocyte differentiation. (A) Expression of RARα, -β, and -γ. (B) RA prevents the down-regulation of RARγ1 as well as the induction of PPARγ, C/EBPα, and aP2. RA or ethanol was added simultaneously with DM on day 0.

RARγ variant in 3T3-L1 cells was studied by using RT-PCR with primers specific for the 5′ ends of RARγ1 and RARγ2 and 3′ primers which could distinguish between the traditional RARs and the endogenous dominant negative forms (Fig. 3A). The dramatic reduction in RARγ1 mRNA was confirmed by RT-PCR (Fig. 3B, lanes 1 and 2), indicating that the technique was at least semiquantitative. RARγ2 mRNA was also easily detectable (Fig. 3B, lanes 5 and 6) but, consistent with the Northern analysis discussed above, was not affected by differentiation. In contrast, the dominant negative forms of RARγ1 (Fig. 3B, lanes 3 and 4) and RARγ2 (lanes 7 and 8) were undetectable in this assay. To increase sensitivity, RT-PCR was done with the same 3′ primers but with a single 5′ primer common to RARγ1 and RARγ2. Figure 3B shows that a long autoradiographic exposure revealed the expression of the endogenous dominant negative RARγ at levels which were far

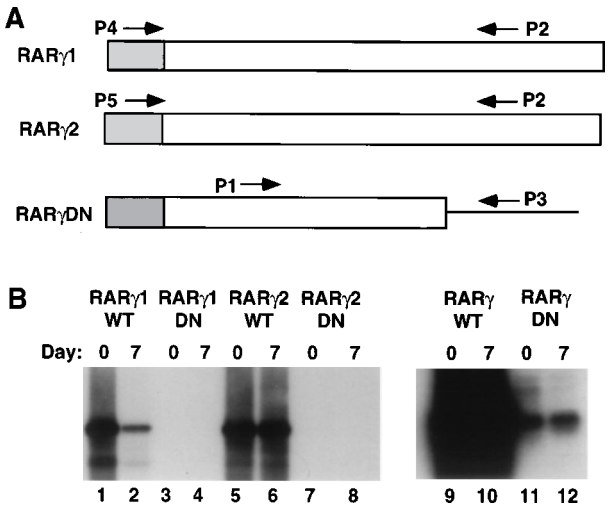

FIG. 3. 3T3-L1 cells express endogenous dominant negative (DN) RARγ forms at very low levels. (A) Schematic of RT-PCR strategy (see Materials and Methods). (B) Southern analysis of PCR products, with an RARγ cDNA probe. The 5′ primers were P4 (RARγ1 [lanes 1 through 4]), P5 (RARγ2 [lanes 5 through 8]), and P1 (RARγ common [lanes 9 through 12]). The autoradiogram of lanes 1 through 8 was exposed for 18 h, and the autoradiogram of lanes 9 through 12 was exposed for 4 days. WT, wild type.

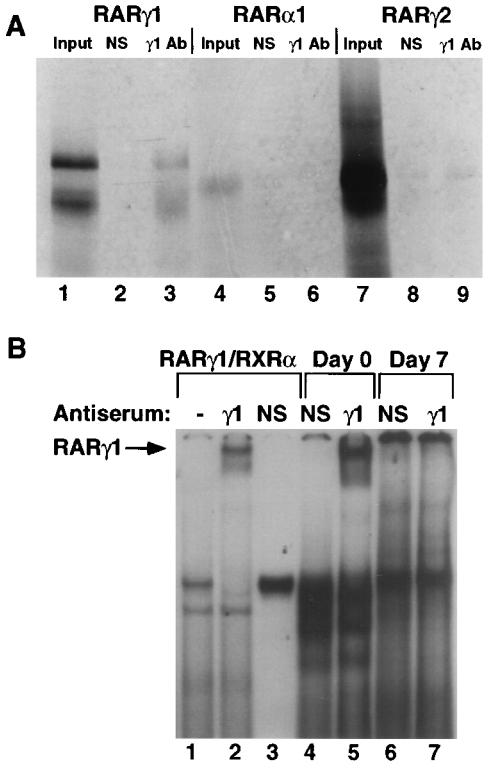

FIG. 4. RARγ1 protein is down-regulated in adipocytes. (A) Characterization of RARγ1-specific antiserum raised against amino acids 40 to 57 of RARγ1. Reticulocyte lysate-translated RARγ1, not RARα1 or RARγ2, was immunoprecipitated by this antiserum. NS, nonimmune serum; Ab, antibody. (B) Gel supershift studies using the ^{32}P-labeled RARE from the RARβ gene as the probe (runoff gel to increase the resolution of more slowly migrating complexes). Lanes 1 through 3, RARγ1-RXRα synthesized in reticulocyte lysate (heterodimer complexes in lanes 1 and 3 and supershifted complex [arrow] in lane 2); lanes 4 through 7, 3T3-L1 nuclear extracts from cells harvested at the indicated times after exposure to DM were used as sources of protein. −, absence of antiserum.

below those of traditional RARγ species (compare lanes 11 and 12 with lanes 9 and 10). Thus, we concluded that the vast majority of RARγ transcripts detected by Northern analysis encoded bona fide RARγ, not the variant species.

We next sought to determine whether the decrease in RARγ1 gene expression early in adipocyte differentiation corresponded to a change in RARγ1 protein levels. An antiserum specific for the polypeptide from the unique N-terminal A domain of RARγ1 was generated and found to specifically immunoprecipitate in vitro-translated RARγ1 (Fig. 4A, lane 3) but not RARα1 (lane 6) or RARγ2 (lane 9). This antiserum also supershifted an RARγ1-RXR heterodimer bound to the RARE from the RARβ gene (Fig. 4B, lane 2). The supershift assay was used to detect endogenous RARγ1 in nuclear extracts of 3T3-L1 cells. Figure 4B shows that the antiserum, but not nonimmune serum, supershifted an RARE-binding complex in preadipocyte nuclear extracts but not adipocyte nuclear extracts (compare lanes 5 and 7). A differentiation time course indicated that the amount of RARγ1 protein was reduced after 24 h and was virtually undetectable at 48 h (73), which correlated well with RARγ1 mRNA levels.

The finding that RARγ1 is specifically down-regulated during adipocyte differentiation suggested two potential functional consequences. First, it was possible that RARγ1 down-regulation is necessary for preadipocytes to differentiate even in the absence of RA. A second possibility was that RARγ1 down-

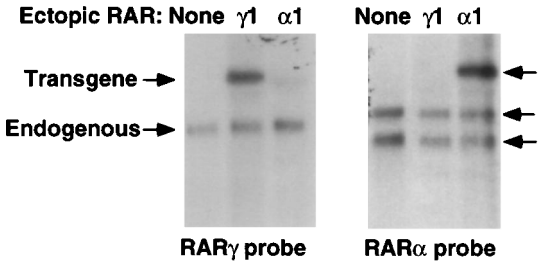

FIG. 5. Ectopic expression of RARγ1 and RARα1 in 3T3-L1 cells. Northern analysis of preadipocyte mRNAs, using either an RARγ1-specific probe or an RARα1-specific probe. Ectopically expressed transcripts are larger than endogenous transcripts, as indicated by arrows.

regulation is related to the abilities of cells to respond to RA. The latter hypothesis was strengthened by the earlier observation that RA is effective at inhibiting adipogenesis only when added shortly after the exposure of preadipocytes to DM, i.e., at times when RARγ1 is still present in cells (38, 63) (see below). For both of these hypotheses, it was also of interest to determine whether the effects of RARγ1 were isoform or subtype specific.

Ectopic expression of RARγ or RARα does not prevent adipocyte differentiation. The potential roles of RAR subtypes in adipocyte differentiation were tested by ectopically expressing RARγ1 and RARα in 3T3-L1 preadipocytes. These experiments utilized retroviral vectors, with analysis of pools of cells selected in G418. Control retrovirus with no insert was used as the control in all experiments. Retroviral infection rather than stable transfection was used because we found that cell lines derived from single colonies after stable transfection were occasionally unable to differentiate even after transfection with control vector expressing only the selectable marker, presumably because of integration effects, making it difficult to assess whether phenotypic changes were due to the transgene (data not shown). Similar experiences have been described by others (20, 67). In contrast, by using a retroviral-infection strategy, pools of cells selected in G418 after infection with control virus differentiated normally by morphological and molecular criteria (see below). Figure 5 shows that ectopic RARγ1 and RARα genes were expressed as larger mRNAs at levels similar to or greater than those of the endogenous genes.

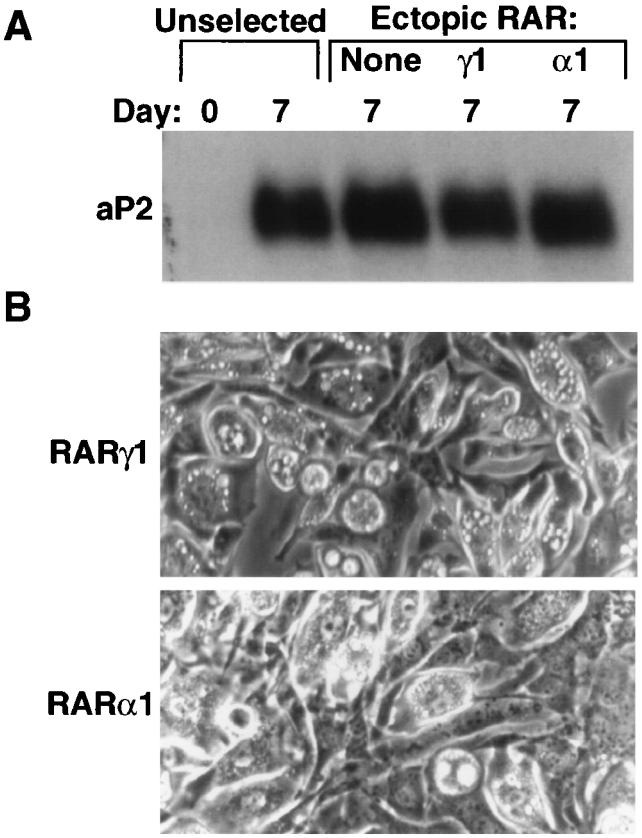

FIG. 6. Adipocyte differentiation of 3T3-L1 cells that ectopically express RARγ1 and RARα1. (A) aP2 mRNA expression at the indicated times after exposure to DM. Unselected, uninfected control cells, compared with cells selected in G418 after infection with virus expressing no RAR (None) or RAR, as indicated. (B) Cell morphology at 7 days after exposure to DM.

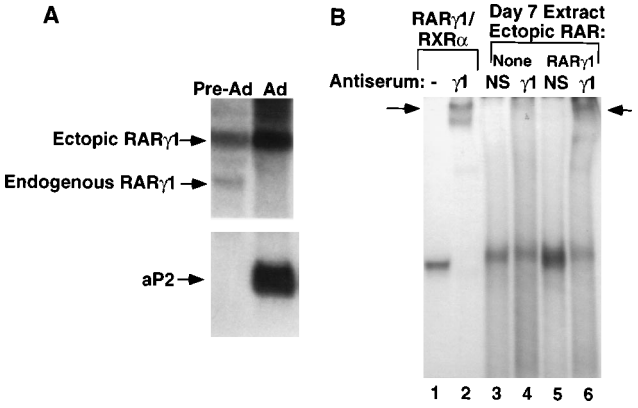

FIG. 7. Expression of transgenic RARγ1 mRNA and protein. (A) Northern analysis of RARγ1 mRNAs in preadipocytes (Pre-Ad; day 0) and adipocytes (Ad; day 7 after exposure to DM). Ectopic and endogenous RARγ1 mRNAs are indicated, and aP2 expression consistent with adipocyte differentiation is also shown. (B) Gel supershift analysis, using the ^{32}P-labeled RARβ RARE as the probe (runoff gel). Lanes 1 and 2, reticulocyte lysate-translated RAR-RXR heterodimer (with the supershifted heterodimer in lane 2 indicated by an arrow); lanes 3 through 6, 3T3-L1 cell nuclear extracts from the indicated cells at 7 days after exposure to DM were used as sources of protein. NS, nonimmune serum; −, absence of antiserum.

We first assessed the expression of ectopic RARγ1 and RARα expression on adipocyte differentiation. Figure 6 shows that adipocyte differentiation was observed in both control cells and RAR-expressing cells. This was confirmed by measuring aP2 expression (Fig. 6A) and by observing the cells morphologically (Fig. 6B). As shown in Fig. 7A, endogenous RARγ1 mRNA was down-regulated appropriately in cells which expressed ectopic RARγ1. To be certain that the ectopic RNA was in fact translated into protein, we took advantage of the gel supershift assay by using ectopic RARγ1-expressing cells. Figure 7B shows that RARγ1 protein was detectable in ectopic-RARγ1-expressing cells, despite the absence of endogenous RARγ1 mRNA in differentiated adipocytes (compare lanes 4 and 6), indicating that the RARγ1 transgene was translated into protein. Although regulation of the endogenous RARγ1 and aP2 genes in the control and ectopic-RAR-expressing cells was essentially indistinguishable, ectopic expression of either RAR had a reproducible, modest (10 to 50% in five experiments) inhibitory effect on adipogenesis. Given the likely role of PPARs in adipocyte differentiation, it is possible that this effect of the unliganded wild-type RAR was due to competition with PPARγ for RXR (21, 33, 37) or for other transcriptional coregulators, such as putative corepressors (11, 26), especially on DR1 DNA binding sites bound by both

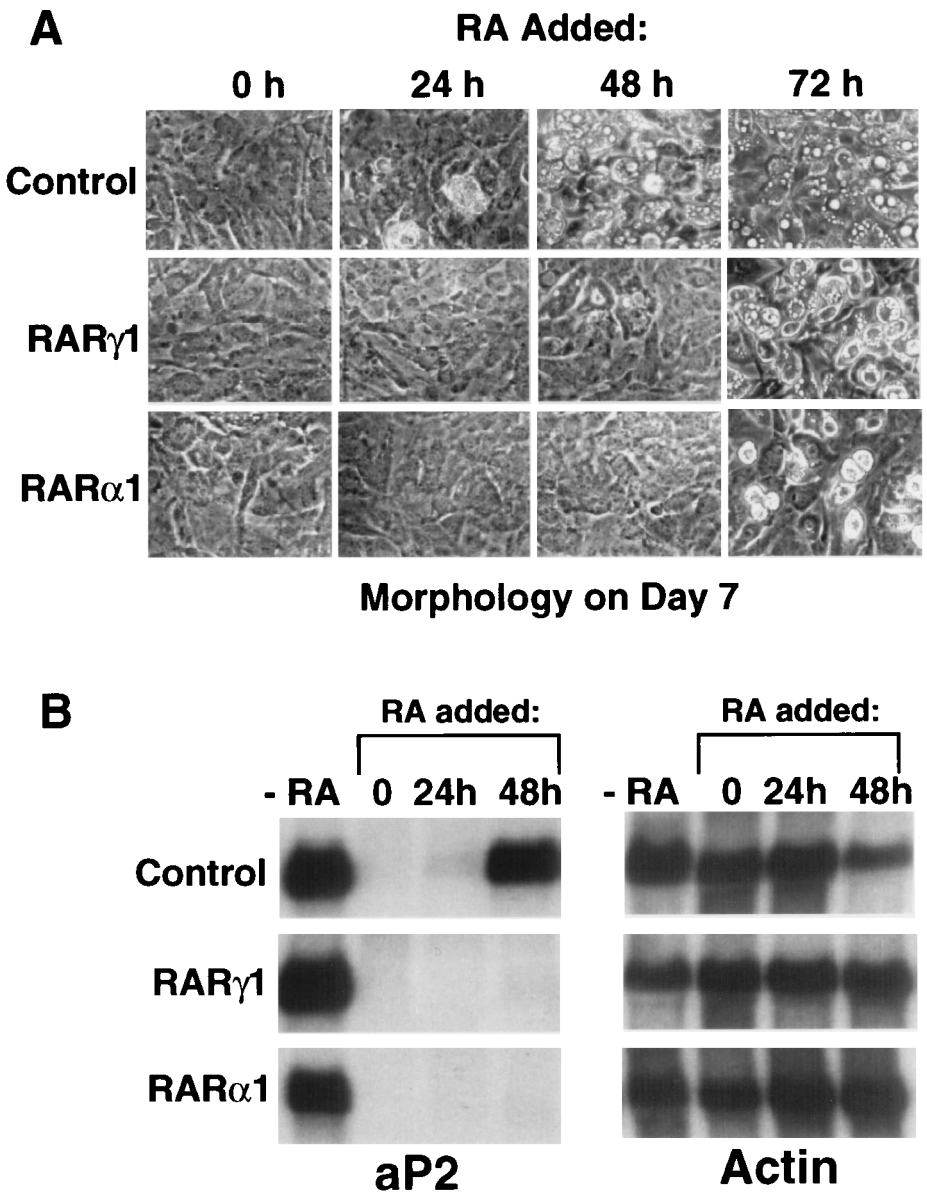

FIG. 8. Overexpression of RARγ1 or RARα1 prolongs the RA sensitivity of 3T3-L1 cells. 3T3-L1 cells were treated with DM on day 0 (0 h), and RA was added at the indicated times. (A) Morphology of cells at 7 days after exposure to DM, with RA treatment at the indicated times; (B) expression of aP2 and actin at 7 days after the exposure of confluent preadipocytes to DM, with RA treatment at the indicated times. −RA, absence of RA.

PPAR and RAR (39, 40). This is unlikely to be the main purpose of down-regulation of endogenous RARγ1 during normal adipocyte differentiation, though, because ectopic expression of RARγ1 (or RARα1) did not prevent adipocyte differentiation in the majority of cells.

Ectopic expression of RARγ1 or RARα extends the window of efficacy of RA. We next tested the hypothesis that ectopic expression of RARγ1 would have an effect upon the period during which cells were responsive to RA in terms of the inhibition of adipogenesis. Figure 8 shows that RA nearly completely inhibited the adipocyte differentiation of preadipocytes selected after infection with control vector when RA was provided at the same time as was DM (0 h) or 24 h after DM addition. However, these cells resembled wild-type 3T3-L1 cells in that RA failed to prevent adipocyte differentiation when provided at 48 h after DM addition. This was apparent by using morphological criteria (Fig. 8A) and by Northern analysis of aP2 gene expression (Fig. 8B). In contrast, RA blocked the differentiation of cells which ectopically expressed RARγ1 even when added 48 h after DM addition. At 72 h, however, RA no longer blocked the adipocyte differentiation of cells which ectopically expressed RARγ1 (Fig. 8A). In some experiments, ectopic RAR expression extended the window of RA efficacy by as much as 36 to 48 h (not shown), but in all experiments, the ability of RA to inhibit differentiation was lost by 96 h after exposure to DM. Thus, the failure of wild-type cells to respond to RA between 24 and 48 h after exposure to DM was likely due to the loss of endogenous RARγ1 from these cells. However, between 48 and 96 h after the induction of adipogenesis, cells became irreversibly committed to adipocyte differentiation even when abundant amounts of RARγ1 and RA were present.

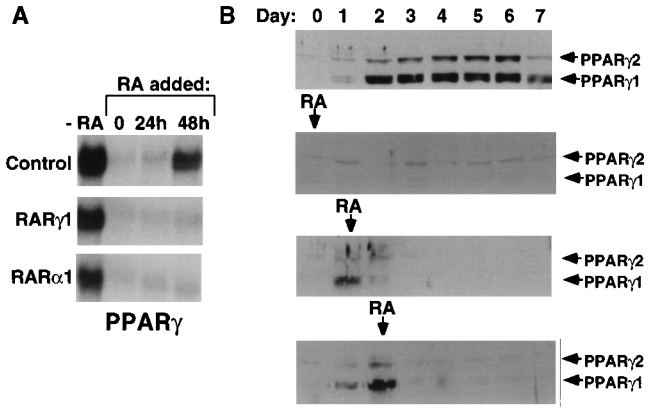

FIG. 9. Effects of RA on PPARγ expression. (A) Expression of PPARγ mRNA at 7 days after the exposure of confluent preadipocytes to DM, with RA treatment at the indicated times. −RA, absence of RA. (B) PPARγ1 and PPARγ2 protein expression after the treatment of ectopic-RARγ1-expressing 3T3-L1 cells with DM. Top panel: Standard differentiation protocol. The three panels below show, from top to bottom, the effects of RA added simultaneously with DM, 24 h later, and 48 h later, respectively. PPARγ1 begins at the second methionine (amino acid 31) of PPARγ2. PPARγ antiserum was raised against amino acids 118 to 134 of PPARγ2 (amino acids 87 to 103 of PPARγ1).

The ability of ectopic expression of RARγ1 to prolong the RA-responsive period of 3T3-L1 cells suggested a potential function for down-regulation of endogenous RARγ1. To test whether this was RAR subtype specific, similar experiments were performed with cells which ectopically expressed RARα. Even though the transgene was expressed at levels similar to those of endogenous RARα, ectopic expression of RARα had an effect similar to that of ectopic expression of RARγ1, prolonged responsiveness to RA, as assessed both by morphology (Fig. 8A) and by using aP2 expression as a molecular marker of differentiation (Fig. 8B). Thus, ectopic expression of RARα was able to functionally complement the reduction in endogenous RARγ1.

PPARγ induction is prevented as well as reversed by RA. As shown previously, RA blocked the induction of both C/EBPα and PPARγ. The prevention of adipocyte differentiation by RA occurs prior to the induction of C/EBPα mRNA and protein (5, 13). In contrast, PPARγ mRNA levels have been shown to be increased within 24 h of exposure to differentiating conditions (9, 66), at which time RA is still effective in inhibiting adipocyte differentiation. Figure 9A shows that the addition of RA at 24 to 48 h after DM addition resulted in very low PPARγ mRNA levels on day 7. This was consistent with the lack of other features of the adipocyte phenotype but somewhat surprising since PPARγ mRNA had already been induced at the time RA was added. Thus, RA not only prevented PPARγ gene expression but antagonized it as well.

Therefore, it was of great interest to know whether PPARγ proteins as well as PPARγ mRNA were present at times when RA was effective in preventing adipocyte differentiation. PPARγ exists in two forms, PPARγ1 and PPARγ2, which are identical except for an additional 31 amino acids at the N terminus of PPARγ2. We raised a PPARγ-specific antiserum which recognizes a peptide common to PPARγ1 and PPARγ2. Figure 9B shows that this antibody detected both PPARγ1 and PPARγ2 by Western analysis. Very little PPARγ protein was detected in preadipocytes at 2 days postconfluency (day 0), although in some experiments both PPARγ1 and PPARγ2 were detectable, with the levels of PPARγ2 somewhat higher than those of PPARγ1. Both PPARγ1 and PPARγ2 proteins were found to be greatly induced during adipocyte differentiation of 3T3-L1 cells, with the levels of PPARγ1 consistently greater. PPARγ expression during the adipocyte differentiation of ectopic-RARγ1-expressing cells was similar to that of wild-type 3T3-L1 cells (60). Interestingly, PPARγ2 mRNA has been reported to be more abundant than PPARγ1 mRNA is in adipocytes (66). However, PPARγ1 protein may result both from PPARγ1 mRNA and from translational initiation at the second in-frame AUG (codon 31) in PPARγ2 mRNA. The induction of PPARγ proteins occurred within 24 h and was nearly maximal by 48 h after exposure to DM. Thus, RA was effective in inhibiting adipocyte differentiation at times when 3T3-L1 cells had already expressed PPARγ protein (e.g., at 24 h), indicating that PPARγ expression was not sufficient to induce adipocyte differentiation in the presence of RA.

This raised the question of whether the PPARγ proteins present prior to RA treatment remained in undifferentiated cells. The induction of PPARγ proteins was prevented when RA was added simultaneously with DM (day 0), in agreement with the lack of PPARγ mRNA (Fig. 9A). RA also prevented adipocyte differentiation and PPARγ mRNA induction when added 24 to 48 h later, as in the experiments shown in Fig. 8 and 9A, respectively. In the absence of RA, PPARγ1 and PPARγ2 proteins were induced at 24 h, as expected. Interestingly, Fig. 9B shows that the addition of RA at 24 h after DM addition resulted not only in the prevention of additional accumulation of PPARγ proteins but also in the loss of existing PPARγ proteins from cells. This effect was even more pronounced when RA was added at 48 h. Despite the near-maximal induction of PPARγ isoforms at this time, RA treatment caused a rapid loss of PPARγ from cells, down to levels similar to those in undifferentiated preadipocytes. Thus, RA inhibition of adipocyte differentiation involves the prevention of PPARγ expression and the loss of PPARγ protein from cells which have already expressed this protein prior to the addition of RA.

DISCUSSION

The results presented here are consistent with the hypothesis that RARγ1 down-regulation contributes to the RA unresponsiveness which occurs 24 to 48 h after the exposure of 3T3-L1 cells to differentiation conditions. Ectopic expression of RARα1 was equally as effective as ectopic expression of RARγ1 in extending the RA-responsive period. Furthermore, an RA antagonist with specificity for RARα, Ro 41-5253 (2), prevented retinoid inhibition of adipocyte differentiation (30, 73). Therefore, it is likely that there is functional redundancy of RARα1 and RARγ1 at least with respect to the ability to mediate the inhibition of adipocyte differentiation by RA. Functional redundancy of RAR subtypes has been previously observed in F9 cells (64) and is consistent with the conclusions from studies of mice lacking specific RAR isoforms (47, 50). The present results suggest that the efficacy of RA in inhibiting adipocyte differentiation requires a threshold level of RAR; both RARα and RARγ contribute to this level in the preadipocyte and at times shortly after the induction of differentiation. It is not clear why RARγ1 is selectively down-regulated, rather than RARα1 (and RARγ2) being coordinately reduced. However, independent regulation of the expression of RARγ1 and RARγ2 isoforms is consistent with their differential tissue and developmental expression (31). It is possible that the RARs have subtype- or isoform-specific effects in differentiated adipocytes, which continue to express RARα and RARγ2.

The process of adipocyte differentiation is associated with the induction of a large number of genes (57, 61), including

transcription factors (70). The regulation of different genes at different times after the exposure of cells to differentiation conditions suggests the existence of a regulatory hierarchy or cascade of events (5, 74). The findings that the inhibition of C/EBPα expression prevents differentiation (48) and that ectopic expression of C/EBPα induces adipogenesis (20, 49, 74) underscore the importance of C/EBPα in this process. However, C/EBPα is not induced until 2 to 3 days after exposure to differentiating conditions (5, 12) and is therefore likely to be induced by transcription factors induced earlier in adipocyte differentiation, such as other C/EBP subtypes (74) or PPARγ (9, 66). In agreement with the cascade model of adipocyte differentiation is the observation that RA is effective at much earlier times, suggesting that while the prevention of C/EBPα induction by RA is probably important for inhibiting adipogenesis, this is likely to be an indirect effect.

PPARγ is a member of the nuclear hormone receptor superfamily which, like C/EBPα, has the ability to induce adipocyte differentiation of fibroblasts (67). PPARγ mRNA is induced earlier than is C/EBPα, and we have shown here for the first time that the PPARγ1 and PPARγ2 proteins are also induced by the first day of the differentiation protocol. This is important because it indicates that RA can block the differentiation of cells which have already expressed PPARγ1 and PPARγ2. Therefore, the effect of RA cannot be solely to prevent PPARγ induction. Furthermore, PPARγ mRNA and protein disappear from cells shortly after RA treatment, indicating that RA interferes with the mechanisms which maintain as well as induce PPARγ expression. Thus, it is possible that RA works by interfering with the function(s) of the responsible transcription factors. One candidate is C/EBPβ, since the mouse PPARγ gene has a putative C/EBP binding site (76) and C/EBPβ is induced very early in adipogenesis (15). Another possible target of RAR is PPARγ itself. RAR could compete with PPAR for RXR, either off DNA or on DR1 sites where RAR-RXR is inactive (39); although there is no evidence that RAR-RXR interaction is stimulated by a ligand in vitro (21, 33, 37), in vivo footprinting studies suggest that RA may stabilize RAR binding to genomic targets (15). Alternatively, RA binding to RAR could squelch PPAR activation off DNA, since both RAR and PPAR contain an autonomous activation domain (3, 17, 42) that interacts with potentially limiting coactivators in a ligand-dependent manner (6, 25, 42). This hypothesis would explain the need for a threshold level of RAR. In fact, the required RAR concentration might actually increase as the PPARγ concentration dramatically increases during the first 2 days of adipogenesis.

Our results have important implications for defining stages along the adipocyte differentiation pathway. 3T3-L1 cells are committed to adipocyte differentiation, in that they differentiate into adipocytes under conditions which do not cause adipogenesis of the fibroblasts from which they were derived (23, 24). However, an additional restriction to adipogenesis must exist because 3T3-L1 cells do not differentiate spontaneously into adipocytes. Stimulation with standard differentiation cocktail or PPAR activators, such as thiazolidinediones (34, 44) or eicosanoids (18, 36, 75), is required to move cells further along the differentiation pathway, but passage to this second stage of the differentiation process is prevented by RA (8). RA is effective prior to the induction of C/EBPα and PPARγ, as well as after PPARγ has already been induced. The time after which RA can no longer prevent adipocyte differentiation defines a third stage of differentiation, which is normally irreversible. Moreover, the present studies show that this RA unresponsiveness can be subdivided into two additional stages (Fig. 10). Initially, the inability to respond to RA is due to limiting

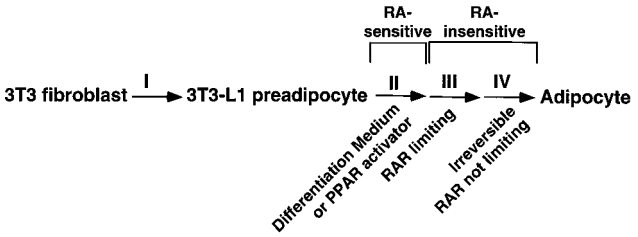

FIG. 10. Stages of progression to the adipocyte phenotype. Stage I refers to the unknown event(s) that creates 3T3-L1 cells from parent 3T3 fibroblasts. The 3T3-L1 preadipocytes are predisposed to adipogenesis, but DM or PPAR activators lead to events which are sufficient for adipocyte differentiation but reversible by RA (stage II). From the present results, we propose two additional stages occurring ~48 h after exposure to DM when RA can no longer reverse the process. Stage III refers to the next ~24 to 48 h, when RA insensitivity is due to the reduced RAR concentration. Thereafter, the cells in stage IV differentiate into mature adipocytes despite ectopic expression of RAR and the presence of RA.

concentrations of RARs, as shown by the ability of ectopic expression of RAR to prolong the period of RA responsiveness. Only at 72 to 96 h after exposure to differentiating conditions do cells reach a stage at which they are irreversibly committed to adipocyte differentiation, even in the presence of high intracellular concentrations of RA and RARs. This transition could be due to a quantitative change in the level of one or more adipogenic factors, including PPARγ and C/EBPα, or a more qualitative change, reflecting the induction or loss of a key regulator of differentiation. Analysis of the molecular mechanism of this transition will be essential to fully understand the different stages of progression to the adipocyte phenotype.

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