Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs

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To define a mechanism by which retinoblastoma protein (Rb) functions in cellular differentiation, we studied primary fibroblasts from the lung buds of wild-type ($RB^{+/+}$) and null-mutant ($RB^{-/-}$) mouse embryos. In culture, the $RB^{+/+}$ fibroblasts differentiated into fat-storing cells, either spontaneously or in response to hormonal induction; otherwise syngenic $RB^{-/-}$ fibroblasts cultured in identical conditions did not. Ectopic expression of normal Rb, but not Rb with a single point mutation, enabled $RB^{-/-}$ fibroblasts to differentiate into adipocytes. Rb appears in murine fibroblasts to activate CCAAT/enhancer-binding proteins (C/EBPs), a family of transcription factors crucial for adipocyte differentiation. Physical interaction between Rb and C/EBPs was demonstrated by reciprocal coimmunoprecipitation, but occurred only in differentiating cells. Wild-type Rb also enhanced the binding of C/EBP to cognate DNA sequences in vitro and the transactivation of a C/EBP β -responsive promoter in cells. Taken together, these observations establish a direct and positive role for Rb in terminal differentiation. Such a role contrasts with the function of Rb in arresting cell cycle progression in G_1 by negative regulation of other transcription factors like E2F-1.

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Functions of the tumor suppressor protein Rb have been explored in many systems. Rb is fundamental in suppressing the formation of certain neoplasms, because all retinoblastomas and many other tumors harbor important *RB* gene mutations. Considerable evidence is accumulating to show that Rb is also involved in pathways of growth and differentiation in normal cells (Riley et al. 1994; Chen et al. 1995a). The protein is crucial for example in development, as determined from studies of mice in which Rb has been inactivated in the germ-line. $RB^{-/-}$ mice die during mid- to late gestation, at a stage when many highly specialized cell types begin to form. At the time of their demise at gestational day 14.5, the fetuses have significant abnormalities in neuronal and hematopoietic development: Certain neurons and erythrocyte precursors fail to exit the cell cycle and continue to divide in aberrant locations (Jacks et al. 1992; Lee et al. 1992). Moreover, some nondividing cells also fail to differentiate terminally, whereas others undergo unscheduled apoptosis (Lee et al. 1992; 1994).

Cells that express no functional Rb cycle faster and more autonomously than normal cells. In human tumor

cells expressing only mutated Rb, expression of normal Rb suppresses the cells' neoplastic phenotypes, including tumorigenicity in nude mice (Huang et al. 1988; Bookstein et al. 1990). Furthermore, in both *RB*-deficient and normal cells, Rb has been shown to be a gatekeeper for entry into S phase of the cell division cycle (Goodrich et al. 1991). Taken together, the observations in human tumors, experimental animals, and cultured cells demonstrate the importance of Rb in cell differentiation, as well as in regulating cell cycle progression and tumorigenesis.

Many of the functions of Rb depend on association with other proteins at crucial phases in the life of the cell. The identification of Rb-associated proteins has shed considerable light on the molecular pathways involved in the deregulated growth characteristic of cells with inactivated Rb. Several viral oncoproteins, including simian virus 40 large T antigen and adenovirus E1A, have been found to bind strongly to hypophosphorylated Rb (DeCaprio et al. 1988; Whyte et al. 1988). By binding to strategic domains they functionally inactivate Rb, probably by displacing cellular proteins important for normal functions in negative cell cycle regulation. A key cellular protein with which Rb associates is one of the transcription factors known as E2F (Nevins 1992). Rb is

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known to inactivate E2F-1, which plays a critical role in promoting S phase entry (Johnson et al. 1993; Shan and Lee 1994). For example, expression of excess E2F-1 in G₁ phase initiates premature S phase entry and leads to unscheduled apoptosis (Shan and Lee 1994). In normally cycling cells, phosphorylation of Rb serves to release free E2F-1, and thereby to allow progression to committed phases of the cell cycle (Nevins 1992).

Although the molecular mechanisms by which the retinoblastoma protein functions to restrict passage past G₁ phase of the cell cycle have been reported extensively (Riley et al. 1994), the functions of Rb in cellular differentiation have only begun to be explored. Correlative evidence suggests that Rb is important in mice for muscle differentiation in culture (Gu et al. 1993) and neuronal differentiation (Lee et al. 1994). However, the more precise molecular roles of Rb in differentiation have remained elusive. In the course of establishing embryonic lung fibroblasts from Rb-deficient mice, we discovered that *RB*^{+/+} cells could be induced to differentiate into adipocytes whereas otherwise equivalent *RB*^{-/-} fibroblasts could not. These primary fibroblasts thus closely resemble the well-studied 3T3-L1 preadipocyte cell line and are therefore suitable for addressing in depth the potential roles of Rb in adipocyte differentiation. The differentiation of preadipocytes is known to involve a group of transcription factors, CCAAT/enhancer-binding proteins (C/EBPs) (McKnight et al. 1989; Cao et al. 1991; Samuelsson et al. 1991; Umek et al. 1991), that transactivate the promoters of several adipocyte-specific genes, including the fatty acid-binding protein 422/aP2 (Christy et al. 1989; 1991). In this communication, we show that Rb directly interacts with and activates C/EBPs to regulate the transcription of genes involved in adipocyte differentiation.

Results

Normal embryonic fibroblasts differentiate into adipocytes but Rb-deficient cells do not

Embryonic lung fibroblasts (ELFs) were prepared from postcoital day 14.5 C57BL/6J/129Sv mouse embryo lung buds, and checked by a PCR method for the presence of wild-type or mutant *RB* genes (Fig. 1B). These cells were subsequently cultured in DME medium supplemented with 10% fetal bovine serum until density arrested. Medium was changed periodically but the cells were not further passaged. When confluent for 4–6 weeks, *RB*^{+/+} (ELF1) cells spontaneously differentiated to a fat-laden phenotype but *RB*^{-/-} (ELF7) cells derived from an embryo in the same litter did not (Fig. 1A,B). Characteristic fat droplets in the cytoplasm stained with Oil Red EGN and marked the adipogenic phenotype. With a reliable hormone induction protocol established for 3T3-L1 murine preadipocytes (Green and Kehinde 1974; Student et al. 1980), *RB*^{+/+} ELF1 cells could be induced to differentiate much like 3T3-L1 cells into fat-laden cells after 8

Figure 1. Spontaneous differentiation of murine embryonic fibroblasts into adipogenic cells. Primary fibroblasts lines ELF1 (*RB*^{+/+}) or ELF7 (*RB*^{-/-}) were cultured in DMEM with either 5% bovine serum (BS) or 10% fetal bovine serum (FBS). The cultures were allowed to be confluent for 4 weeks with medium changes twice weekly. (A) Photomicrographs under plane-polarized light (*left*) show the morphology of fibroblasts when maintained quiescent in 5% BS and when cultured in 10% FBS but contact inhibited. The panels on the *right* show the cells maintained in 10% FBS and stained with Oil Red EGN and hematoxylin to show the accumulation of fat droplets. (B) Genotyping for *RB* by a PCR method. A 151-bp fragment is derived from wild-type *RB* genomic DNA (W) and the 236-bp fragment from mutated *RB*^{A20} DNA (M) (Nikitin and Lee 1996). (Lanes 1–3) Controls: genomic DNA extracted from tail biopsies of mice with the genotypes *RB*^{+/+}, *RB*^{+/-}, and *RB*^{-/-}; (lane 4) ELF7; (lane 5) ELF1.

days, but similarly treated *RB*^{-/-} ELF7 cells could not (Fig. 2). From these observations we conclude that *RB*-null fibroblasts cannot differentiate in culture to the adipogenic phenotype, either spontaneously or in response to appropriate hormonal stimuli.

Figure 2. Adipocyte differentiation of ELF1 and 3T3-L1 cells after hormone treatment. Mouse fibroblasts lines ELF1, ELF7, and 3T3-L1 were induced with hormones for 8 days following the treatment protocol described in Materials and Methods. The morphologies of each cell line before and after hormonal induction are shown. The *left* and *middle* panels are photomicrographs taken under plane-polarized light. At day 8 after induction of differentiation, cells were stained with Oil Red EGN and counterstained with hematoxylin (*right* panels).

Expression of wild-type Rb in $RB^{-/-}$ fibroblasts restores their ability to differentiate into adipocytes

To test whether Rb expression is the key factor in determining the potential of embryonic lung fibroblasts to differentiate into fat-laden cells, $RB^{-/-}$ fibroblasts were transfected with plasmids containing wild-type or point mutant (Cys⁷⁰⁶→Phe, Bignon et al. 1990) *RB* cDNA to express either Rb protein. Both wild-type and mutant Rb proteins were expressed in the appropriate cells after transfection (Fig. 3B). The fibroblasts transfected with the plasmid expressing wild-type Rb differentiated into fat-laden cells in response to hormonal treatment (Fig. 3A). In contrast, untransfected fibroblasts and those transfected with mutant *RB* failed to differentiate along the same lineage (Fig. 3A). Eight hygromycin-resistant colonies from a bulk population of ELF7 fibroblasts transfected with wild-type *RB* were tested individually for expression of Rb protein and for their potential to

Figure 3. Ectopic expression of the wild-type but not point mutated Rb in $RB^{-/-}$ ELF7 cells restores the capacity to differentiate. ELF7 cells were cotransfected with two plasmids, one which expresses the wild-type Rb under the tetracycline-controllable promoter, and one which expresses the tet regulator along with a hygromycin selection marker (Gossen and Bujard 1992). The H209 mutant Rb expression was similarly driven by a tetracycline-inducible promoter. Low-level expression from this leaky promoter in the presence of tetracycline supplied detectable Rb but did not arrest cell growth. After selection with hygromycin, the transfected cells were grown to confluence and subjected to the hormonal treatment protocol previously described. (A) Photomicrographs show the morphological changes in ELF7 cells transfected with the wild-type Rb: They acquire the adipogenic phenotype. Identical cells untransfected or transfected with the H209 mutant RB do not differentiate similarly. (B) Rb protein is expressed in cells transfected with both wild-type and mutant RB constructs. Lysates of the transfected cells were immunoprecipitated with an anti-Rb mAb (11D7), separated by SDS-PAGE, transferred to membranes, and probed with the same antibody. Cycling T24 cells (lane 1) serve as a control for the positions at which hypo- and hyperphosphorylated Rb species migrate. Expression of both wild-type (lane 2) and mutant Rb (lane 3) was apparent in both populations of transfected cells, but not in untransfected cells (lane 4).

Figure 4. Differentiation of ELF7 clones into adipocytes depends on the expression of Rb protein. Eight hygromycin-resistant clones were propagated until confluent, and treated with the hormone combination described previously. (A) Entire populations of cells in 60-mm plates were first stained with Oil Red EGN. (B) The cells were then counterstained with hematoxylin and photomicrographs were taken from random sections of the plates in A. Clones C10, C14, C26, C34, and C35 differentiated into adipocytes as described for Fig. 3. (C) Expression of Rb detected by immunoprecipitation and Western blotting. Only clones expressing Rb differentiated in response to hormonal induction. (Lane 1) Untransfected ELF7 cells; (lane 2) T24 bladder carcinoma cells (growth arrested and expressing predominantly hypophosphorylated Rb) as a positive control for the position of p110^{RB}; (lane 3) clone 2 (C2); (lane 4) C10; (lane 5) C14; (lane 6) C26; (lane 7) C29; (lane 8) C32; (lane 9) C34; (lane 10) C35.

differentiate into fat-storing cells with hormone treatment. Only those clones expressing Rb gained the ability to differentiate; those that failed to express Rb also failed to acquire the adipogenic phenotype (Fig. 4). To test whether the cells rescued by Rb and differentiated by hormone treatment were adipocytes, we examined them for expression of the fatty acid-binding protein 422/aP2 (Christy et al. 1991). 422/aP2 messenger RNA was detected in all fat-laden cells that expressed Rb, but not in undifferentiated fibroblasts without functional Rb (Fig. 5). Taken together, these results suggest that Rb is a critical factor for the differentiation of embryonic lung fibroblasts into fat-storing cells.

Rb and C/EBPs interact in vitro

Previous studies have shown convincingly that expression of CCAAT/enhancer-binding protein transcription factors sequentially activates terminal adipocyte differentiation (Cao et al. 1991; Yeh et al. 1995). In particular C/EBP α directly regulates terminal adipocyte differentiation by turning on a battery of genes required for the synthesis, uptake, and storage of long-chain fatty acids (Samuelsson et al. 1991; Umek et al. 1991; Freytag et al.

Figure 5. 422/aP2 mRNA expression in morphologically differentiated adipocytes. 3T3-L1, ELF1, ELF7, and three ELF7 clones transfected with RB were treated with the hormone induction protocol for 8 days, after which RNA was extracted from each individual line. Ten μ g of total RNA were analyzed and probed with 422/aP2 cDNA or with G β -like protein cDNA. Only those lines that expressed Rb (3T3-L1, ELF1, C26, and C35) also expressed 422/aP2 mRNA.

1994; Lin and Lane 1994). C/EBP δ and β appear to play earlier, catalytic roles in the differentiation pathway by relaying hormonal and other events, leading to activation of C/EBP α (Yeh et al. 1995). To begin to address how Rb influences adipocyte differentiation, the interaction between Rb and C/EBPs was examined in vitro. The cDNAs encoding C/EBP α , β , and δ were each individually inserted into glutathione *S*-transferase (GST) expression plasmids and the resultant fusion proteins were expressed in bacteria. In the case of C/EBP β , we took advantage of a specific *Nco*I site at codon 22 (see Materials and Methods) to create a construct that would preferentially produce the transcriptionally active form of C/EBP β , termed *liver-enriched transcriptional activator protein* (LAP), rather than the smaller, inactive form, termed *liver-enriched transcriptional inhibitory protein* (LIP) (Descombe and Schibler 1991).

All three GST-C/EBP fusion proteins bound specifically to the hypophosphorylated isoform of Rb, the ac-

tive form that predominates in the G₀ and G₁ phases of the cell cycle (Fig. 6A). GST-SV40 T antigen also bound Rb, but GST alone did not. To determine which region of Rb is required for binding to LAP, various Rb-deletion mutant proteins were expressed using the pET system (Shan et al. 1992). By passing the bacterial lysates over GST-LAP or GST-SV40 T antigen fusion protein columns, we determined that the same two domains required for binding T antigen (Hu et al. 1990; Huang et al. 1990) are also required for Rb to bind to LAP (Fig. 6B,C). The interaction of LAP with Rb may occur in a manner similar to that used by the nuclear factor-interleukin 6 (NF-IL6) (P.-L.Chen et al. 1996), because a sequence in C/EBP β , from amino acids 66 to 102, is nearly identical to NF-IL6 amino acids 78 to 122. Similar critical residues also were found in C/EBP α and C/EBP δ , as well as in the Rb-binding region of E2F-1 (Shan et al. 1996), as shown in Fig. 6D. This sequence conservation is consistent with the results from in vitro binding assays.

Rb and C/EBPs interact in terminally differentiating cells

Having established an interaction between Rb and C/EBPs in vitro, we next explored the interaction in cells during terminal differentiation. In these experiments, *RB*^{+/+} ELF1 cells were again employed, for they could be reliably and synchronously differentiated into adipocytes by hormonal treatment. At intervals after the beginning of induction of adipocyte differentiation, the interaction between Rb and C/EBP β (as an example for other C/EBPs) was examined by coimmunoprecipitation. In quiescent *RB*^{+/+} ELF1 cells, expression of both C/EBP β and hypophosphorylated Rb was observed (Fig. 7, lane 6), and the cells were subsequently able to differentiate into adipocytes. Attempts to induce adipocyte differentiation in *RB*^{-/-} ELF7 cells were unsuccessful, despite the presence and relative abundance of C/EBP β

Figure 6. Hypophosphorylated Rb protein binds specifically to C/EBPs in vitro through an SV40 large T antigen binding domain. (A) GST, GST-T, or GST-C/EBP α , GST-LAP (GST-C/EBP β), and GST-C/EBP δ fusion proteins immobilized on glutathione beads were used to bind Rb in *RB*-reconstituted (WR2E3) cell lysates. Lane 1 shows both hypo- and hyperphosphorylated forms of Rb protein immunoprecipitated (IPP) with anti-Rb antibody 11D7 in cycling cells. Similar cell extracts were incubated with beads containing various GST fusion proteins (lanes 2–6). The three forms of GST-C/EBP specifically bound hypophosphorylated Rb (lanes 4–6), as did the positive control, GST-SV40 T antigen (lane 3). (B) Both wild-type and

a series of mutant Rb proteins, as shown in C, were used to bind beads containing either GST-T (lanes 1–5) or GST-LAP (lanes 6–10). The proteins bound to the beads were then released, separated by SDS-PAGE, and detected with antibody 11D7, as described above. (C) The binding sites for C/EBP in Rb protein are similar to those used by the SV40 T antigen. The shaded areas represent the A and B regions of T-antigen binding domain. (D) Comparison revealed sequence homology in the Rb-binding motifs of C/EBP proteins with those of NF-IL6 (from residues 105 to 128) and E2F-1 (from residues 411 to 425). The consensus motif is shown.

Figure 7. Interaction of Rb with C/EBP β occurs specifically when ELF1 cells differentiate into adipocytes. (A) $RB^{-/-}$ ELF7 and $RB^{+/+}$ ELF1 cells were treated with the aforementioned hormone protocol for 6 days. Cells lysates were prepared at different time points for reciprocal coimmunoprecipitations with anti-C/EBP β antibody C-19 (A) or anti-Rb antibody 0.47 (B) D0 is day zero with respect to the beginning of hormone induction, D2 is day two, etc. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to Immobilon-P, and immunoblotted with either 0.47 antibody for Rb or C-19 for C/EBP β , as indicated. p84, a protein expressed constitutively and constantly during differentiation (Durfee et al. 1994), was used as an internal standard to control for cell lysate loading (C).

(Fig. 7A, lanes 1–5). In contrast, hormonal treatment of $RB^{+/+}$ ELF1 cells resulted in acquisition of the morphologic characteristics of mature adipocytes. Concomitant with this differentiation, C/EBP β expression was slightly increased by day 2, and a definite interaction between C/EBP β and the hypophosphorylated form of Rb was detected by coimmunoprecipitation (Fig. 7, lanes 7–9). By day 6 after hormone induction, the interaction between C/EBP β and Rb was no longer evident (lane 10). Rb protein could not be detected by coimmunoprecipitation in $RB^{-/-}$ ELF7 cells, because any Rb protein produced in these cells is truncated and nonfunctional (Lee et al. 1992) (Fig. 7B, lanes 1–5). Note that the coimmunoprecipitations were not attributable to antibody cross-reactivity, because antibodies to C/EBP β epitopes did not recognize Rb (Fig. 7A) and antibodies to Rb epitopes did not recognize C/EBP β (Fig. 7B). Like the interaction between Rb and C/EBP β , binding between Rb and C/EBP α could also be demonstrated by coimmunoprecipitation during the terminal differentiation of ELF1 cells to adipocytes (data not shown).

Rb enhances the binding of LAP to specific DNA

The consequence of Rb binding to C/EBPs cannot be attributed to a negative role in inactivation of C/EBPs, because the expression of C/EBPs actually activates the adipogenic differentiation process in a sequential cascade (Cao et al. 1991; Samuelsson et al. 1991; Yeh et al. 1995). To address the influence of Rb on the DNA-binding activity of a C/EBP in vitro and to compare it with

the established role of Rb in regulating another well-characterized transcription factor, a DNA electrophoretic mobility shift assay (EMSA) was designed specifically for LAP and for E2F-1. The latter protein is known to be inactivated when Rb prevents progression of the cell cycle to S phase (Nevins 1992). The DNA probe (E2F100) used in our assay contains both LAP and E2F-1-responsive sequences (P.-L. Chen et al. 1996), and forms complexes with either LAP or E2F-1. As expected, bacterially expressed GST-LAP specifically recognized the appropriate DNA sequence and retarded the migration of a labeled DNA probe (Fig. 8A, lane 2). However, addition of purified, amino-terminally truncated p56^{RB}, which still has intact T antigen, NF-IL6, and C/EBP-binding domains (Hensley et al. 1994; P.-L. Chen et al. 1996), increased by >10-fold the binding of LAP to its recognition sequences, in an Rb dose-dependent manner (Fig. 8A, lanes 5–9). In contrast, the binding of E2F-1 to the same DNA probe was either unaffected or actually decreased by Rb (Fig. 8B). Rb thus had opposite effects on the DNA-binding activity of two important transcription factors.

Physical interaction with Rb is a prerequisite for the DNA-binding activity of LAP to be enhanced. A point-mutated Rb (H209, Cys⁷⁰⁶→Phe) (Bignon et al. 1990), which cannot bind C/EBPs, did not enhance the binding of LAP to DNA in the same assay (Fig. 8C). To determine whether Rb protein was present in the LAP–DNA complexes, three anti-Rb antibodies, 3C8 (Lee et al. 1992), 11D7 (Shan et al. 1992), and 0.47 (Huang et al. 1990), which recognize three distinct epitopes, were added individually to the EMSA. None of the antibodies had any effect on the mobility of the LAP–DNA complexes (Fig. 8D, lanes 9–11). This observation indicates that Rb is not present continuously or stably in complexes with LAP and sequence-specific DNA. The presence of LAP in complexes with DNA, however, was demonstrated by supershift with an anti-C/EBP β /LAP antibody (Fig. 8D, lane 12). The specificity of the antibodies was confirmed by inability of anti-T antigen antiserum to supershift the protein–DNA complex (Fig. 8D, lane 13). Moreover, the effect of Rb on the DNA-binding activity of LAP was not particular to the amino-terminally truncated p56^{RB}, because full-length p110^{RB}, purified from the pET system or from a baculovirus expression system, also enhanced the binding of LAP to specific DNA sequences (data not shown).

Rb enhances transactivation by C/EBP in transient transfection assays

To explore the functional significance of the interaction between Rb and C/EBPs, the effect of Rb expression on transcriptional activation by LAP was examined. The chloramphenicol acetyl transferase reporter construct [pP_(D)9CAT] (Mueller et al. 1990), which is regulated specifically by LAP, was used for testing the effect of Rb on adipocyte-specific gene expression. Human retinoblastoma WERI-Rb27 cells, which do not express functional Rb, were chosen as the recipient cells because they pro-

Figure 8. Rb enhances binding of the transcription factor LAP to DNA-containing LAP recognition sequences. (A) E2F100, an oligonucleotide which contains consensus sequences with which both LAP and E2F-1 interact specifically, was labeled with 32 P and served as probe. Bacterially expressed GST-LAP was added into the EMSA and retarded the mobility of E2F100 (lane 2). Upon addition of excess of unlabeled oligo DNA containing the wild-type recognition sequence of LAP (Wt, lane 3) as a competitor, the binding of E2F100 was abolished (lane 3). Upon the addition of mutant oligo DNA, which LAP cannot recognize, the binding of E2F100 by GST-LAP was not changed (lane 4). Increasing concentrations of purified p56^{RB} protein enhanced binding to E2F100 in a dose-dependent manner, ultimately by more than 10-fold when present at a dose of 0.3 μ g (lane 9). (B) A similar experiment demonstrates that Rb inhibits binding to the same E2F100 oligonucleotide to E2F-1. Wild-type and mutant E2F-1 oligo DNA were used to demonstrate the binding specificity of E2F-1 (lanes 1–4). Upon addition of increasing amounts of purified p56^{RB} to the EMSA, the binding of E2F-1 to E2F100 was reduced or abolished (lanes 6–9). Equivalent amounts of the fu-

sion proteins (50 ng/lane) GST-LAP (A) and GST-E2F-1 (B) were added for each mobility shift assay. (C) Enhancement of the binding of GST-LAP to its DNA sequence is specific for wild-type p56^{RB} (GST-RBS, lanes 5–10) but not mutated p56^{RB} (GST-RBH209, lanes 13–19). Instead of the purified p56^{RB} protein, GST fusion proteins with the wild-type p56^{RB} (GST-RBS) and the corresponding point mutant p56^{RB} (GST-RBS20, Cys⁷⁰⁶→Phe) were used in the EMSA. GST-RBS enhanced the binding activity of LAP, but the mutant GST-RBS20 did not. Each reaction contains 50 ng of GST-LAP. Alone, neither GST-RBS (lane 11) nor GST-RBS20 (lane 20) bind to the probe. (D) Rb is not present in the DNA-LAP protein complex. In this set of experiments, 50 ng of GST-LAP was added to each reaction. The binding to DNA of the activated LAP could be abolished by competition with wild-type but not mutant oligo DNA (lanes 7 and 8). This suggests that activated LAP binds specifically to its cognate sequences, as it does even before activation by p56^{RB} (lane 2). Various anti-Rb antibodies (3C8, 11D7, 0.47, lanes 9–11) and a nonspecific, anti-T antigen antibody (419) (lane 13) had no effect on the mobility of these complexes. A supershift was detected, however, when antibody C-19, which recognizes LAP, was added (lane 12).

vide a background on which the effects of Rb expression can be measured. As shown in Fig. 9, LAP alone increased CAT activity 10-fold compared with vector alone (lane 2). Coexpression of LAP and Rb in Rb-null cells, however, synergistically increased CAT activity almost 40-fold (lane 4). When compared with LAP expression alone, coexpression of the H209 Rb mutant with LAP (lane 4) had no significant additional effect on basal CAT activity. Furthermore, expression of Rb itself, either wild-type (lane 5) or the H209 mutant (lane 6) had no effect on CAT activity. These results demonstrate that wild-type Rb, in addition to increasing the DNA-binding activity of LAP in vitro, can also enhance the transcriptional activity of LAP in intact cells. A mutant Rb protein that did not interact with LAP also failed to enhance specific transcriptional activity.

Discussion

We have demonstrated that wild-type retinoblastoma protein is essential for mouse embryonic lung fibroblasts to differentiate into adipocytes. Without Rb, syngenic fibroblasts fail to undergo terminal differentiation despite hormonal induction. The process of adipocyte dif-

ferentiation is regulated by the interaction between Rb, through its SV40 T antigen-binding domains, and the family of transcription factors known as C/EBPs. The interaction occurs in certain preadipocytes precisely when they begin to differentiate. Furthermore, Rb appears to activate C/EBP β /LAP directly by enhancing its binding to cognate DNA sequences. Taken together, these observations suggest a function for Rb that has only begun to be explored: In addition to negatively regulating transcription factors like E2F-1 to prevent quiescent cells from passing a restriction point in G₁, it positively regulates transcription factors important for differentiation.

The development of distinct cell types from multipotent precursors can be viewed as a two-step process. In the first step, termed commitment or determination, the stem cell becomes limited to one particular lineage. In the second step, terminal differentiation, the cell develops along its determined lineage to become a functional specialized cell such as an adipocyte (Sager and Kovac 1982). Rb is not required in general for all steps in the differentiation of every cell type. In Rb-deficient embryos by the time of their demise in utero, certain cells have already substantially committed to terminal differ-

Figure 9. Rb activates C/EBP β in transcription of a specific promoter. $RB^{-/-}$ human retinoblastoma WERI-Rb27 cells were transfected with one or more of four DNA constructs: 20 μ g of the a C/EBP β -specific promoter driving the CAT reporter [pP(D)9CAT] (lanes 1–6); 20 μ g of a C/EBP β expression construct (MSV-C/EBP β) (lanes 2–4); 4 μ g of the plasmid that expresses wild-type Rb (CMV-NeoRb) (lanes 3 and 5); and 4 μ g of the construct that expresses a mutant Rb (CMVNeoH209) that fails to bind to C/EBP β (lanes 4 and 6). The histograms represent mean CAT activities from three separate transfections. Standard error bars are shown.

entiation. For example, the formation of somites, cardiac and skeletal muscle, bone, and several other tissues has already been accomplished in $RB^{-/-}$ mice by day 14.5 of embryonic development. However, clear abnormalities in the differentiated phenotypes of diverse cells types have also been observed. Erythrocytes retain their nuclei and neuronal cells in the developing hind brain and spinal ganglia fail to differentiate further into mature neurons (Lee et al. 1994). A role for the tumor suppressor protein Rb in normal development is consistent in many ways with the failure in terminal differentiation seen as a distinctive phenotype of cancer. The positive role of Rb in terminal differentiation may be critical for tumor suppression functions.

In our current study, the interaction between Rb and C/EBP β did not take place in $RB^{+/+}$ ELF1 cells before hormonal treatment. Syngenic $RB^{-/-}$ ELF7 cells, despite expressing C/EBP proteins in a similar temporal pattern, did not differentiate even when induced by appropriate hormones. These results suggest that the presence of the transcription factors that regulate adipocyte differentiation is not by itself sufficient to allow acquisition of the final adipogenic phenotype. However, it was noted that the elevated expression of C/EBP β was observed at D2 after interaction with Rb (D1). This is consistent with the finding that C/EBP β mRNA was induced at the beginning of adipocyte differentiation (Cao et al. 1991). Hormonal treatment may activate C/EBPs through the interaction with Rb, and may be the major force for initiation of subsequent events in the differen-

tiation cascade. Congruous findings have been reported in the differentiation of 3T3-L1 cells, although even the expression of C/EBPs in these cells was not evident until after hormonal induction (Yeh et al. 1995). The detailed downstream mechanisms by which glucocorticoids, adenylate cyclase activators, and insulin transduce signals to allow the interaction between Rb and C/EBPs is unknown at present, but is actively being pursued. It is likely that C/EBP β and other C/EBPs may require hormone-mediated signal transduction pathways that ultimately result in post-translational modifications or changes in subcellular compartmentalization and allow them to interact with Rb *in vivo*. In addition to C/EBPs, another transcription factor, peroxisome proliferation-activator receptor γ (PPAR γ), has also been shown to be directly involved in adipocyte differentiation (Hu et al. 1995; Tontototz et al. 1994a,b). Interestingly, known members of the C/EBP family not only all interact with Rb, they each contain Rb-binding motifs similar to the one used by E2F-1. In contrast, PPAR γ , although it has no recognizable Rb-binding motif (Tontototz et al. 1994a), can be regulated by C/EBP β (Wu et al. 1995). We therefore would not expect it to be regulated by Rb. Rb probably instead acts directly on C/EBPs, which play distinct and somewhat earlier roles in the cascade of events in adipocyte differentiation.

Previous studies in *ex vivo* culture systems have shown that Rb may also be important for muscle differentiation by interacting with myoD (Gu et al. 1993). Early muscle development in $RB^{-/-}$ embryos, however, proceeds normally well past the stage when myoD begins to initiate muscle developmental patterns, so the significance of the interaction between myoD and Rb in muscle differentiation *in vivo* remains correlative and circumstantial. Myotube differentiation from $RB^{-/-}$ myoblasts may be mediated in part by Rb-related proteins such as p107 (Schneider et al. 1994). In a different but analogous cell culture system, another correlation was observed between the process of monocyte-macrophage differentiation and the positive regulation of the transcription factor NF-IL6 by Rb (P.-L. Chen et al. 1996). The significance of this correlation *in vivo* is likewise still unknown; no immediate monocyte-macrophage precursors with the genotype $RB^{-/-}$ are currently available to test the isolated effects of Rb expression on macrophage differentiation. Our results in this communication, which show lack of adipocyte differentiation in $RB^{-/-}$ mouse embryonic fibroblasts unless they are reconstituted by ectopic Rb expression, go beyond correlation. They suggest clearly that functional Rb is critical for the terminal differentiation of certain specialized cells. Mature adipose tissues have not been observed in either wild-type or Rb-deficient mouse fetuses because the animals depend entirely on maternal energy sources and do not store fat until birth (Wang et al. 1995).

It is important to note that Rb itself does not bind to C/EBP-specific DNA sequences in the mobility shift assays (Fig. 8), but nonetheless does bind to C/EBPs both *in vitro* and in cells during the differentiation process (Fig. 7). The interaction between Rb and C/EBP β molecules

appears to have a function in allowing LAP to bind DNA, and in the transactivation of LAP target genes: The H209 (Cys⁷⁰⁶→Phe) Rb that fails to bind to LAP also fails to enhance transactivation of relevant promoters in the *in vitro* systems used. These observations suggest that the Rb–C/EBP interaction occurs during a defined window only in differentiating cells, as it would if Rb has a function akin to a molecular chaperone (Ellis and van der Vies 1991). Our experiments suggest that there may be three forms of LAP: one inactivated prior to interaction with wild-type Rb, a second transiently complexed with Rb during differentiation, and a third activated after dissociation from Rb. The precise conformational or other change by which Rb directly activates LAP and other C/EBPs is not yet known. An alternative explanation for the observed transience of the Rb–C/EBP interaction only in differentiating cells may be the instability of the Rb–C/EBP–DNA complex during the particular conditions of the DNA electromobility shift assay. The dependence of C/EBP activation on Rb protein under physiological conditions remains to be defined. Nonetheless, the *in vivo* transfection experiments clearly show that the Rb–C/EBP interaction, demonstrated biochemically, is also important functionally, because Rb positively activates transcription by C/EBP β /LAP.

The mechanism by which Rb binds to similar motifs in two different classes of proteins (C/EBPs and E2Fs) and results in two seemingly opposite functions remains unknown. Rb apparently functions both to prevent progression of G₁, when E2F-1 is bound and inactivated, as well as to promote differentiation when C/EBPs are bound and activated. The T-antigen-binding domain is the major region for the interaction of Rb with a variety of cellular proteins (Chen et al. 1995a). The motifs recognized by this domain include LxCxE, as in SV40 T-antigen, and Y(x7-37)E(x3)DLF, as in E2F-1. Binding of Rb to E2F-1 results in inactivation of target gene transcription, perhaps because Rb binds to the carboxy-terminal transactivation domain of E2F-1. The main Rb-binding motifs in C/EBPs, however, are also located within their amino-terminal transactivation domains, and the consequence of Rb binding to C/EBPs is nonetheless activation. This apparent contradiction cannot be resolved without further detailed molecular characterization of the interactions between Rb, C/EBPs, and E2F-1. Rb may use subtly different motifs for interaction with the two classes of molecules. Alternatively, the binding of Rb to given transcription factors may alter their conformations and expose inherent elements remote from Rb-binding sites. Altered conformations after interaction with Rb may then result in the binding of each transcription factor to different downstream target proteins and DNA sequences.

Other members of Rb protein family such as p107 and p130 (Ewen et al. 1991; Li et al. 1993) could play similar roles in differentiation of cell types other than the few observed to be grossly abnormal in *RB*^{−/−} embryos. Our preliminary data suggest that these other proteins have similar chaperone-like activities in C/EBP EMSA assays. Because the developmental expression patterns of p107

and p130 in mice (G. Chen et al. 1996) are quite different from that of Rb (Szekely et al. 1992), distinct but related roles for Rb, p107, and p130 in the acquisition of specific, differentiated cell phenotypes should be expected. Further studies in the differentiation of fibroblasts to adipocytes and in other model systems of cellular differentiation will be helpful in exploring such roles.

Materials and methods

Mouse embryonic lung fibroblasts and culture conditions

C57BL/6J/129Sv mice heterozygous for an *RB* gene disrupted at exon 20 (Lee et al. 1992) were crossed. On postcoital day 14.5, pregnant females were sacrificed. Lung buds were dissected individually from harvested embryos, minced, and cultured in Dulbecco's modified Eagle medium (DMEM, GIBCO BRL, Life Technologies, Inc., Gaithersburg, Maryland) containing 10% fetal calf serum (Hyclone, Logan, UT). Cells propagated *en masse* under identical conditions, with medium changes every 3 to 4 days, were genotyped using murine *RB* and *Neo* gene oligomers designed for PCR (Nikitin and Lee 1996).

Hormone treatment and determination of terminal differentiation

Fibroblasts were induced to differentiate as described previously by Student et al. (1980). This induction involved first growing cells to confluence, then at day 0 of the differentiation induction program exposing them to fresh DMEM containing 10% FBS, 1 μ M dexamethasone (DEX), 10 μ M forskolin, and 10 μ g/ml insulin for 48 hr to initiate adipogenesis. The medium was then replaced with DMEM containing 10% FBS and 10 μ g/ml insulin, and cells were refed every other day until day 8. To confirm the appearance of the adipogenic phenotype, particularly the accumulation of fat droplets in the cytoplasm, cells were fixed at particular time points in 3% glutaraldehyde/100 mM sodium phosphate (pH 7.4) and stained with Oil Red EGN (Preece 1972).

RNA blotting analysis

Total RNA was prepared from cells as described previously (Shan et al. 1992). For Northern blotting, 10 μ g of RNA from cells in each condition were used. cDNAs used as probes were those encoding 422/aP2 (Spiegelman et al. 1983) and G β -like protein (Shan et al. 1992).

Expression of GST fusion proteins

The C/EBP cDNAs were expressed as GST fusion proteins in bacteria as described previously for other fusions (Shan et al. 1992). To construct GST–C/EBP α , the MSV–C/EBP α plasmid (Christy et al. 1991) was digested with *Hind*III and then partially digested with *Nco*I. Blunt ends were created in the resultant 1939-bp insert, and the fragment was then subcloned into the *Sma*I site of pGEX-2T. GST–LAP was constructed in several steps. First, the 1-kb *Eco*RI–*Nhe*I fragment containing the LAP coding sequence from MSV–LAP was inserted into pBSK, then cleaved with *Eco*RI and *Xba*I to create pBSK–LAPR. pBSK–LAPR was then digested with *Not*I, extended to create blunt ends, and engineered with an *Eco*RI linker to generate pBSK–LAPR2. The 1-kb *Nco*I–*Eco*RI fragment from pBSK–LAPR2 was then inserted into pGEPKIII, a derivative of pGEX-2T, and finally cleaved with *Nco*I and *Eco*RI to create pGST–LAP. To generate pGST–C/EBP δ , *Bam*HI-digested pGEX2T (a 218-bp

*Bam*HI–*Nco*I fragment generated by PCR) was inserted along with a 790-bp *Nco*I–*Bam*III fragment of MSV–C/EBP δ in a three-way ligation into the amino-terminal fragment of C/EBP δ . Primers 4-1 (5'-CAAGGATCCATGAGCGCCGCGCTTTTC-3') and 4-2 (5'-TGGGCACGGCGGCCTGGAGTCAATGT-3') were used to generate the 218-bp PCR fragment; absence of base substitution errors was confirmed by DNA sequencing. All GST–C/EBP constructs were used to transform *Escherichia coli* strain BL21-LysS and express the GST fusion proteins. Preparation of GST fusion proteins and Rb mutant proteins from bacteria has been described (Chen et al. 1995b).

Coimmunoprecipitation

Nuclei from terminally differentiated or undifferentiated fibroblasts were first prepared and disrupted in Lysis 250 buffer by subjecting them to three freeze/thaw cycles (liquid nitrogen/37°C) and clearing by centrifugation (14,000 rpm, 2 min at room temperature). The supernatant was diluted with equal volumes of lysis buffer (without NaCl) and used for coimmunoprecipitation as described (P.-L. Chen et al. 1996). Briefly, to each clarified supernatant was added 1 μ g of rabbit polyclonal anti-Rb antibody (0.47) or 5 μ l anti-C/EBP β polyclonal (C-19, Santa Cruz Biotech. Santa Cruz, California) antibody. Antigens and antibodies were incubated together for 1 hr before the addition of protein A–Sepharose beads (80 μ l). After another 1-hr incubation, the beads were collected and washed five times with lysis buffer containing 125 mM NaCl. Beads were then boiled in SDS loading buffer for immunoblotting analysis.

Cell lysate preparation, in vitro binding assay, and Western blotting

Extracts made either from 2×10^6 WR2E3 cells or from bacterial lysates were incubated with beads containing 2–3 μ g of GST or GST fusion proteins in Lysis 150 buffer (identical to Lysis 250 buffer except that it contains 150 mM NaCl) for 30 min at room temperature. Complexes were washed extensively with Lysis 150 buffer and analyzed by Western blotting using the primary antibodies, anti-Rb (11D7) or anti-C/EBP β (C-19) as described (Chen et al. 1995b).

DNA EMSAs

GST fusion proteins, including GST–LAP, GST–RBS, GST–RBH209 and GST–E2F-1, were prepared as described above. E2F100, which contains sequences with which both LAP and E2F-1 interact specifically, was labeled with [α - 32 P]dCTP and served as probe (P.-L. Chen et al. 1996). Unlabeled oligo DNA (5'-GGACGTCACATTGCACAATCTTAATAA-3') containing the wild-type (Wt) recognition sequence of LAP was used at 100-fold molar excess as a competitor, as was a mutant oligo DNA (5'-GGACGTCACACTACAACTCTTAATAA-3') (Mt) which cannot be recognized by LAP. The binding reaction mixtures for the LAP–DNA complex contained 10 mM HEPES (pH 7.9), 60 mM KCl, 5 mM Tris at pH 7.0, 0.1 mM EDTA, 10% glycerol, 5 mM MgCl₂, 2 mM DTT, 2 mg/ml bovine serum albumin (BSA), and 4 ng 32 P-labeled probe. The binding reactions for the E2F–DNA complexes have been described previously (Shan et al. 1992), except that BSA (2 mg/ml) was included in the reactions here. Analysis of binding complexes was performed by electrophoresis on a 5%, 0.25 $\times$ TBE polyacrylamide gel at 4°C.

Transfection and CAT assays

The reporter construct, pP_(D)CAT, used in the transient assays was a gift from C.P. Mueller (Queen's University, Kingston, Ontario, Canada). To generate pCMVLAP, an *Nco*I–*Bam*HI fragment containing the LAP coding sequence was inserted into an engineered CMVNeoBam vector. The *Nco*I site corresponds to the position of the initiating methionine of LAP (Cao et al. 1991). The construction of plasmids pCMVNeoRB and pCMVNeoRBH209 has been described (P.-L. Chen et al. 1996). Transfections were carried out by conventional calcium phosphate/DNA coprecipitation in 5×10^6 WERI–RB27 cells. The precipitates were removed 12 hr after transfection and the cultures were refed with fresh medium containing 10 μ g/ml regular insulin. The cells were collected after 48 hr and CAT activity was determined as previously described (Shan et al. 1992).

Lipofection and cloning of RB-reconstituted ELF7 cells

Wild-type and H209 mutant RB were individually inserted into the tetracycline-controllable vector (pUHD10-3) (Gossen and Bujard 1992). The tTA gene of the regulatory plasmid (pUHD15-1) was inserted into a new plasmid, pCMV-hygro, which provides a hygromycin selection marker, to form pCHTV. About 2×10^6 ELF7 cells were either cotransfected with 1 μ g pCHTV and 10 μ g pUHD10-3/RB (or 10 μ g pUHD10-3/RBH209) by lipofection (GibcoBRL, Gaithersburg, MD). The cultures were refed with medium containing 200 μ g/ml hygromycin (Sigma), 24 hr after lipofection. The cells were refed every 3–4 days with medium containing hygromycin until distinct colonies formed. Drug-resistant colonies were isolated 3 weeks later and propagated into mass cultures.

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