Activation of Peroxisome Proliferator-activated Receptor γ Bypasses the Function of the Retinoblastoma Protein in Adipocyte Differentiation*  

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The retinoblastoma protein (pRB) is an important regulator of development, proliferation, and cellular differentiation. pRB was recently shown to play a pivotal role in adipocyte differentiation, to interact physically with adipogenic CCAAT/enhancer-binding proteins (C/EBPs), and to positively regulate transactivation by C/EBPβ. We show that PPARγ-mediated transactivation is pRB-independent, and that ligand-induced transactivation by PPARγ1 present in RB+/− and RB−/− mouse embryo fibroblasts is sufficient to bypass the differentiation block imposed by the absence of pRB. The differentiated RB−/− cells accumulate lipid and express adipocyte markers, including C/EBPα and PPARγ2. Interestingly, adipo conversion of pRB-deficient cells occurs in the absence of compensatory up-regulations of the other pRB family members p107 and p130. RB+/− as well as RB−/− cells efficiently exit from the cell cycle after completion of clonal expansion following stimulation with adipogenic inducers. We conclude that ligand-induced activation of endogenous PPARγ1 in mouse embryo fibroblasts is sufficient to initiate a transcriptional cascade resulting in induction of PPARγ2 and C/EBPα expression, withdrawal from the cell cycle, and terminal differentiation in the absence of a functional pRB.

The retinoblastoma protein (pRB) is a key regulator of the mammalian cell cycle. Through repression of the growth-promoting E2F transcription factors, pRB controls the transition from the G1 to the S phase (1). pRB function is regulated by cyclin-dependent kinases, which phosphorylate pRB in a characteristic cell cycle-dependent manner (2). In addition, pRB plays a pivotal role during development and differentiation. The multifunctional character of pRB has been demonstrated by targeted disruption of the retinoblastoma gene in mice. Homozygous mutant embryos die in utero and show abnormalities in hematopoiesis and neurogenesis (3).

Numerous ex vivo studies have established the importance of pRB in myocyte differentiation (4). pRB has been shown to interact physically and functionally with members of the myogenic MyoD family of basic helix-loop-helix transcription factors (5), and pRB-deficient cells fail to undergo terminal myogenesis (6, 7). This included a defect in expression of late differentiation markers, reduced myoblast fusion, a failure to terminally withdraw from the cell cycle, and an increased incidence of apoptosis (6–8). These observations have also been seen in vivo when pRB is expressed at subphysiological levels (9).

Adipocyte differentiation is a complex process regulated by CCAAT/enhancer-binding proteins (C/EBPs), peroxisome proliferator-activated receptors (PPARs), and the adipocyte determination and differentiation-dependent factor-1/sterol regulatory element-binding protein-1 (ADD1/SREBP1) (10–12). C/EBPβ and C/EBPα are induced very early during differentiation and have been shown to promote adipogenesis, possibly through induction of C/EBPα and PPARγ (13–15), and abrogation of their activity blocks adipose conversion (15, 16). Ectopic expression of C/EBPα is adipogenic in fibroblasts, and abrogation of C/EBPα expression blocks adipocyte differentiation (11). PPARs are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors. Ligands for PPARγ include the antidiabetic thiazolidinedione drugs and certain prostaglandin J2 derivatives (17–19). PPARγ is induced early in adipocyte differentiation (20), and addition of ligands to fibroblasts expressing PPARγ endogenously or ectopically induces or promotes adipose conversion (17–19, 21). ADD1/SREBP1 is also induced early in the differentiation program and promotes adipogenesis (22). Ectopic expression of ADD1/SREBP1 was recently shown to induce the synthesis of an unidentified PPARγ-activating ligand (23).

Adipocyte differentiation ex vivo requires growth arrest, usually obtained by growing cells to confluence. Following stimulation with adipogenic factors, density-arrested preadipocytes undergo several rounds of postconfluent cell divisions (clonal expansion), followed by terminal withdrawal from the cell cycle, expression of adipocyte markers, and accumulation of intracellular lipid (24). The importance of pRB in adipocyte differentiation has been amply demonstrated. It was shown that the ability of a truncated simian virus 40 large T antigen to block adipocyte differentiation is dependent on its ability to...
sequester the pRB family (pRB, p107, p130) (25), and recently it was demonstrated that lung fibroblasts from RB−/− mouse embryos are unable to undergo adipose conversion unless rescued by an RB transgene (26). Furthermore, pRB was shown to physically interact with C/EBPs, promote the binding of C/EBPβ to its cognate DNA response element, and increase its transactivation capacity (26, 27). The functional interaction with C/EBPβ suggests that pRB plays an important role early in the adipocyte differentiation program. Finally, regulated phosphorylation and expression of the three pRB family members during adipose conversion have recently been demonstrated (24, 28).

In this study, we used fibroblasts from normal and RB−/− mouse embryos to further characterize the importance and functions of pRB in adipocyte differentiation. We show that transactivation by PPARγ is not dependent on pRB. Mouse embryo fibroblasts (MEFs) express PPARγ1 in the predifferentiated state, and the inability of pRB-deficient MEFs to differentiate is efficiently bypassed by addition of PPARγ ligands. The differentiated RB−/− MEFs accumulate lipid and express adipocyte markers. Surprisingly, adipocyte differentiation of RB−/− MEFs was found not to be accompanied by compensatory up-regulation of p107 and p130 expression, and RB−/− as well as RB−/− MEFs effectively withdraw from the cell cycle following clonal expansion.

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation—The preparation of wild-type and RB−/− mouse embryo fibroblasts (MEFs) has been described previously (29). MEFs were grown in AmnioMax basal medium (Life Technologies, Inc.) supplemented with 7.5% fetal bovine serum (FBS), 7.5% Amni- oMax-C100 supplement, 2 mM glutamine, 62.5 μg/ml penicillin, and 100 μg/ml streptomycin (growth medium) in a humidified atmosphere of 5% CO2 at 37 °C. Medium was changed every other day. For differentiation, 2-day postconfluent cells (day 0) were treated with growth medium containing 1 μM dexamethasone (Sigma), 0.5 mM methylisobutyxanthine (Aldrich), 5 μg/ml insulin (Boehringer Mannheim), and BRL49653 (0.5 μM unless otherwise indicated) or vehicle (0.1% Me2SO) for 2 days. From day 2, medium contained 5 μg/ml insulin and BRL49653 or vehicle. MEFs were not used beyond passage 10. 3T3-L1 cells were cultured to confluence in DMEM (10% FBS) (kindly provided by Mitchell A. Lazar), and rabbit antibodies against mouse C/EBPγ and C/EBPβ (kindly provided by Mitchell A. Lazar), and rabbit antibodies against mouse C/EBPα and C/EBPβ (kindly provided by Mitchell A. Lazar). Secondary antibodies were horse radish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Dako). Enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech) was used for detection. Stripping of membranes was done by boiling for 5-10 min in water.

PCR Analysis of RB Gene Status—RB gene disruption via insertion of the hygromycin resistance gene in exon 19 was detected as described previously (29). Briefly, 50 ng of genomic DNA from individual MEFs were used for PCR amplification. To detect disruption of RB alleles, the following upstream and downstream primers were used: CGATCTGACCGAGCCGCGG (within the hygromycin resistance gene) and TGAAGCTGTGCTTGTGTCCTG (within exon 19 of RB). To detect wild-type RB alleles, the downstream exon 19 primer was used in combination with the following upstream primer: GACTGTAGGAAAGGTGAGGAG. Whole cell extracts were stored at −80 °C. After DNA synthesis, reactions were diluted with 50 μl of water and frozen at −80 °C.

Multiple RT-PCR—Multiple reverse transcription-polymerase chain reaction (RT-PCR) was performed essentially as described (40) with minor changes. PCR was done in 25-μl reactions containing 1 μg of total RNA, 3 μg of random hexamers (Amersham Pharmacia Biotech), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 40 units of RNAguard (Amersham Pharmacia Biotech), 0.9 μM dNTPs (Amersham Pharmacia Biotech), and 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies). Reactions were left 10 min at room temperature, followed by incubation at 37 °C for 1 h. After CDNA synthesis, reactions were diluted with 50 μl of water and frozen at −80 °C.

Cell Purification and Reverse Transcription—Total RNA was purified as described (39). The integrity of all RNA samples was confirmed by electrophoresis in denaturing formaldehyde-containing gels. Reverse transcription were performed in 25-μl reactions containing 1 μg of total RNA, 3 μg of random hexamers (Amersham Pharmacia Biotech), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 40 units of RNAguard (Amersham Pharmacia Biotech), 0.9 μM dNTPs (Amersham Pharmacia Biotech), and 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies). Reactions were left 10 min at room temperature, followed by incubation at 37 °C for 1 h.
were: C/EBPα, 5′-GAAGCAGCAGGATTGGTGTA, 3′-GGCCATTCCGCTGCAG (225 bp); GPDH, 5′-GGTTGACCCCTCACTGTGA, 3′-GTTCCCTGGAGAAGGTCCT (264 bp); pPPARγ1, 5′-GACGTCGAGGAAAGTAAGT, 3′-GCCAGCCCCAGCTGCTGAT (288 bp); pPPARγ2, 5′-CCAGGACGCTGCCTGCTGCT, 3′-GCAACCTGTTGGCATGTC (241 bp); TBP, 5′-CCCTCCACAATGACTCTATG, 3′-ATGATGACTGCAAGAATCCG (190 bp). Ten μl of each reaction were dried and resuspended in formamide dye mix (98% deionized formamide, 10 mM EDTA (pH 8.0), 0.2% bromophenol blue, 0.2% xylene cyanol) and loaded onto 0.4 mm, 8 μl urea, 1X TBE, 6% polyacrylamide gels. Electrophoresis was for 3 h at 50 watts. Gels were dried and exposed overnight to phosphorimage storage screens. Screens were scanned on a PhosphorImager (Molecular Dynamics).

Preparation of Cells for Flow Cytometry—At the indicated time points, bromodeoxyuridine (BrdUrd) (Sigma) was added to the plates to a final concentration of 10 μM, and incubation was continued for 20 min. Cells were then harvested by trypsinization, washed in 0.9% NaCl, fixed in 75% ethanol, and stored at 4 °C until further analysis. Cells were treated with pepsin before incubation with a monoclonal anti-BrdUrd antibody (Becton Dickinson), followed by incubation with a fluorescein isothiocyanate-conjugated rabbit anti-mouse secondary antibody (DAKO). Cells were RNase-treated and stained with propidium iodide before loading onto an Epics Profile I flow cytometer.

RESULTS

Transactivation by PPARγ Is Independent of pRB—Transcriptional activation by nuclear receptors is dependent on recruitment of coactivator proteins. pRB has recently been shown to modulate the activity of the thyroid hormone and glucocorticoid receptors by interaction with coactivators (41, 42). Trip230 was shown to be a thyroid hormone receptor (TR) coactivator and a pRB-interacting protein (41). pRB was able to sequester Trip230 from TR, thereby down-regulating the activity of TR. Contrary to the effect on TR, pRB was found to potentiate glucocorticoid receptor (GR)-mediated transactivation by direct interaction with hBrm (42), a previously identified GR coactivator (35). To examine whether the transcriptional activity of PPARγ was modulated by pRB, we analyzed the transactivation potential of full-length PPARγ in the human cell line C33A which does not express functional pRB. C33A cells have previously been used to demonstrate potentiation of GR transactivation by pRB (42). As shown in Fig. 1A, pPARγ is transactivating the reporter in C33A cells (column 3), and addition of the high affinity PPARγ ligand BRL49653 further enhances its activity (column 4). Coexpression of pRB has little or no effect on either BRL49653-dependent or -independent transactivation by PPARγ (compare columns 3 and 7 and columns 4 and 8). As expected, no effect on PPARγ transactivation was observed by coexpressing the nonfunctional pRB mutant pRB(H209) (compare columns 3 and 11 and columns 4 and 12). Since C33A cells express no hBrm (35), we wanted to rule out the possibility that pRB affected PPARγ transactivation via this coactivator. This appeared not to be the case, as we observed no effect on PPARγ transactivation by coexpression of hBrm, neither in the presence nor in the absence of pRB (data not shown). As a positive control, we tested the effect of pRB on E2F-mediated transactivation. Fig. 1B shows that pRB represses basal reporter activity, probably by repressing endogenous E2F in C33A cells (compare columns 1 and 3). Furthermore, E2F-1-induced transactivation of the reporter (column 2) was partially repressed by coexpression of pRB (column 4). The pRB mutant failed to repress either basal reporter activity or E2F-1-induced reporter activity (compare columns 1 and 5 and columns 2 and 6). From these experiments, we conclude that transactivation by PPARγ is independent of pRB in C33A cells.

Activation of PPARγ Bypasses the Function of pRB in Adipocyte Differentiation—The use of embryonic fibroblasts (MEFs) from mice with targeted disruptions of specific genes is a powerful tool in deciphering the importance and functions of proteins in cellular differentiation. By using lung fibroblasts from mouse embryos with targeted disruption of the Rb gene, the importance of pRB in adipocyte differentiation was demonstrated (26). pRB and C/EBPs were shown to interact, and it was demonstrated that pRB potentiated transactivation by C/EBPβ (26, 27).

To further characterize the importance and function of pRB in adipose conversion, we examined the potential of different known adipogenic inducers to support adipocyte differentiation of fibroblasts from normal and Rb−/− mouse embryos (29). These experiments were performed with MEFs from one wild-type (MEFα) and two pRB-deficient (ME3 and ME8) mouse embryos. Genotypes were validated by genomic PCR (Fig. 2A), and the absence of pRB expression in Rb−/− MEFs was confirmed by Western blotting (Fig. 2B) and immunostaining (data not shown). Using a standard differentiation protocol including treatment with dexamethasone, methylisobutylxanthine, and insulin (DMI treatment), only the Rb+/− MEFs differentiated to a significant degree (Fig. 3, A and B (a and c)). This is in agreement with previous results (26). However, we consistently observed some pRB-deficient cells accumulating lipid in response to the DMI treatment (Fig. 3B, c). In one of the Rb+/− MEFs (ME8), only a few cells accumulated lipid in response to standard inducers, whereas, in the other (ME3), approximately 1% of the cells accumulated lipid. By RT-PCR and Western

FIG. 1. Transcriptional activation by PPARγ is not dependent on pRB. A, C33A cells were transfected with the PPREx3-luc reporter (0.7 μg) and CMV-β-galactosidase (0.7 μg), together with combinations of expression vectors for mPPARγ2 (0.7 μg), wild-type human pRB (0.7 μg), and pRB(H209) (0.7 μg). Empty expression vector was added to ensure equal promoter load. Cells were subsequently treated with medium containing either BRL49653 (0.5 μM) or vehicle (0.1% MeSO4) for approximately 48 h before harvest. Reporter activity was normalized to β-galactosidase values. Transfections were performed in triplicate, measured in duplicate and repeated three times. B, pRB represses E2F-mediated transactivation. C33A cells were transfected with the 6xE2F-luc reporter (0.35 μg) and CMV-β-galactosidase (0.35 μg), together with combinations of expression vectors for E2F1 (0.35 μg), wild-type human pRB (0.35 μg), and pRB(H209) (0.35 μg). Cells were harvested after approximately 48 h. Reporter activity was normalized to β-galactosidase values. Transfections were performed in triplicate, measured in duplicate, and repeated three times.
Fig. 2. Characterization of the mouse embryo fibroblasts. A, PCR was performed on genomic DNA from the MEFs. Primers amplifying mutated or wild-type RB alleles were used. MEFA contains only wild-type RB alleles whereas ME3 and ME8 contain only mutated alleles. Primers for GAPDH were used as a control. PCR products were resolved on 1.5% agarose gels. MW, marker DNA ladder (1000, 750, 500, and 300 bp); C, negative control without template. B, expression of pRB in MEFs analyzed by Western blotting. One hundred µg of protein from confluent MEF cultures were loaded in each lane. As expected, no expression of pRB is observed in the RB"−/−" MEFs (ME3 and ME8).

Fig. 3. Morphological differentiation of normal and pRB-deficient MEFs. Cells were induced to differentiate as described under "Experimental Procedures." Dishes were photographed on day 10. A, representative dishes of RB"+/+" and RB"−/−" MEFs stained with Oil Red O. Cells were differentiated in the absence (top) or presence (bottom) of BRL49653 (0.5 µM). B, micrographs of Oil Red O stained dishes containing wild-type (a and b) or RB"−/−" (c and d) fibroblasts differentiated in the absence (a and c) or presence (b and d) of BRL49653, and (e) micrograph showing a small cluster of lipid-accumulating RB"−/−" MEFs after a standard differentiation induction (DMI treatment) without addition of BRL49653.

blotting, we found that all three MEFs express PPARγ1 mRNA and protein in the predifferentiated state (see Fig. 4). Therefore, we hypothesized that addition of a high affinity ligand for PPARγ might be able to bypass the block in adipose conversion imposed by the absence of pRB. Addition of BRL49653 to the standard differentiation medium efficiently promoted differentiation of RB"−/−" as well as RB"+/+" MEFs (Fig. 3, A and B (b and d)). The ligand concentration needed to bypass the defective differentiation in RB"−/−" MEFs was in agreement with the Kd of BRL49653 binding to PPARγ (17) in that differentiation was promoted with 50 nM BRL49653 (data not shown). Furthermore, even though predifferentiated MEFs express low levels of PPARα and PPARα mRNA (data not shown), the concentration of BRL49653 used in this report (0.5 µM) is sufficient to activate only the PPARγ subtype (17, 18). Therefore, we conclude that PPARγ is the target receptor in the BRL49653-induced differentiation of pRB-deficient MEFs.

To characterize the differentiation of RB"+/+" and RB"−/−" MEFs in more detail, gene expression was examined by multiplex RT-PCR and Western blotting. Treatment of MEFs with adipogenic inducers (DMI) resulted in a transient induction of C/EBPβ with expression levels peaking on day 1, irrespective of RB status and supplementation of BRL49653 (Fig. 4B). A transient up-regulation of C/EBPβ is also seen during differentiation of 3T3-L1 cells (15, 43). Treatment of RB"+/+" MEFs with either DMI or DMI together with BRL49653 resulted in the induction of PPARγ, C/EBPα, C/EBPβ, aP2/ALBP, and TBP was analyzed by Western blotting. Equal loading/transfer was confirmed by Ponceau S staining of membranes and by incubation with anti-TBP antibody.
ferentiating \( RB^{+/+} \) cells in absence of the PPAR\( \gamma \) ligand, C/EBP\( \alpha \) protein was detected only in cells treated with BRL49653 (Fig. 4B, left). Even extended exposure of the blots of protein from the DMI-treated \( RB^{+/+} \) cells challenged with antibodies against C/EBP\( \alpha \) revealed no signals above background (data not shown). Considering the relatively strong induction of C/EBP\( \alpha \) mRNA on day 6 in the DMI-treated cells, the absence of detectable C/EBP\( \alpha \) protein suggests a posttranscriptional regulation of C/EBP\( \alpha \) expression in MEFs. In pRB-deficient MEFs, PPAR\( \gamma \), C/EBP\( \alpha \), and GPDH mRNAs were very weakly induced when cells were treated with DMI in the absence of BRL49653 (Fig. 4A, right). Treatment of \( RB^{-/-} \) MEFs with DMI plus BRL49653, however, led to an induction of PPAR\( \gamma \), C/EBP\( \alpha \), and GPDH mRNAs similar to that observed in \( RB^{+/+} \) MEFs (Fig. 4A). Robust induction of PPAR\( \gamma \), C/EBP\( \alpha \), and p2AALBP proteins in pRB-deficient cells was also dependent on the PPAR\( \gamma \) ligand (Fig. 4B, right). As mentioned above, PPAR\( \gamma \) (but not PPAR\( \gamma \)-2) mRNA and protein were expressed in confluent MEFs (day 0 in Fig. 4, A and B). Therefore, it appears that ligand-activation of endogenous PPAR\( \gamma \) induces PPAR\( \gamma \)2 expression and differentiation of \( RB^{-/-} \) MEFs.

The Effect of pRB on C/EBP\( \alpha \) and C/EBP\( \beta \)-Mediated Transactivation of the Proximal PPAR\( \gamma \) Promoter—pRB has been demonstrated to potentiate C/EBP\( \beta \)-mediated transactivation of reporter plasmids containing multimeric C/EBP binding sites, possibly by acting as a chaperone to induce binding of C/EBP\( \beta \) to its cognate DNA response element (26, 27). Whether pRB was capable of regulating the activity of natural promoters via C/EBP sites was not addressed in these studies. To investigate this, we analyzed the importance of pRB in the transactivation of a C/EBP-regulated gene which is induced during adipose conversion. The proximal part of the PPAR\( \gamma \)2 promoter contains two C/EBP sites, which confer C/EBP-dependent activation in transient transfection studies (36). Using the proximal PPAR\( \gamma \)2 promoter as a reporter plasmid (36), coexpression with both C/EBP\( \alpha \) and C/EBP\( \beta \) expression vectors was found to transactivate the reporter in the pRB-deficient C33A cells (Fig. 5, columns 3 and 7). The effect of coexpression of pRB on C/EBP\( \alpha \)- or C/EBP\( \beta \)-mediated transactivation is shown in Fig. 5. In the case of C/EBP\( \alpha \), coexpression of pRB was found to have little or no effect (compare columns 3 and 4) in accordance with the previously noted pRB insensitivity of C/EBP\( \alpha \)-dependendent transactivation (44), and similarly, pRB only modestly increased the C/EBP\( \beta \)-mediated transactivation (compare columns 7 and 8). The pRB pocket mutant pRB(H209) did not significantly affect transactivation mediated by the C/EBPs (Fig. 5, compare columns 3 and 6 and columns 7 and 9). The absent or very moderate effect of pRB on C/EBP-mediated transactivation of the proximal PPAR\( \gamma \)2 promoter is in contrast to the pronounced effect on reporters containing multimeric C/EBP binding sites (26, 27). The experiments in Fig. 5 were performed with the same ratio of transcription factor to pRB expression vectors as in Fig. 1, where pRB significantly repressed E2F-mediated transactivation. pRB has been shown to potentiate GR-mediated transactivation in C33A cells (42), but it cannot be excluded that the chaperone-like effect of pRB on C/EBP proteins may be sensitive to relative protein levels. Furthermore, cell lines may differ in their ability to support a functional pRB-C/EBP interaction. In conclusion, however, our results suggest that pRB may regulate adipogenesis through pathways in addition to those controlled by C/EBP proteins.
cell extracts were prepared on the indicated days and the expression of pRB, p107, and p130 was analyzed by Western blotting. One hundred μg of protein were loaded in each lane. Equal loading/transfer was confirmed by Ponceau S staining of membranes. DNA synthesis are apparent, the first peaking on day 1 and the second peaking on day 2.5. Supplementation of 5 μM BRL49653 to the standard inducers did not significantly affect the distribution of neither RB+/+ nor RB−/− MEFs in S phase (data not shown). A similar distribution of cells in S phase was seen during adipocyte differentiation of 3T3-L1 cells, again with peaks on days 1 and 2.5.2 The percentage of cells in S phase was consistently higher in wild-type cells compared with RB−/− cells (Fig. 7). The flow cytometric analysis was performed with only one of the RB−/− MEFs (ME8), so whether the reduced number of cells in S phase extend to other pRB-deficient MEFs is not known at present. However, the fact that both RB+/+ and RB−/− MEFs underwent two rounds of DNA replication with approximately the same time course indicates that pRB is not critical for the timing of the clonal expansion phase. In addition, Fig. 7 shows that pRB is not essential for cell cycle exit during adipose conversion.

A comparison of the time course of DNA synthesis (Fig. 7) and the expression profiles of pRB, p107, and p130 (Fig. 6) indicates that hyperphosphorylation of pRB in RB+/+ MEFs is coinciding with the first round of DNA replication on day 1. The peaks of DNA synthesis coincide approximately with the induction and hyperphosphorylation of p107 on days 1 and 3 in both pRB-positive and pRB-negative cells. The transient down-regulation of p130 after stimulation with adipogenic inducers in both RB+/+ and RB−/− MEFs indicate that the level of p130 is low during clonal expansion, followed by an up-regulation after the clonal expansion phase. Similar results have been reported for differentiating 3T3-L1 cells (24, 28).

DISCUSSION

In this report we show that a high affinity PPARγ ligand effectively bypasses the block in adipocyte differentiation imposed by pRB-deficiency. To show this we used fibroblasts from normal and RB−/− mouse embryos. A significant fraction of the RB+/+ cells differentiated in response to a standard differentiation protocol as determined by lipid accumulation and expression of adipocyte markers, whereas only few RB−/− cells differentiated when subjected to the same treatment. This is in agreement with previous work showing the importance of pRB in adipose conversion (25, 26). Addition of the high affinity PPARγ ligand BRL49653 dramatically increased adipose conversion of RB+/+ as well as RB+/− MEFs. At the molecular level this was accompanied by induction of adipocyte markers, including the key transcription factors C/EBPα and PPARγ.2 The ability of BRL49653 to induce differentiation in pRB-deficient cells was not strictly dependent on the standard inducers (dexamethasone, methylisobutylxanthine, and insulin) since exposure to PPARγ ligand alone induced significant lipid accumulation (data not shown).

C/EBPβ along with C/EBPδ play crucial roles in adipocyte differentiation, as revealed by targeted disruptions (16), and are considered important for induction of PPARγ2 and C/EBPα expression (36, 45). In our experiments, BRL49653 did not significantly affect the level of C/EBPβ protein but was required for induction of both PPARγ2 and C/EBPα mRNAs in RB−/− MEFs. Wild-type and pRB-deficient MEFs express PPARγ1, but no PPARγ2, in the predifferentiated state. Therefore, it is conceivable that the critical steps regulated by pRB early in the differentiation program are bypassed by ligand activation of PPARγ1. The differentiation-promoting effect of ligand-induced PPARγ activation in the pRB-deficient fibroblasts is in agreement with our observation that PPARγ transactivation is independent of pRB in C33A cells.

pRB was shown to stimulate binding of C/EBPβ to DNA without being present in the C/EBP-DNA complex (26, 27). This indicates that pRB acts as a chaperone to enhance specific DNA binding of C/EBPs. Furthermore, pRB was shown to potentiate C/EBPβ-mediated transactivation of a reporter construct containing multimerized C/EBP binding elements in the promoter (26, 27). Thus, it could be hypothesized that the lack of adipocyte differentiation of RB−/− MEFs was related to a severely reduced level of C/EBPβ-dependent transactivation. We found that C/EBPβ-mediated transactivation of the proximal PPARγ2 promoter was rather insensitive to the level of pRB expression. This indicates that the inability of pRB-deficient MEFs to undergo adipose conversion in response to a treatment that is sufficient to induce adipocyte differentiation of normal embryo fibroblasts may reflect impairment of additional processes involving pRB. Glucocorticoids play decisive roles during differentiation of most preadipocyte cell lines (15, 43), but surprisingly little is known about the molecular func-

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tions of GR in adipocyte differentiation. pRB has been shown to potentiate transactivation mediated by GR (42, 46, 47), suggesting that impaired GR function in the pRB-deficient cells may contribute to the refractoriness of these cells to undergo adipocyte conversion. Finally, our finding that addition of a high affinity PPARγ ligand is required to induce adipose conversion of RB−/− MEFs leaves open the possibility that pRB participates in a pathway leading to the production of an endogenous ligand for PPARγ. Such a pathway may involve ADD1/SREBP1, which was recently shown to play an important role in the production of an unidentified PPARγ ligand (23). Both RB−/− and RB−/− MEFs express ADD1/SREBP1 mRNA (data not shown), but whether pRB modulates the activity of ADD1/SREBP1 is not known.

To examine whether BRL49653-induced differentiation of RB−/− MEFs was accompanied by a compensatory regulation of p107 and p130, we compared the expression of these genes during differentiation of both RB−/− and RB−/− cells. We found little or no difference in the expression pattern of p107 and p130 between normal and RB−/− MEFs. In the later stages of the differentiation program, we found that the level of p107 was lower in cells treated with BRL49653 compared with control cells. This indicates that down-regulation of p107 is related to the degree of adipose conversion, even in RB−/− MEFs. This is in contrast to the observed up-regulation of p107 during myocyte differentiation of RB−/− cells, an up-regulation not seen in RB−/− cells (6, 7). However, even though p107 is down-regulated in the terminal stages of adipose conversion, it is transiently up-regulated during clonal expansion (Ref. 28 and this study). Furthermore, p130 is up-regulated during the late stages of adipocyte differentiation (Ref. 28 and this study). This raises the question as to whether p107 and p130 are important regulators of adipose conversion. Recent evidence from other differentiation systems suggests that members of the pRB family may differ in their ability to regulate differentiation. Using the myocyte differentiation system, cells from wild-type, RB−/−, p107−/−, and p130−/− mouse embryos were compared (7). Only RB−/− cells had defects in expression of late differentiation markers and terminal cell cycle withdrawal. Furthermore, pRB was significantly more potent in activating MyoD-mediated transactivation than p107 and p130 (7). A similar increased activity of pRB compared with p107 and p130 was observed in flat cell formation of Saos-2 cells, a phenotype indicative of osteoblast differentiation (46). The in vitro importance of the three pRB family members has been addressed by gene targeting in mice. Whereas RB−/− embryos die in utero with defects in neurogenesis and erythropoiesis (3), p107−/− and p130−/− mice are viable, fertile, and show no apparent abnormalities (48, 49). These observations show that pRB is unique among the three pRB family members in the regulation of differentiation of many lineages. Whether pRB is the key pocket protein positively regulating adipogenesis remains to be established, but evidence obtained so far indicates that this may very well be the case.

Terminal withdrawal from the cell cycle is an essential step in differentiation of many cell lineages. Little is known about the regulation of cell cycle withdrawal in adipocyte differentiation. Recent evidence suggests that hypophosphorylation of pRB is important for the commitment of cells to undergo adipose conversion (24, 50). Both of the major regulators of adipocyte differentiation, C/EBPα and PPARγ, have been shown to inhibit cell proliferation (51, 52). Inhibition of proliferation by C/EBPα does not require the presence of pRB but is dependent on a functional activation domain (44). C/EBPα inhibits proliferation via transcriptional stimulation and posttranslational stabilization of the p21 cyclin-dependent kinase inhibitor (53, 54). Activation of PPARγ has been shown to inhibit proliferation by down-regulation of the P21A phosphatase, which in turn is accompanied by a decrease in E2F activity (52). The inhibition of cell proliferation by PPARγ was also observed in cells expressing the simian virus 40 large T antigen, indicating that PPARγ-mediated growth arrest does not require a functional pRB (52). In adipocyte differentiation, both C/EBPα and PPARγ are present at the time when clonal expansion ceases, and therefore, they are both possible effectors of the cell cycle withdrawal. Evidently, the PPARγ/C/EBPα initiated cell cycle withdrawal and adipocyte differentiation of MEFs may proceed in the absence of a functional pRB-dependent pathway, a notion in keeping with the finding that a certain cell cycle control prevails in pRB deficient cells (29, 55). How PPARγ and C/EBPα function in such regulatory circuits remains to be established.

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REFERENCES

1. Helin, K. (1998) Curr. Opin. Genet. Dev. 8, 28–35
2. Bartek, J., Bartkova, J., and Lukas, J. (1998) Curr. Opin. Cell Biol. 10, 805–814
3. Vignali, D. A., Lee, E. Y.-H., and Lee, W.-H. (1994) Annu. Rev. Cell Biol. 10, 1–29
4. Walsh, K., and Perlman, H. (1997) Curr. Opin. Genet. Dev. 7, 597–602
5. Wu, Z., Bucher, N. L. R., and Farmer, S. R. (1996) Genes Dev. 10, 3226–3237
6. Schneider, J. W., Gu, W., Zhu, L., Mahdavi, V., and Nadal-Ginard, B. (1993) Cell 72, 309–324
7. Schneider, J. W., Gu, W., Zhu, L., Mahdavi, V., and Nadal-Ginard, B. (1994) Science 264, 1467–1471
8. Novitch, B. G., Mulligan, G. J., Jacks, T., and Lassar, A. B. (1996) J. Cell Biol. 135, 441–456
9. Wang, J., Gu, K., Wills, K. N., and Walsh, K. (1997) Cancer Res. 57, 351–354
10. Zuckennhaus, E., Jiang, Z., Zeh, D., Smith, D. J., Phillips, R. A., and Gallie, B. L. (1996) Genes Dev. 10, 3051–3064
11. Brun, R. P., Kim, J. B., Hu, E., Altiek, S., and Spiegelman, B. M. (1996) Curr. Opin. Cell Biol. 8, 826–832
12. Mandrup, S., and Lane, M. D. (1997) J. Biol. Chem. 272, 5367–5370
13. Fajas, L., Fruchart, J.-C., and Auwerx, J. (1996) Curr. Opin. Cell Biol. 10, 165–173
14. Wu, Z., Xie, Y., Bucher, N. L. R., and Farmer, S. R. (1995) Genes Dev. 9, 2350–2363
15. Wu, Z., Bucher, N. L. R., and Farmer, S. R. (1996) Mol. Cell. Biol. 16, 4228–4136
16. Yeh, W.-C., Cao, Z., Classon, M., and McKnight, S. L. (1995) Genes Dev. 9, 168–181
17. Tanaka, T., Yoshida, N., Kishimoto, T., and Akira, S. (1997) EMBO J. 16, 7362–7368
18. Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkinson, W. O., Willson, T. M., and Kliever, S. A. (1995) J. Biol. Chem. 270, 12953–12956
19. Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995) Cell 83, 803–812
20. Kliever, S. A., Lenhard, J. M., Willson, T. M., Patel, I., Morris, D. C., and Lehmann, J. M. (1995) Cell 83, 813–819
21. Chawla, A., Schwarz, E. J., Dimasalangian, D. D., and Lazar, M. A. (1994) Endocrinology 135, 798–800
22. Tontonoz, P., Hu, E., and Spiegelman, B. M. (1994) Cell 79, 1147–1156
23. Kim, J. B., and Spiegelman, B. M. (1996) Genes Dev. 10, 1069–1077
24. Higgins, C., Chatterjee, S., and Cherington, V. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 826–832
25. Chen, P.-L., Riley, D. J., Chen, Y., and Lee, W.-H. (1996) Genes Dev. 10, 2794–2804
26. Chen, P.-L., Riley, D. J., Chen-Kiang, S., and Lee, W.-H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 465–469
27. Richon, V. M., Lyle, R. E., and McGhee, R. E. (1997) J. Biol. Chem. 272, 1117–1124
PPARγ Activation Bypasses pRB Function in Adipogenesis

35. Muchardt, C., and Yaniv, M. (1993) *EMBO J.* 12, 4279–4290
36. Clarke, S. L., Robinson, C. E., and Gimble, J. M. (1997) *Biochem. Biophys. Res. Commun.* 240, 99–103
37. Cao, Z., Umek, R. M., and McKnight, S. L. (1991) *Genes Dev.* 5, 1538–1552
38. Gao, X., and Huang, L. (1991) *Biochem. Biophys. Res. Commun.* 179, 280–285
39. Checzymska, P., and Szeczi, N. (1987) *Anal. Biochem.* 162, 156–159
40. Jensen, J., Serup, P., Karlson, C., Nielsen, T. F., and Madsen, O. D. (1996) *J. Biol. Chem.* 271, 18749–18758
41. Chang, K.-H., Chen, Y., Chen, T.-T., Chou, W.-H., Chen, P.-L., Ma, Y.-Y., Yang-Feng, T. L., Leng, T., Tsai, M.-J., O’Malley, B. W., and Lee, W.-H. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 9040–9045
42. Hendricks-Taylor, L. R., and Darlington, G. J. (1995) *Nucleic Acids Res.* 23, 4726–4733
43. Legraverend, C., Antonson, P., Flobdy, P., and Xanthopoulos, K. (1993) *Nucleic Acids Res.* 21, 1735–1742
44. Alcalay, M., Tomassoni, L., Colombo, E., Stoldt, S., Grignani, F., Fagioli, M., Szekely, L., Helin, K., and Pelicci, P. G. (1998) *Mol. Cell. Biol.* 18, 1084–1093
45. Altiok, S., Xu, M., and Spiegelman, B. M. (1997) *Genes Dev.* 11, 1987–1998
46. Timchenko, N. A., Wilde, M., Nakanishi, M., Smith, J. R., and Darlington, G. J. (1996) *Genes Dev.* 10, 804–815
47. Timchenko, N. A., Harris, T. E., Wilde, M., Biyleu, T. A., Burgess-Beusse, B. L., Finegold, M. J., and Darlington, G. J. (1997) *Mol. Cell. Biol.* 17, 7553–7561
48. Herrera, R. E., Sah, V. P., Williams, B. O., Makela, T. P., Weinberg, R. A., and Jacks, T. (1996) *Mol. Cell. Biol.* 16, 2402–2407
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