Deregulated MAPK Activity Prevents Adipocyte Differentiation of Fibroblasts Lacking the Retinoblastoma Protein*

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A functional retinoblastoma protein (pRB) is required for adipose conversion of preadipocyte cell lines and primary mouse embryo fibroblasts (MEFs) in response to treatment with standard adipogenic inducers. Interestingly, lack of functional pRB in MEFs was recently linked to elevated Ras activity. Ras-dependent signaling plays a significant, although incompletely understood, role in adipocyte differentiation, because activated Ras has been reported to either promote or inhibit adipogenesis depending on the cellular context. In various cell types activation of Ras leads to activation of the mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase 1/2 (ERK1/2), and protein kinase B (PKB/Akt), which exert opposing effects on adipogenesis, with ERK1/2 inhibiting and PKB/Akt promoting terminal differentiation. Here we report that the levels of activated ERK1/2 and PKB/Akt are significantly increased in pRB-deficient MEFs both before and after the addition of adipogenic inducers. Consistently, we detected higher levels of activated Ras in MEFs lacking pRB. Suppression of ERK1/2 activation by the MEK inhibitor U0126 restored the ability of pRB-deficient MEFs to undergo adipocyte differentiation, as manifested by expression of adipocyte marker genes and lipid accumulation. Furthermore and reflecting the elevated levels of activated PKB/Akt in the pRB-deficient MEFs, differentiation proceeded in an insulin-independent manner. In conclusion, we suggest that pRB plays a pivotal role in adipogenesis by suppressing MAPK activity.

The conversion of fibroblast-like precursor cells to fully differentiated adipocytes is a process controlled by a complex network of signaling pathways and is tightly regulated by numerous transcription factors, including members of the peroxisome proliferator-activated receptor (PPAR)1 and CCAAT/enhancer-binding protein (C/EBP) families, and the adipocyte determination and differentiation-dependent factor-1/sterol regulatory element-binding protein-1 (for review, see Refs. 1–3). Ras-dependent signaling has been shown to have a significant impact on adipogenesis, although the reported results are difficult to reconcile (4–7). In a series of studies, it was shown that expression of oncogenic Ras in 3T3-L1 preadipocytes induces adipocyte differentiation, whereas expression of dominant-negative Ras abrogates adipose conversion of these cells (4, 5). In contrast, it was reported that expression of an activated Ras mutant abolished differentiation of both C3H10T1/2 and certain subclones of 3T3-L1 cells (6, 7). Terminal adipocyte differentiation is promoted by or dependent on the activity of a number of kinases, most notably kinases of the phosphatidylinositol 3-kinase-protein kinase B (PKB/Akt) pathway (8, 9) and kinases leading to activation of p38 mitogen-activated protein kinases (MAPKs) (10, 11). In contrast, sustained activation of the ERK1/2 MAPKs has been demonstrated to inhibit adipogenesis (12), at least in part, by mediating an inhibitory phosphorylation of PPARγ, a crucial regulator of terminal adipocyte conversion (13, 14).

The retinoblastoma protein (pRB), encoded by the retinoblastoma tumor suppressor gene (Rb), is a key regulator of proliferation, development, and the differentiation of multiple cell types, including skeletal muscle and fat cells (reviewed in Ref. 15). The role of pRB in development and differentiation relates to the ability of pRB to repress E2F transcription factors and/or enhance the activity of transcription factors promoting differentiation. Examples of such pRB targets are MyoD and C/EBPβ in myogenensis and adipogenesis, respectively (16–19).

A recent report indicated an alternative function of pRB in cellular differentiation by showing that the absence or the inactivation of pRB led to an increased activity of Ras (20). Expression of dominant-negative Ras in pRB-deficient fibroblasts partially restored the transactivation capacity of MyoD but did not restore repression of E2F. However, the downstream effectors of Ras-dependent repression of MyoD activity were not identified (20). Although it was not reported whether dominant-negative Ras normalized the defective myocyte differentiation of Rh−/− cells, these data indicate that deregulated Ras signaling may be an important event responsible for the defects in differentiation associated with the absence of a functional pRB, at least in the case of skeletal muscle. Recent work in Caenorhabditis elegans further underscores the important link between pRB and Ras signaling in regulating embryonic asymmetry and vulval development (21, 22).

Simian virus 40 TAg has been shown to inhibit 3T3-L1 mouse embryo fibroblast; MLB, magnesium lysate buffer; PKB, protein kinase B; pRB, retinoblastoma protein; Rh, retinoblastoma gene; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase.
preadipocyte differentiation in a manner partly dependent on the ability of TAg to bind and inactivate pRB (23). Similarly, pRB−/− MEFs do not undergo significant adipocyte differentiation in response to treatment with standard adipogenic inducers (19), but the block in differentiation caused by pRB deficiency can be bypassed by administration of the potent PPARγ agonist BRL49653 (24). Here we show that the activities of two targets of Ras signaling, the ERK1/2 MAPKs and PKB/Akt, are up-regulated in pRB−/− MEFs compared with wild-type MEFs. This is most likely due to a pRB-dependent difference in Ras signaling, as we observed an increased amount of active Ras in pRB−/− cells. We show that BRL49653-induced differentiation is accomplished by down-regulation of ERK1/2 activities. In contrast, treatment of pRB−/− MEFs with standard adipogenic inducers even led to an elevation of the levels of activated ERK1/2 during the first 6 days of treatment. We hypothesized that the up-regulation of ERK1/2 activities might prevent adipocyte differentiation of pRB-deficient MEFs and, furthermore, that blocking ERK1/2 activation would allow adipogenesis even in the absence of exogenously added insulin-like growth factor-1/insulin due to the elevated levels of active PKB/Akt. In keeping with these hypotheses, we demonstrate that suppression of ERK1/2 activation by UO126 to approximately the level of activation observed in wild-type MEFs indeed restored adipogenesis of pRB−/− MEFs in response to a standard adipogenic treatment and, furthermore, that differentiation of the pRB-deficient MEFs proceeded independently of the addition of insulin.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Differentiation**—Wild-type and pRB−/− MEFs were grown and differentiated as described (24). Standard adipogenic treatment included the addition of dexamethasone (1 μM) (Sigma), methyl-

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sobutyrate/2-hydroxy acid (0.5 mM) (Sigma), and insulin (5 μg/ml) (kindly provided by Novo Nordisk A/S) (MDI treatment) to confluent MEF cultures (designated day 0). At days 2, 4, 6, and 8, the cells were refed with medium containing insulin (5 μg/ml). UO126 (10 μM unless otherwise indicated) (Promega), BRL49653 (1 μM) (kindly provided by Novo Nordisk A/S), or LY294002 (10 μM) (Cell Signaling Technology) was added at equal concentrations beginning at day 0. Cells not treated with UO126, BRL49653, or LY294002 received similar volumes of vehicle (0.1% MeSO). For oil red O staining, the cells were fixed and stained at day 10 as described (24).

**Western Blotting**—Whole cell extracts, SDS-polyacrylamide gel electrophoresis, blotting, and enhanced chemiluminescence were performed as described (24). Primary antibodies used were rabbit anti-p44/p42 MAP kinase (Thr-202/Tyr-204), rabbit anti-Akt, rabbit anti-phospho-Akt (Thr-308), rabbit anti-p38 MAP kinase, and rabbit anti-phospho-p38 MAP kinase (Thr-180/Tyr-182) (all primary antibodies from New England Biolabs). Secondary antibodies were horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (DAKO). In Vitro Kinase Assays—The p44/42 MAP kinase and Akt kinase assays were performed as recommended by the manufacturer (Cell Signaling Technology). Briefly, cells lysates were incubated with immobilized primary antibodies (monoclonal antibodies against phospho-p44/42 MAP kinase (Thr-202/Tyr-204) and Akt, respectively). After the washing of immunoprecipitates, the immunoprecipitated phospho-p44/42 MAP kinase and Akt were incubated with their substrates (Elk-1 and GSK3α/β cross-tide, respectively) in kinase buffer in the presence of ATP. Kinase reactions were subsequently subjected to Western blot analyses using antibodies recognizing phospho-Elk-1 (Ser-383) and phospho-GSK3α/β (Ser-21/9), respectively.

**Activated Ras Interaction Assay**—The activated Ras interaction assay (ARIA) allows a measure of the amount of active (GTP-bound) Ras present in whole cell extracts and was performed essentially as described (25), with minor modifications. Briefly, glutathione S-transferase-Raf-1 (amino acids 1–149) was expressed in Escherichia coli and purified on glutathione-Sepharose beads. Approximately 20 μg of fusion protein were used per assay. The fusion protein was washed twice in magnesium lysis buffer (MLB) (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 10% glycerol, 1 mM EDTA, 10 mM MgCl₂, 0.5 mM phenylmethylsulfonyl fluoride, and Complete™ (Roche Molecular Biochemicals)). One 15-cm dish of confluent cells was lysed in 1.5 ml of MLB on ice for 10 min, after which the cells were scraped off, vortexed, and incubated on ice for another 10 min. After centrifugation (14000 rpm, 15 min, 4 °C) the protein concentration of the supernatant was determined according to the Bradford procedure. An aliquot of total protein was used per assay. The cell lysate was incubated with the fusion protein at 4 °C for 60 min with rotation. After the incubation, the beads were washed 3 times with 1 ml of MLB, boiled in SDS sample buffer, run on 12.5% SDS-polyacrylamide gels, and blotted. Interacting Ras was visualized with chemiluminescence using a pan anti-Ras mouse monoclonal antibody (Quality Biotech, clone LA045). As a control for equal input, 0.5% of the cell lysate from confluent Rb+/+ and Rb−/− MEFs used for the ARIA experiments was loaded on a gel, and the amount of total Ras was determined by Western blotting using the anti-Ras antibody described above. Therefore, the input represents the combined amount of GTP- and GDP-bound Ras, whereas the ARIA method provides a measure of the amount of Ras bound to GTP.

**Reverse Transcription–PCR**—RNA purification, reverse transcription, and multiplex reverse transcription–PCR were performed as described (24). Primer sets have been described (24, 26). The number of cycles used for the different primer sets is described in the legend to Fig. 5.

**RESULTS**

MEFs lacking pRB are blocked in their ability to undergo adipocyte differentiation in response to treatment with standard adipogenic inducers (19) but undergo efficient adipocyte differentiation when treated with the potent PPARγ agonist BRL49653 (24). Recently, it was reported that the level of activated Ras was elevated in Rb−/− compared with wild-type cells (20). It is well established that Ras-dependent signaling generally activates the ERK1/2 MAPKs and that activation of Ras in certain cellular settings also leads to activation of the phosphatidylinositol 3-kinase/PKB/akt pathway (for review, see Ref. 27). Because both the ERK1/2 kinases and the phosphatidylinositol 3-kinase/PKB/akt pathway have been shown to play pivotal roles in the control of adipogenesis (9, 12, 13), we decided to determine whether the activities of these kinases were deregulated in Rb−/− cells. Initially, we performed Western blot analyses using antibodies recognizing either ERK1/2, irrespective of phosphorylation status, total ERK1/2, or antibodies specifically recognizing the phosphorylated (and active) forms. To allow comparison between the different blots, we applied a day 0 sample from the opposite genotype on each gel. Such analyses revealed that the levels of activated ERK1/2 were considerably higher in Rb−/− than in wild-type cells, whereas the levels of total ERK1/2 were comparable (Fig. 1A). In wild-type MEFs, treatment with the standard adipogenic inducers methylisobutylxantine, dexamethasone, and insulin (MDI treatment) did not significantly change the levels of activated ERK1/2 (Fig. 1A, panel labeled Me2SO for Rb+/+ cells). In contrast, MDI treatment of the pRB-deficient MEFs resulted in a pronounced increase in the levels of activated ERK1/2 up to day 6 (Fig. 1A, panel labeled Me2SO for Rb−/− cells). To validate the use of Western blotting using a phospho-specific anti-ERK1/2 antibody, we also performed an immunoprecipitation-coupled kinase assay on whole cell extracts from confluent cells. The immunoprecipitation-coupled kinase assay with Elk-1 as the ERK1/2 substrate confirmed the increased activity of ERK1/2 in Rb−/− MEFs (Fig. 1B). This assay revealed an ~3-fold higher activity of ERK1/2 in pRB-deficient compared with wild-type MEFs, consistent with the results in Fig. 1A. Therefore, we conclude that Western blotting using the phospho-specific anti-ERK1/2 antibody fairly accurately determines the ERK1/2 activity in the MEFs. BRL49653-induced adipocyte differentiation of pRB-deficient MEFs was accompanied by a marked reduction in the level of activated ERK1/2 after day 2 (Fig. 1A). This is likely to be an indirect effect of the efficient BRL49653-driven differentiation (see below), because
BRL49653 had no acute effect on the activities of ERK1/2 in either undifferentiated or differentiated cells (data not shown).

Similarly, significantly more active PKB/Akt was present at day 0 in Rb−/− MEFs compared with wild-type MEFs as determined by Western blotting using a phospho-specific anti-PKB/Akt antibody (Fig. 2A). The difference in PKB/Akt activity between Rb+/+ and Rb−/− MEFs was, however, not as prominent as the difference in ERK1/2 activity described above. The higher abundance of active PKB/Akt in Rb−/− compared with Rb+/+ MEFs was detected only in the earlier stages of the differentiation, i.e. at days 0, 2, and 4. Consistently, an immunoprecipitation-coupled kinase assay with GSK3α/β cross-tide as a substrate demonstrated a 1.5–2-fold higher PKB/Akt activity in day 0 Rb−/− compared with wild-type MEFs, thereby validating the use of Western blotting using the phospho-specific anti-PKB/Akt antibody to determine PKB/Akt activity in the MEFs (Fig. 2C). To determine the activity of PKB/Akt in the very early stages of differentiation, we also measured PKB/Akt activity 1 and 4 h after the addition of adipogenic hormones. Also, at these time points the activity of PKB/Akt was higher in Rb−/− cells compared with wild-type cells (Fig. 2B, panel labeled Me2SO). The presence of active PKB/Akt appeared to decrease at later time points during differentiation independent of Rb status (Fig. 2A). Activation of p38 MAPKs has been shown to be required for terminal differentiation of 3T3-L1 cells (10). However, we observed no differences between wild-type and Rb−/− MEFs in the activation profiles of p38 MAPKs during differentiation, as determined by Western blotting using antibodies specifically recognizing phosphorylated (active) p38 MAPKs (data not shown).

To investigate whether the higher levels of active ERK1/2 and PKB/Akt in Rb−/− MEFs correlated with the presence of more active Ras, we measured the level of activated Ras in confluent MEFs by the ARIA method (25). This assay measures the amount of Ras in cell lysates that is able to bind recombinant glutathione S-transferase-Raf-1. This is an indirect measure of Ras activity, as only GTP-bound (active) Ras binds Raf-1 with high affinity. As shown in Fig. 3, significantly more active Ras was present in day 0 Rb−/− relative to Rb+/+ MEFs. This is consistent with previous results (20) and suggests that the deregulated activities of ERK1/2 and PKB/Akt in pRB-deficient MEFs are due to increased Ras signaling.

So far, we have demonstrated that Ras activity and the activities of two downstream targets of Ras signaling, ERK1/2 and PKB/Akt, were increased in Rb−/− MEFs. Next, we were interested in analyzing if these deregulated downstream kinases were responsible for the different adipogenic capacities of wild-type and Rb−/− MEFs. We speculated that the deregulated ERK1/2 activity might enforce an inhibitory effect on adipoic differentiation of the pRB-deficient cells. Therefore, we analyzed the effect of suppressing ERK1/2 activation by the addition of the MEK inhibitor UO126. Initially, we performed a titration with UO126 to determine which concentration would suppress the activities of ERK1/2 in Rb−/− MEFs to approximately the levels observed in Rb+/+ cells. This concentration of UO126 was found to be 10 μM (Fig. 1A, compare panel Me2SO for Rb+/+ cells with panel UO126 for Rb−/− cells; data not shown). It is important to notice that this concentration of UO126 was sufficient to reduce the levels of activated ERK1/2 to those observed in the wild-type cells without causing complete ablation of ERK1/2 activity. Moreover, UO126 did not suppress the increased activity of PKB/Akt in the pRB-defi-
Fig. 3. Detection of the amount of activated Ras present in confluent Rb+/+ and Rb−/− MEFs by the ARIA method. The amount of GTP-bound Ras present in confluent MEF lysates (day 0) was determined by the amount of cellular Ras retained on glutathione S-transferase (GST)-Raf-1 (1–149) glutathione-Sepharose beads. Retained cellular Ras was eluted in SDS-sample buffer and subjected to Western blotting using a pan anti-Ras antibody. Input represents 0.5% of the cell lysate used for the interaction assay and represents the combined amounts of the GST- and GDP-bound forms of Ras. In agreement with previous reports, this assay results in two bands, which represent either different posttranslationally modified forms of Ras or K-Ras and N-Ras (upper and lower bands, respectively) (Refs. 20 and 25).

Fig. 4. Effect of inhibiting ERK1/2 activation by UO126 on adipose conversion of Rb−/− cells. Oil red O staining of Ras+/+ or Ras−/− cells at day 10. Shown are whole stained dishes. Cells were induced to differentiate with dexamethasone and methylisobutylxanthine in combination with insulin (Ins), BRL49653, UO126, or LY294002 as indicated in the figure. Cells not treated with BRL49653, UO126, or LY294002 received similar volumes of vehicle (0.1% Me2SO).

Fig. 5. Expression of adipocyte marker genes in cells treated with UO126 or BRL49653. Cells were induced to differentiate as described in the legend to Fig. 4. Multiplex reverse transcription-PCR was used to analyze the expression of PPARγ (20 cycles), adipocyte lipid-binding protein (ALBP)/aP2 (18 cycles), glycerol-3-phosphate dehydrogenase (GPDH) (20 cycles), β-actin (18 cycles), and TATA-binding protein (TBP) (20 cycles). β-Actin and TATA-binding protein primer sets were included as internal standards.

DISCUSSION

Ras is a multifunctional protein affecting several signaling pathways, and this signaling influences processes such as proliferation, apoptosis, and differentiation (for review, see Ref. 28). Conflicting results have been reported on the function and importance of Ras in adipocyte differentiation (4–7). However, it has been demonstrated that two downstream targets of Ras signaling, ERK1/2 and PKB/Akt, exert opposing effects on adipogenesis, with ERK1/2 inhibiting and PKB/Akt promoting differentiation (9, 12–14). In this study we describe a novel mechanism through which pRB promotes adipogenesis, which relates to the ability of pRB to suppress the activities of ERK1/2, possibly by suppressing Ras signaling. This is a surprising finding, as it suggests that the role of pRB in promoting adipocyte differentiation apparently is independent of its binding to E2F and/or adipogenic transcription factors. It is not clear at present how pRB regulates Ras signaling, but several ways in which this deregulated signaling might inhibit adipose conversion are discussed below.

pRB was reported to promote C/EBPβ-mediated transactivation via a direct interaction between the two proteins, with pRB acting as a chaperone-like factor to promote binding of C/EBPβ to its response elements (19). According to this model, the defective differentiation of Ras−/− cells in response to standard adipogenic inducers was suggested to be caused by a reduced C/EBPβ-dependent activation of target genes due to the ab-
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