A pRb-independent mechanism preserves the postmitotic state in terminally differentiated skeletal muscle cells

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In skeletal muscle differentiation, the retinoblastoma protein (pRb) is absolutely necessary to establish definitive mitotic arrest. It is widely assumed that pRb is equally essential to sustain the postmitotic state, but this contention has never been tested. Here, we show that terminal proliferation arrest is maintained in skeletal muscle cells by a pRb-independent mechanism.

Acute Rb excision from conditional knockout myotubes caused reexpression of E2F transcriptional activity, cyclin-E and -A kinase activities, PCNA, DNA ligase I, RPA, and MCM2, but did not induce DNA synthesis, showing that pRb is not indispensable to preserve the postmitotic state of these cells. Muscle-specific gene expression was significantly down-regulated, showing that pRb is constantly required for optimal implementation of the muscle differentiation program. Rb-deleted myotubes were efficiently reactivated by forced expression of cyclin D1 and Cdk4, indicating a functionally significant target other than pRb for these molecules. Finally, Rb removal induced no DNA synthesis even in pocket-protein null cells. Thus, the postmitotic state of myotubes is maintained by at least two mechanisms, one of which is pocket-protein independent.

Introduction

The defining property of terminal cell differentiation is the permanent withdrawal from the cell cycle also known as postmitotic state. This state is the most typical cellular condition, characterizing the majority of cells in adult mammals, yet too little is known about the mechanisms underlying the irrevocable decision to exit the cell cycle during differentiation and the maintenance of such permanent mitotic arrest.

Early work demonstrated that the retinoblastoma protein (pRb), a key regulator of proliferation, differentiation, and apoptosis, is absolutely required to establish the postmitotic state in a number of cell types, prominently including skeletal muscle cells (Schneider et al., 1994). In the absence of pRb, skeletal muscle cells and numerous other histotypes show ectopic DNA synthesis and/or apoptosis, both in vitro and in vivo (Lipinski and Jacks, 1999). Indeed, wild-type myoblasts enter the postmitotic state before differentiating into myotubes, whereas $Rb^{-/-}$ myoblasts can only undergo nonterminal differentiation, as they convert into myotubes that continue to synthesize DNA (Novitch et al., 1996; Zacksenhaus et al., 1996).

It is widely assumed that, because pRb is essential for the establishment of the postmitotic state, it must be also responsible for its maintenance (Novitch et al., 1996; Lipinski and Jacks, 1999; Lowenheim et al., 1999; Mal et al., 2000; Ruiz et al., 2004). However, this view is challenged by several observations. In unpublished experiments, expression in terminally differentiated (TD) myotubes of various molecules known or likely to inactivate/counteract pRb, such as Id2, MDM2, and cytomegalovirus IE1 and IE2, could not trigger DNA synthesis. In addition, it has been shown that forced expression of E2F transcription factors or active, pRb-phosphorylating Cdk2 kinase is unable to reactivate DNA synthesis in myotubes (Puri et al., 1998; Pajalunga et al., 1999; Latella et al., 2001). Finally, papillomavirus E7 is also incapable of triggering DNA synthesis in these cells, though targeting and degrading pRb (Sacco et al., 2003). Altogether, this evidence strongly suggests that pRb is not responsible for the maintenance of the postmitotic state or

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Abbreviations used in this paper: AdCre, Cre recombinase-carrying adenovirus; ΔRb, Rb-deleted; ΔRb-Mt, ΔRb myotubes; IF, immunofluorescence; KO, knockout; MEF, mouse embryo fibroblasts; m.o.i., multiplicity of infection; MSC, muscle satellite cell; MyHC, myosin heavy chain; pRb, retinoblastoma protein; TD, terminally differentiated.

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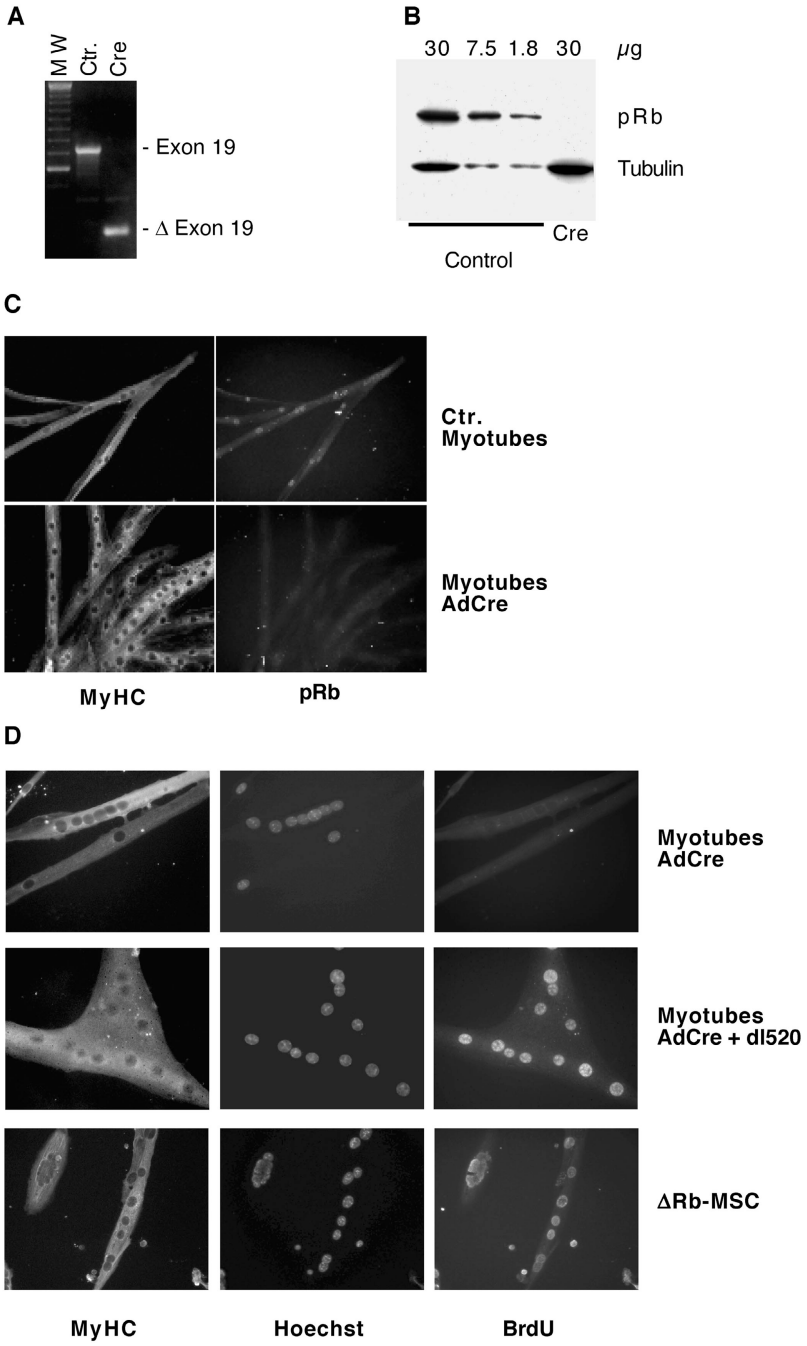

Figure 1. **pRb ablation in myotubes does not induce DNA synthesis.** (A) PCR of myotubes infected with a control virus (Ctr.) or AdCre. Exon 19 denotes the amplification product obtained from undeleted cells; Δ Exon 19, Exon 19 deletion; MW, 100-bp ladder (Invitrogen); PCR performed as in Marino et al. (2000). (B) Western blot analysis of pRb in control- and AdCre-infected myotubes; decreasing amounts of total protein were loaded in the control lanes to assess blot sensitivity. Tubulin indicates loading control. (C and D) IF analysis of myotubes infected with the indicated viruses or derived from ΔRb-MSC.

that additional mechanisms protect the proliferative arrest of these cells.

This work exploits conditional Rb knockout (KO) cells to demonstrate that pRb contributes to but is not required for the preservation of the postmitotic state in TD myotubes. Thus, an additional, pRb-independent, possibly TD-cell specific mechanism maintains the postmitotic state in these cells.

Results and discussion

pRb ablation does not trigger DNA synthesis in myotubes

To investigate the role of pRb in the maintenance of the postmitotic state in TD myotubes, we exploited Rb conditional KO mice (Marino et al., 2000) in which pRb can be ablated via Cre-mediated excision of loxP-flanked Rb exon 19. Myoblasts (muscle satellite cells [MSCs]) from these mice were induced to terminally differentiate while expressing pRb and the resulting myotubes were then subjected to Rb excision by infection with a Cre recombinase-carrying adenovirus (AdCre). Fig. 1 (A–C) shows that Rb gene excision and pRb protein elimination were essentially complete 48 h after AdCre infection. In spite of total ablation of pRb, the myotubes did not synthesize DNA, as assessed by exposure to BrdU for up to 84 h after AdCre infection and subsequent immunodetection of BrdU incorporation (Fig. 1 D).

To ensure that the lack of DNA synthesis was not due to artifacts connected with Cre expression (Loonstra et al., 2001),

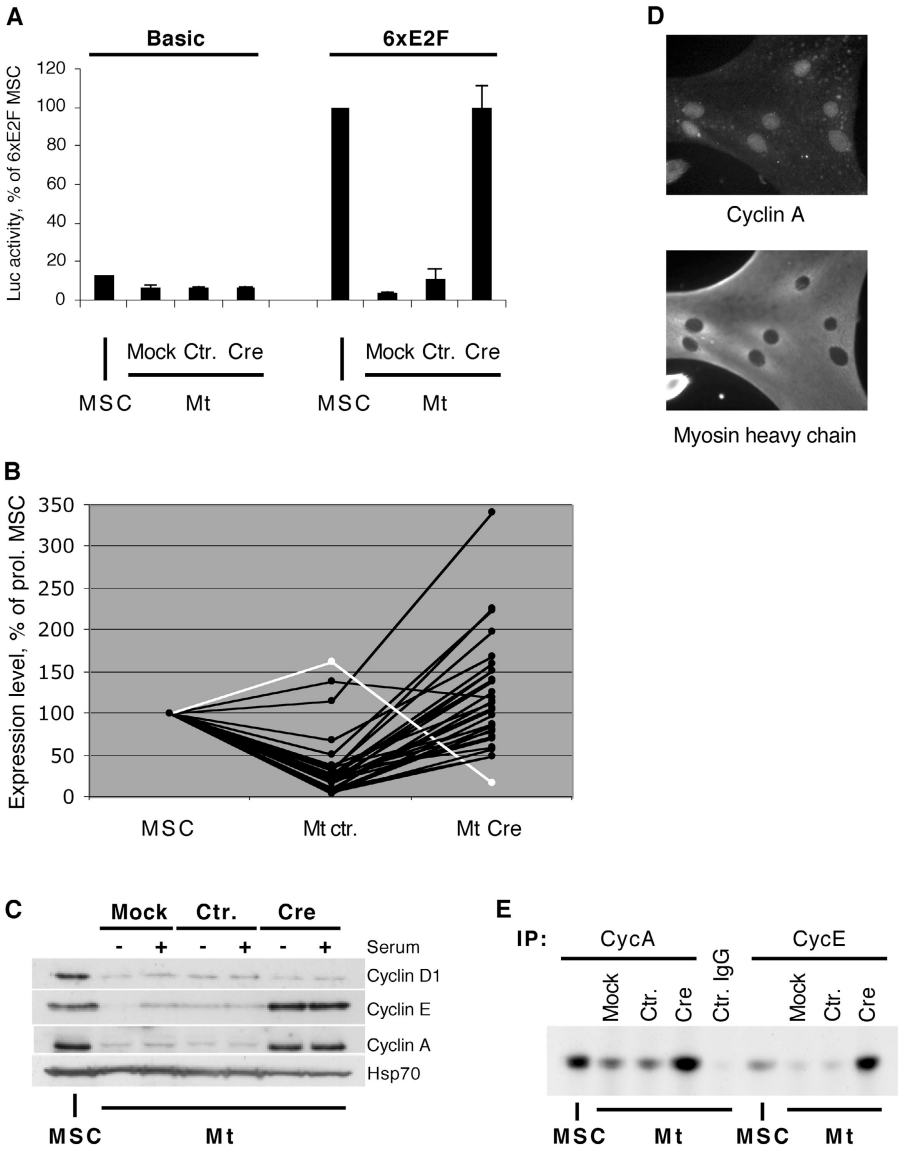

Figure 2. **Reactivation of cell cycle regulators in ΔRb-Mt.** (A) Luciferase activity measured in MSCs stably transfected with the control (Basic) or E2F-responsive (6xE2F) reporter construct and derived myotubes (Mt) infected with the indicated viruses; data are averages of two independent experiments, with SDs, and are expressed as percentage of value obtained in 6xE2F-MSC. (B) DNA microarray analysis of MSCs and myotubes infected with control or Cre adenovirus; each line represents a distinct mRNA; values are averages of two independent experiments and are shown as percentages of measurements obtained in MSCs (see Table S1); white line indicates pRb mRNA. (C) Western blot analysis of the indicated proteins in MSCs or myotubes infected as shown, in the presence or absence of 5% FBS; HSP70 indicates loading control. (D) IF for MyHC and cyclin A on ΔRb-Mt. (E) Cyclin A and cyclin E kinase complexes were immunoprecipitated from MSCs or infected myotubes, reacted with histone H1 in the presence of γ-[^{32}P]ATP, resolved by gel electrophoresis, and autoradiographed; Ctr. IgG indicates control-infected myotubes immunoprecipitated with normal rabbit IgG.

Rb-deleted (ΔRb) myotubes (ΔRb-Mt) were superinfected with the dl520 adenovirus, carrying 12S E1A and shown previously to reactivate the mitotic cycle in TD cells (Crescenzi et al., 1995). E1A expression readily induced 95% of the cells to incorporate BrdU (Fig. 1 D), showing that ΔRb-Mt retained intact DNA-replicating potential.

It has been demonstrated that myoblasts lacking Rb (ΔRb-MSC) cannot enter the postmitotic state upon differentiation and continue to synthesize DNA (Schneider et al., 1994; Novitch et al., 1996). To ensure that our experimental system behaves accordingly, we generated ΔRb-MSC by infection with AdCre. These cells were induced to differentiate for 72 h and exposed to BrdU during the next 24 h. Double immunofluorescence (IF) for muscle-specific myosin heavy chain (MyHC), a differentiation marker, and BrdU revealed that 44.9% of the myotubes incorporated BrdU during the indicated period, in keeping with previous results (Fig. 1 D; Novitch et al., 1996). Thus, in our system, Rb deletion prevents entry into the postmitotic state when performed before differentiation, but cannot reactivate the cell cycle if performed in myotubes.

It has been shown recently that acute Rb deletion reactivates the cell cycle in quiescent and senescent mouse embryo fibroblasts (MEF; Sage et al., 2003). For comparison purposes, we isolated MEF from the Rb-loxP mice. Serum-starved, quiescent MEF showed a fourfold increase in BrdU incorporation upon AdCre-mediated Rb deletion (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200408164/DC1). Thus, the combined performances of our AdCre virus and of the cells derived from the Rb-loxP mice are consistent with previous results.

We noticed that, between 72 and 96 h after AdCre infection, ΔRb-Mt underwent massive TUNEL-positive cell death (unpublished data). To rule out the theoretical possibility that cell death might temporally precede DNA synthesis and prevent its occurrence, we coinfected Rb-loxP myotubes with AdCre and an adenovirus carrying the antiapoptotic Bcl-2 gene. Expression of Bcl-2 dramatically improved cell survival, increasing the number of live myotubes 96 h after infection by 5–10-fold in several independent experiments. However, no BrdU incorporation was observed up to 120 h after infection (unpublished data).

Altogether, these results allow us to conclude that the post-mitotic state of myotubes is preserved by a pRb-independent mechanism. This conclusion distinguishes TD myotubes from quiescent and senescent cells, in which maintenance of mitotic arrest depends on pRb (Sage et al., 2003). These data underscore the uniqueness of the control of the cell cycle in TD myotubes and contribute to explain their remarkable refractoriness to proliferation-promoting stimuli that are otherwise powerful inducers of cell-cycle reentry in non-TD cells (Tiainen et al., 1996).

Rb deletion in myotubes reactivates cell cycle regulators

Although ΔRb-Mt do not synthesize DNA, we investigated the effects of pRb deletion at the biochemical level. First, the transacting activity of the Rb-modulated E2F family of transcription factors was studied using an E2F reporter construct. Fig. 2 A shows that E2F transcriptional activity, readily measurable in MSCs, was much reduced in myotubes. Strikingly, Rb deletion brought this activity back to MSC levels. To determine whether the E2F activity detected in myotubes is effective on endogenous, S phase–promoting, E2F-target genes, cRNA from our cells was hybridized with DNA oligonucleotide arrays. Only small parts of these results are shown here, whereas the microarray experiments will be described in full elsewhere. We looked at genes shown to be E2F regulated by a previous microarray study of murine fibroblasts (Ishida et al., 2001). Fig. 2 B shows normalized expression values of all E2F targets that could be unambiguously identified in our microarrays as coincident with those of Ishida et al. (2001) and yielded meaningful signals in our experiments (see Materials and methods and Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200408164/DC1). In agreement with the results obtained using the E2F reporter construct, most E2F-regulated genes (26 of 28) showed decreased expression in TD myotubes, but their expression levels significantly increased upon Rb excision. Thus, pRb ablation in myotubes brings about reactivation of E2F transcription factors and enhanced expression of their target genes.

Next, we assessed the expression of G1 cyclins regulating the G1/S phase transition. Fig. 2 C shows that the cyclin E and A proteins, normally expressed in the proximity of the G1/S transition, are up-regulated in ΔRb-Mt to levels similar to those found in MSCs, consistent with their genes being E2F targets (Ishida et al., 2001). Conversely, expression of cyclin D1 protein, a cyclin exerting its action at earlier times in G1 phase, was not increased by pRb ablation. Fig. 2 D shows that cyclin A was properly localized to the nuclei of ΔRb-Mt, ruling out the possibility that it is inactive with respect to DNA synthesis due to delocalization. In addition, Fig. 2 E shows that in ΔRb-Mt, cyclin E– and A–associated kinase activities were higher than in MSCs, a finding at least partially explained by the comparison of de facto synchronized myotubes with asynchronous MSCs.

Finally, Rb deletion brought the expression of DNA replication effector proteins, such as PCNA, MCM2, DNA ligase I, and replication protein A, back to MSC levels (Fig. 3 A). Because the levels of PCNA and DNA ligase I increased in ΔRb-Mt, we investigated their subnuclear distribution pattern as a means to distinguish nuclei that are in early, mid, and late S phase

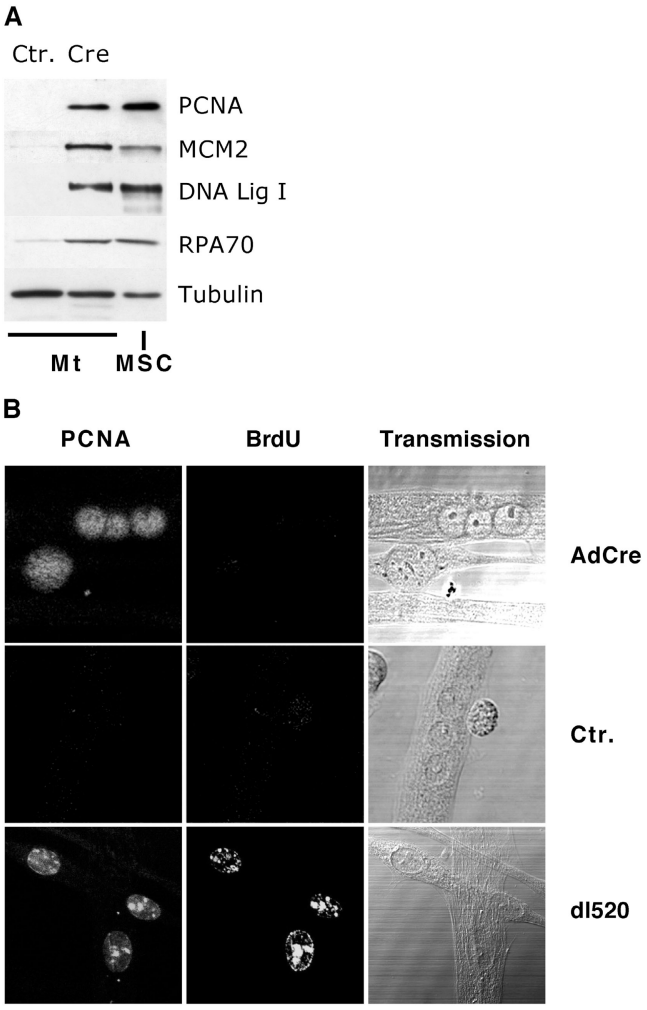

Figure 3. **Reactivation of DNA synthesis effectors in ΔRb-Mt.** (A) Western blot analysis of the indicated proteins in MSCs and infected myotubes. (B) Confocal microscopy of PCNA and BrdU IF in myotubes infected as indicated.

(Hozak et al., 1994; Montecucco et al., 1995). The majority of ΔRb-Mt displayed evenly distributed, fine, punctate PCNA (Fig. 3 B) and DNA ligase I (unpublished data) pattern throughout their nucleoplasms, suggesting an arrest at the G1/S boundary.

These results show that pRb removal activates molecular events usually associated with transition into S phase, indicating that pRb does act in myotubes as a barrier to S phase entry. However, as demonstrated in the preceding paragraph, a second, pRb-independent mechanism prevents DNA synthesis from taking place and arrests ΔRb-Mt in a state best described as accumulation at the G1/S transition.

The constant presence of pRb is necessary for optimal skeletal muscle gene expression

It has been shown that pRb is necessary in the course of differentiation for the full expression of a subset of muscle genes (Novitch et al., 1999). One view is that pRb facilitates such expression by contributing to chromatin remodeling, which might be accomplished once and forever at the onset of terminal differentiation. Hence, pRb might become dispensable once its re-

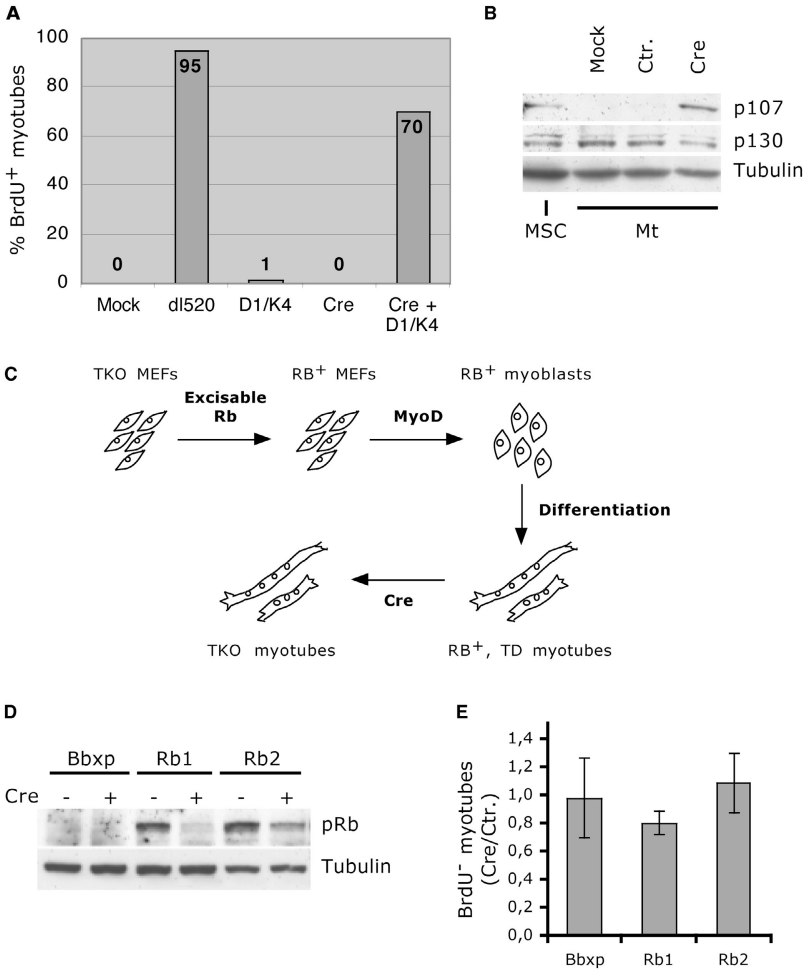

Figure 4. **Roles of cyclin D1 and pocket proteins in controlling the postmitotic state.** (A) BrdU incorporation in myotubes infected as indicated. (B) Western blot analysis of p107 and p130 in MSCs and infected myotubes. (C) Schematic of the generation of TKO myotubes. (D) Myotubes derived from TKO MEF infected with a control retrovirus (Bbxp) or from two independent populations infected with a retrovirus carrying a Cre-excisable Rb (Rb1 and Rb2) were treated with AdCre or a control adenovirus. Total proteins were extracted and Western blot analyzed. (E) Analysis of BrdU incorporation in the same myotube populations as in D. The results are averages and SDs of three independent experiments and are shown as the ratio of $BrdU^-$ myotubes in Cre-infected (Rb^-) over control-infected (Rb^+) populations.

modeling function has been performed. Thus, we asked whether pRb is still required for full muscle gene expression after the establishment of differentiation. To address this question, we interrogated again the microarray data described above. Expression of a number of muscle-specific genes was compared in ΔRb-Mt versus control-infected myotubes. These genes showed reductions in expression ranging from 66 to 15% (Table S2, available at http://www.jcb.org/cgi/content/full/jcb.200408164/DC1) already 48 h after AdCre infection, which probably underestimates the true extent of gene down-regulation due to variable persistence of the different mRNAs.

Thus, although pRb is not necessary to maintain the postmitotic state, its continuous presence is required for full expression of tissue-specific genes.

The pRb-independent block to DNA synthesis can be relieved by cyclin D1/Cdk4

We have shown previously that cyclin D1 and Cdk4 coexpression can reactivate DNA synthesis in TD myotubes (Latella et al., 2001). Because pRb is the most important target of cyclin D, we asked whether the latter would still be able to reactivate the cell cycle in ΔRb-Mt. Therefore, we superinfected ΔRb-Mt with adenoviruses expressing cyclin D1 and Cdk4. Fig. 4 A shows that a subthreshold infection with these viruses, unable to reactivate DNA synthesis in pRb-expressing myotubes, efficiently induced DNA synthesis in 70% of ΔRb-Mt.

Thus, the pRb-independent block to DNA synthesis of TD myotubes is cyclin D1/Cdk4 inactivatable. In addition, these results indicate that at least one significant nonpRb target for these proteins exists in TD myotubes.

The second block of myotubes is pocket-protein independent

It has been shown that, in the absence of pRb, myotubes as well as other cell types express high levels of the pRb-family protein p107 which has been proposed to partially substitute for pRb (Schneider et al., 1994). Western blot analysis of ΔRb-Mt confirmed that p107 protein levels are promptly increased upon pRb removal, whereas expression of the other pRb-family protein, p130 remains essentially unchanged (Fig. 4 B). Because all pRb-family members, referred to as "pocket" proteins, are substrates of cyclin D–associated kinases (Xiao et al., 1996; Farkas et al., 2002; Leng et al., 2002), p107 and/or p130 might conceivably be responsible for the pRb-independent block observed in myotubes and this activity might be removed by Cdk4-mediated phosphorylation. However, several lines of evidence indicate that this is not the case. First, p107/p130 double KO mice show no muscle phenotype (Cobrinik et al., 1996). Second, attempts to curb p107 expression through high serum

treatment (Schneider et al., 1994) in ΔRb-Mt significantly reduced p107 levels but induced no DNA synthesis (unpublished data). Third, expression of HPV E7 in myotubes presumably inactivates all three pRb family members, but cannot induce DNA synthesis (Sacco et al., 2003). Finally, E2F transcriptional activity is fully reactivated in ΔRb-Mt, showing that the two sister proteins cannot replace the fundamental E2F-repressing activity of pRb in this setting (Fig. 2, A and B). These arguments notwithstanding, the potential vicarious role of p107/p130 in the maintenance of the postmitotic state was addressed directly by performing Rb deletion in cells devoid of its sister proteins.

As schematically shown in Fig. 4 C, MEF from mice in which the three Rb-family genes have been targeted (triple KO, TKO; Sage et al., 2000) were infected with a retrovirus expressing a floxed Rb cDNA or the Bbxp control retrovirus. Phleomycin-selected, polyclonal populations were then converted into muscle cells by expressing the muscle-regulatory gene MyoD and induced to differentiate. Myotubes developed within 3 d in both the pRb-expressing and control populations. These myotubes were then infected with AdCre or control adenovirus and DNA synthesis was assessed by double IF for MyHC and BrdU. Although populations homogeneously expressing pRb could not be obtained, in three independent experiments, each performed with two distinct pRb-transduced cell lines, the number of $BrdU^-$ myotubes increased from 39.3 ± 6.6% (Rb^-) to 61.8 ± 12.2% (Rb^+) (average ± SD), indicating that exogenous Rb effectively allowed a significant fraction of cells to reach the postmitotic state. Subsequent infections of the myotubes with AdCre eliminated pRb (Fig. 4 D), but resulted in no significant reduction in the number of $BrdU^-$ myotubes (Fig. 4 E). This experiment shows that pRb can establish the postmitotic state even in the absence of its sister proteins. More important, it shows that deletion of pRb from fully differentiated myotubes does not trigger DNA synthesis even in a pocket-protein null background. We conclude that the newly identified mechanism preventing DNA replication in TD myotubes is pocket-protein independent.

The results summarized in this paper unveil a previously unsuspected pocket-protein independent block prohibiting TD myotubes entry into S phase, setting apart TD myotubes from quiescent and senescent cells, whose proliferation arrest critically depends on pRb (Sage et al., 2003). Whether this block is common to other TD cell types remains to be investigated. We note, however, that in one study functional ablation of the pRb family caused apotosis in cortical neuronal precursors but no phenotype in TD neurons (Slack et al., 1998). This supports the notion that pRb plays different roles before and after the onset of terminal differentiation in other histotypes.

The finding that cyclin D1 and Cdk4 can suppress the newly discovered block is also important. Genetic evidence indicates that the only critical substrate of the cyclin D–associated kinase is pRb (Lukas et al., 1995; Medema et al., 1995), although a few additional targets have been found (Matsuura et al., 2004). However, cyclin D1 is also involved in regulatory activities independent of its Cdk-regulating function (Zwijsen et al., 1997; Cheng et al., 1998) and we do not know whether unlocking the pRb-independent block requires Cdk4 kinase activity. This issue deserves further investigation.

Materials and methods

Antibodies

Antibodies used in IF: BrdU: clone Bu20a (DakoCytomation) or Alexa Fluor 488 conjugate (Molecular Probes); cyclin A: C-19; PCNA: PC10 (Santa Cruz Biotechnology, Inc.); MyHC: rabbit polyclonal (a gift from G. Cossu, Scientific Institute San Raffaele, Milan, Italy). Secondary antibodies were purchased from Molecular Probes. Nuclei were counterstained with Hoechst 33258. Where indicated, confocal microscopy was performed with TCS SP2 digital scanning instrument (Leica). Western blotting: pRb, clone G3-245 (BD Biosciences); p107, clone SD9; p130, C-20; cycD1, M-20; cycA, C-19; cycE, M-20; PCNA, PC10; MCM2, N-19 (Santa Cruz Biotechnology, Inc.); RPA70, 2H10 (NeoMarkers); DNA ligase I, 5H5 (Areta International); β-tubulin, clone 2-28-33 (Sigma-Aldrich); and HSP70, N27F3-4 (StressGen). Detection was performed with HRP-conjugated secondary antibodies (Cappel) and West Dura kit (Pierce Chemical Co.).

Cells and viruses

MSC isolation and culture have been described previously (Tiainen et al., 1996). To obtain myotubes, MSCs were plated on gelatin-coated dishes in DME containing 10% FBS and 12 h later switched to 5% horse serum. After 72 h, myotubes were infected with either control or AdCre virus at multiplicity of infection (m.o.i.) 1,000. The dl520, cyclin D1–, and Cdk4-expressing adenoviruses (Latella et al., 2001) were used at m.o.i. 300, 40, and 500, respectively. In multiple infections, AdCre was used first followed, 24 h later, by dl520 or cycD1/Cdk4. Unless otherwise stated, myotubes were treated with 10% FBS (with 20 μM BrdU if required) starting 24 h after infection and fixed for IF or harvested for biochemical procedures 24 h later.

Luciferase reporter assays

MSCs were transfected with the pGL3TATAbasic or pGL3TATA6xE2F reporter construct and selected in 2 μg/ml puromycin. Stable polyclonal MSCs were infected after differentiation as described above. Luciferase activity was measured with the Bright-Glo Luciferase Assay System (Promega).

DNA microarrays

Total mRNA was converted into cDNA and then biotin-labeled cRNA using the SuperScript II (Invitrogen) and ENZO (Affymetrix) kits. cRNAs were hybridized to MGU74Av2 oligonucleotide microarrays (Affymetrix) according to manufacturer's instructions. Data analysis was performed using Affymetrix Microarray Suite v.5.0 and Microsoft Excel.

Kinase assays

Immunoprecipitation of cyclin-associated kinase activity (Pajalunga et al., 1999) was performed using antibodies to cycA (H-432) or cycE (M-20) (Santa Cruz Biotechnology, Inc.).

Generation of TKO myotubes

To stably express an excisable pRb in TKO MEF, a LoxP site was introduced in the pBabeBleo (Morgenstern and Land, 1990) 3′ LTR as described previously (Berghella et al., 1999), obtaining pBbxp. An HA-tagged Rb cDNA (a gift from P. Amati, European Institute of Oncology, Milan, Italy) was inserted into the EcoRI and SalI sites, obtaining pBbxp/HA-Rb. Control and Rb-expressing retroviruses, produced in Phoenix cells, were used to generate phleomicin-resistant TKO MEF polyclonal populations. These cells were infected with a MyoD adenovirus (m.o.i. 750) and induced to differentiate in serum-free medium for 72 h. Myotubes were infected as described above and assayed for BrdU incorporation.

Online supplemental material

Fig. S1 shows that pRb ablation can reactivate the cell cycle in quiescent MEFs. pRb immunoblot confirmed pRb removal from Cre-infected fibroblasts (A), which increasingly incorporated BrdU in the next 96 h (B). Table S1 shows increased accumulation of mRNAs of unselected E2F target genes upon pRb removal in myotubes, as measured by DNA microarray analysis. Table S2 shows decreased expression of mRNAs of muscle-specific genes after AdCre infection of myotubes. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200408164/DC1.

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