PKCθ signaling is required for myoblast fusion by regulating the expression of caveolin-3 and β1D integrin upstream focal adhesion kinase

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ABSTRACT **Fusion of mononucleated myoblasts to form multinucleated myofibers is an essential phase of skeletal myogenesis, which occurs during muscle development as well as during postnatal life for muscle growth, turnover, and regeneration. Many cell adhesion proteins, including integrins, have been shown to be important for myoblast fusion in vertebrates, and recently focal adhesion kinase (FAK), has been proposed as a key mediator of myoblast fusion. Here we focused on the possible role of PKCθ, the PKC isoform predominantly expressed in skeletal muscle, in myoblast fusion. We found that the expression of PKCθ is strongly up-regulated following freeze injury–induced muscle regeneration, as well as during in vitro differentiation of satellite cells (SCs; the muscle stem cells). Using both PKCθ knockout and muscle-specific PKCθ dominant-negative mutant mouse models, we observed delayed body and muscle fiber growth during the first weeks of postnatal life, when compared with wild-type (WT) mice. We also found that myofiber formation, during muscle regeneration after freeze injury, was markedly impaired in PKCθ mutant mice, as compared with WT. This phenotype was associated with reduced expression of the myogenic differentiation program executor, myogenin, but not with that of the SC marker Pax7. Indeed in vitro differentiation of primary muscle-derived SCs from PKCθ mutants resulted in the formation of thinner myotubes with reduced numbers of myonuclei and reduced fusion rate, when compared with WT cells. These effects were associated to reduced expression of the profusion genes caveolin-3 and β1D integrin and to reduced activation/phosphorylation of their upstream regulator FAK. Indeed the exogenous expression of a constitutively active mutant form of PKCθ in muscle cells induced FAK phosphorylation. Moreover pharmacologically mediated full inhibition of FAK activity led to similar fusion defects in both WT and PKCθ-null myoblasts. We thus propose that PKCθ signaling regulates myoblast fusion by regulating, at least in part, FAK activity, essential for profusion gene expression.**

Monitoring Editor
Mark H. Ginsberg
University of California, San Diego

Received: Oct 13, 2010
Revised: Feb 7, 2011
Accepted: Feb 11, 2011

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E10-10-0821) on February 23, 2011.

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Abbreviations used: ALP, alkaline phosphatase; DM, differentiation medium; EDL, extensor digitorum longus; eMyHC, embryonic myosin heavy chain; FAK, focal adhesion kinase; GM, growth medium; H&E, hematoxylin and eosin; IL-4, interleukin 4; mAb, monoclonal antibody; MMP, matrix metalloproteinase; NCAM, neural cell adhesion molecule; NIH, National Institutes of Health; SC, satellite cell; TA, tibialis anterior; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; WT, wild type.

INTRODUCTION

During muscle development, myoblasts (the muscle-committed cell population) fuse together to form muscle fibers. When the muscle is built, postnatal muscle growth and regeneration are guaranteed by satellite cells (SCs), the muscle stem cells, recognized as located between the basal lamina and the sarcolemma, and expressing the SC marker Pax7; these cells are able to recapitulate myogenesis (Holterman and Rudnicki, 2005; Le and Rudnicki, 2007; Kuang and Rudnicki, 2008). A large number of factors regulate muscle precursor differentiation. Pax7-positive cells must escape from their stem cell program through the activation of myogenin and MRF4, which regulate the expression of muscle-specific protein to build the contractile apparatus. An initial phase of myoblast fusion (primary fusion) is required to form nascent myofibers. A secondary fusion

wave (secondary fusion), involving recruitment of mononucleated cells to the nascent myofibers, then completes myofiber growth. Several molecules contribute to myoblast fusion, such as M-cadherin, neural cell adhesion molecule (NCAM), β1D integrin, and caveolin 3. Among them, β1D integrin and caveolin 3 expression has been recently shown to be required for "secondary" myoblast fusion, because reduction of any of them dramatically impairs myoblast fusion; their expression appears to be regulated by focal adhesion kinase (FAK) signaling, which has thus been proposed as a key player in the fusion process (Quach *et al.*, 2009).

Many studies suggested that PKC is one of the key intermediates in integrin-mediated signaling in many cell types (Disatnik and Rando, 1999; Mostafavi-Pour *et al.*, 2003). Indeed inhibition of PKC activity results in the inhibition of cell attachment and spreading as well as of FAK phosphorylation. PKCs may also directly phosphorylate focal adhesion proteins like talin and filamin (Tigges *et al.*, 2003), allowing the interactions of integrins with the actin cytoskeleton. Moreover activation of PKC can promote the cellular changes mediated by integrin/matrix interactions (Disatnik and Rando, 1999; Disatnik *et al.*, 2002; 2004; Connors *et al.*, 2007; Tulla *et al.*, 2008). These observations together demonstrate that PKC plays a specific role in integrin-mediated signal transduction. Because the PKC family of serine/threonine protein kinases comprises at least 12 isoforms, it is still unclear whether different PKC isoforms may play distinct and specific role. PKCs can be subdivided in three subgroups, according to their structure and enzymatic activity: the "conventional" PKCs (PKCα, β1, β2, and γ), the enzymatic activity of which is calcium and phospholipid dependent, the "novel" PKCs (δ, ε, η, θ, and μ/PKD), the activity of which is calcium independent but phospholipid dependent, and the "atypical" PKCs (ζ, ι/λ, and τ), the activity of which is calcium and phospholipidindependent. In skeletal muscle, PKCθ is the PKC isoform predominantly expressed (Osada *et al.*, 1992; Zappelli *et al.*, 1996). In lymphocytes, it acts as a key regulator in their activation and proliferation (Pfeifhofer *et al.*, 2003; Manicassamy *et al.*, 2006; Boschelli, 2009). We recently showed that it is also required for cardiomyocyte survival and cardiac remodeling (Paoletti *et al.*, 2010). Its role in skeletal muscle is not as clear yet, however. We and others have shown that PKCθ expression in skeletal muscle is developmentally and nerve regulated, and that it mediates various cellular responses (Hilgenberg *et al.*, 1996; Zappelli *et al.*, 1996; Serra *et al.*, 2003; D'Andrea *et al.*, 2006; Gao *et al.*, 2007; Tokugawa *et al.*, 2009; Messina *et al.*, 2010). It is noteworthy that PKCθ starts to be expressed during the fetal period of development, when muscle mass is built, and it peaks during the first few weeks of postnatal life, when muscle mass needs to grow extensively (Hilgenberg *et al.*, 1996; Zappelli *et al.*, 1996; Messina *et al.*, 2010), suggesting that PKCθ may be involved in skeletal muscle growth and remodeling. To test this hypothesis, experimentally induced muscle regeneration, which recapitulates most developmental and histogenetic events, may represent a reliable approach. Earlier studies have suggested that multiple PKC isoforms are implicated in muscle-regenerative process, acting differently in times and location and suggesting that individual isoform may fulfill distinct functions (Moraczewski *et al.*, 2002). Indeed during regeneration, an earlier expression of PKCθ than of the other members of PKC was observed in rats (Moraczewski *et al.*, 2002). More recently, restriction of PKCθ immunoreactivity in SCs was described in regenerating rat TA, suggesting a role in SC maintenance and/or activation (Tokugawa *et al.*, 2009). We here aimed to investigate the role of PKCθ in muscle regeneration in vivo and SC differentiation in vitro, using two different models of PKCθ-null mice: a PKCθ knockout model, in which the PKCθ gene was inactivated in all cells (Sun *et al.*, 2000) and the mPKCθK/R transgenic model, in which a dominant-negative mutant form of PKCθ is expressed under the control of a muscle-specific promoter (Serra *et al.*, 2003).

RESULTS

Lack of PKCθ delays mouse growth

We initially observed that juvenile mice lacking PKCθ appeared smaller than age- and sex-matched WT mice. We thus systematically measured body weight of PKCθ$^{-/-}$ at different ages during postnatal growth, as compared with that of age- and sex-matched WT mice. As shown in Figure 1A, during the first 4–5 wk of age, the body weight of PKCθ$^{-/-}$ mice was significantly lower than that of WT mice, raising the level of WT body weight only by 5–8 wk. Because skeletal muscle is the tissue that contributes most to body weight, we aimed to verify whether the observed decrease in body weight was due to reduction in skeletal muscle mass. As first, lack of PKCθ expression in skeletal muscles (tibialis anterior [TA] and extensor digitorum longus [EDL]) of the mutant mice was confirmed by Western blot analysis (Figure 1A). Indeed muscle mass, as well as muscle fiber size, of 2-mo-old PKCθ$^{-/-}$ hind limb was apparently reduced, as compared with that of WT (Figure 1B). In fact, morphometric analysis of TA muscle showed that muscle fiber cross-sectional area (CSA) was reduced in PKCθ$^{-/-}$ with respect to WT during the first weeks of postnatal life (Figure 1B), whereas no significant differences were observed in the total number of fibers (unpublished data). By 3 mo, however, muscle fiber CSA in PKCθ$^{-/-}$ was similar to that of WT mice (Figure 1B), suggesting that lack of PKCθ$^{-/-}$ delayed postnatal mouse growth, mostly by delaying postnatal skeletal muscle fiber growth.

Lack of PKCθ impairs muscle regeneration in vivo

It is well known that postnatal muscle growth is due to SC (muscle stem cell) differentiation and fusion to preexisting muscle fibers, as well as to each other, to form new fibers. Thus the reduction in muscle mass and myofiber CSA observed during the first weeks of postnatal growth in PKCθ$^{-/-}$ mice suggests that PKCθ may indeed be required for these processes. To verify this possibility, we first analyzed whether the expression and activation/phosphorylation of PKCθ was modulated in WT mice during muscle regeneration, a process which recapitulates muscle formation and growth. Muscle regeneration was induced by freeze injury in TA, and protein extracts from TA muscles, dissected at different periods of time (2, 4, and 7 d) after injury, were analyzed by Western blot. As shown in Figure 2A, PKCθ expression and phosphorylation peaked in regenerating muscle 4–7 d after injury, thus during the period of major fusion and growth of regenerating fibers. Muscle regeneration was then induced in age- and sex-matched PKCθ$^{-/-}$. Muscle reorganization was analyzed at morphological level, 4 d after injury when new myofibers started to form (unpublished data), and 7 d after injury, when most regenerating myofibers had formed but were still immature, and compared with WT regenerating muscle (Figure 2B). Analysis of muscle sections revealed that muscle from mice in which PKCθ was deleted displayed, at both period of times, the characteristics of delayed regeneration, such as smaller regenerating centronucleated myofibers, heterogeneity in myofiber size, increased number of interstitial cells, and increased interstitial space between myofibers, as compared with time-matched regenerating WT muscle (Figure 2B, a and b). In keeping with these observations at day 7, although the number of embryonic myosin heavy chain (eMyHC; used as a marker of regenerating myofibers) expressing myofibers in PKCθ$^{-/-}$ mice was similar to that in WT mice (Figure 2B, c and d), their median CSA was significantly smaller (Figure 2B, e). As a result,

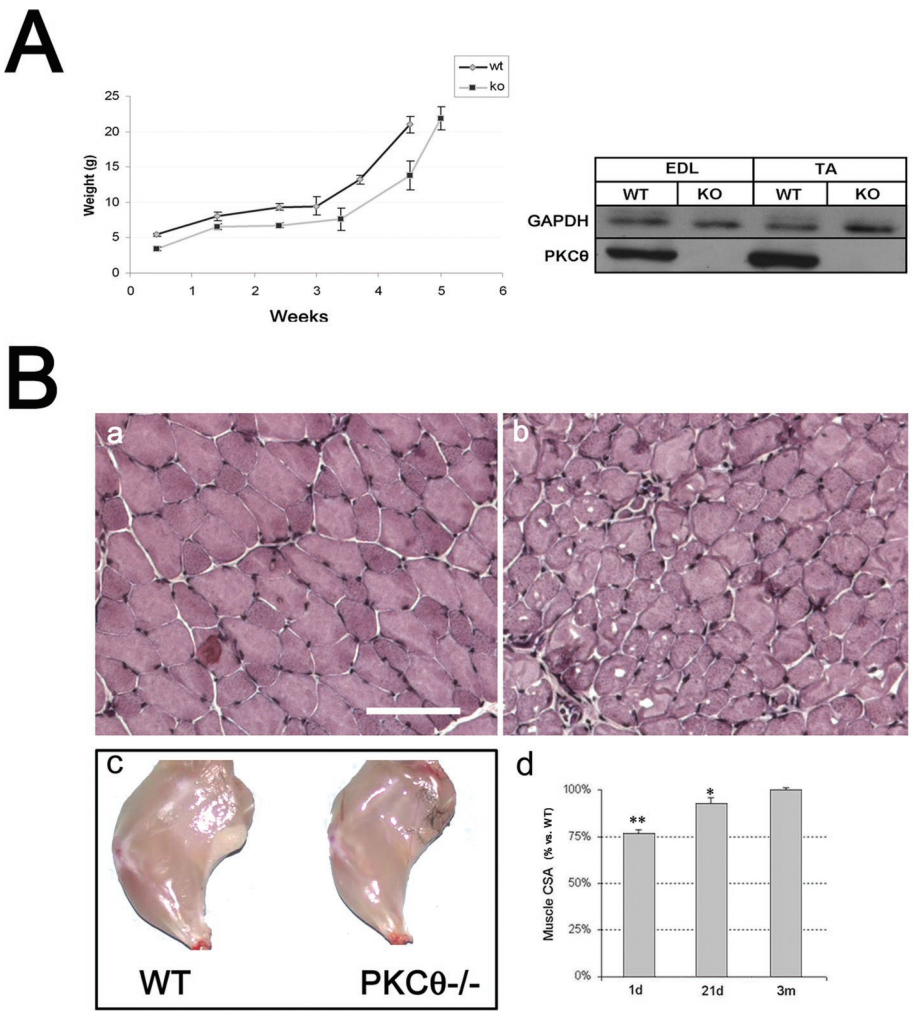

FIGURE 1: Lack of PKCθ delays mice growth. (A) Mean body weight in WT and PKCθ$^{-/-}$ mice at different time points during postnatal growth (left panel) (n ≥ 5 per genotype/age). Right panel, Western blot analysis of PKCθ expression in EDL and TA muscle from 2-mo-old WT and PKCθ$^{-/-}$ mice. (B) H&E staining of TA muscle sections from 2-mo-old WT (a) and PKCθ$^{-/-}$ mice (b) (bar = 100 μm); c: representative picture of hind limbs derived from 2-mo-old WT and PKCθ$^{-/-}$ mice, as indicated; d: mean myofiber CSA in PKCθ$^{-/-}$ (gray bars) TA muscles at different periods of time during postnatal growth, expressed as percentage of myofiber CSA in WT, assumed as 100% for each time point; n ≥ 3 per genotype/age (**p < 0.01, *p < 0.05 vs. time-matched WT).

the amount of eMyHC content in PKCθ$^{-/-}$ regenerating muscle was significantly lower than that in WT. In fact, Western blot analysis revealed that, whereas in WT muscle eMyHC expression was increasing by the time of regeneration, in PKCθ$^{-/-}$ its expression increased at 4 d after injury, but then it did not further increase (Figure 2C). To verify whether the observed alterations in muscle regeneration were due to impairment of SCs activation and/or differentiation, the expression of the SC marker Pax7 and of the myogenic differentiation executor myogenin was analyzed by Western blot. As shown in Figure 2C, Pax7 expression was highly increased in both mutant and WT mice at day 4 after injury, at a similar extent, and then decreased by day 7 similarly in both mutant and WT mice. In contrast, the strong up-regulation of myogenin expression in WT mice at day 4 after injury was prevented in PKCθ$^{-/-}$ mice; moreover the following down-regulation observed in WT mice 7 d after injury, as an indicator of the resolution of the differentiation process, was not observed in PKCθ$^{-/-}$, where the level of expression of myogenin was only slightly lower than that observed at day 4 after injury. One month after injury, muscle morphology seemed similar in control and PKCθ$^{-/-}$ muscles, in terms of muscle fiber CSA and muscle organization (unpublished data), showing that there clearly are compensatory mechanisms that ultimately result in effective regeneration even if significantly delayed compared with control muscle.

Lack of PKCθ impairs in vitro myogenesis

To test whether PKCθ may regulate myoblast fusion, the expression and activation of PKCθ were analyzed in in vitro differentiating primary myoblasts derived from WT mice. As shown in Figure 3A, PKCθ expression was strongly up-regulated within 8 h in differentiation medium (DM), and most of the protein was associated to the particulate fraction, as a feature of PKC activation. In vitro differentiation of primary myoblasts derived from PKCθ mutant mice was then compared with that of WT myoblasts. The cells were fixed after 48 h in DM and were either stained with Wright's solution or immunostained with the anti–sarcomeric myosin heavy chain MF20 antibody. As shown in Figure 3B, by 48 h in DM, WT myoblasts had formed elongated myotubes containing a large number of nuclei; in contrast, PKCθ$^{-/-}$ myoblasts exhibited minimal fusion. To quantify these observations, fusion rate was evaluated by counting the number of nuclei included in myosin-positive myotubes (containing ≥ 3 nuclei) divided by the total number of nuclei. As shown in Figure 3B, the fusion rate in PKCθ$^{-/-}$ muscle cell cultures was almost 50% with respect to WT cells. To verify whether "secondary" myogenesis (addition of nuclei to already formed myotubes) was actually impaired, the number of nuclei per myotube was evaluated; as shown in Figure 3B, the mean number of nuclei per myotube was significantly lower in PKCθ-null cultures, as compared with WT. After culturing the cells for longer periods of time, the number of myotubes was approximately the same, as both cultures already reached the plateau stage by 48 h in DM. Fusion continued in both genotypes, but the fusion rate differences were still maintained (unpublished data). To verify whether similar defects may account for the reduced myofiber CSA observed in vivo, isolated myofibers were prepared from EDL muscle of 2-mo-old mice, and nuclei were counted by means of TO-PRO-3 staining. Nuclei included in myofiber-associated SCs were identified as located outside the sarcolemma. Indeed the number of nuclei in freshly isolated PKCθ$^{-/-}$ myofibers was significantly lower than that in WT ones, whereas the number of SCs per myofiber was not altered (Figures 3C and Supplemental Figure S1).

To verify whether the in vitro observed defects might depend on different cell populations obtained from the two genotypes, the expression of alternative markers of muscle-specific resident precursors, such as PW1 (Mitchell *et al.*, 2010), as well as markers of early or middle stages of myogenesis, such as MyoD and desmin, was

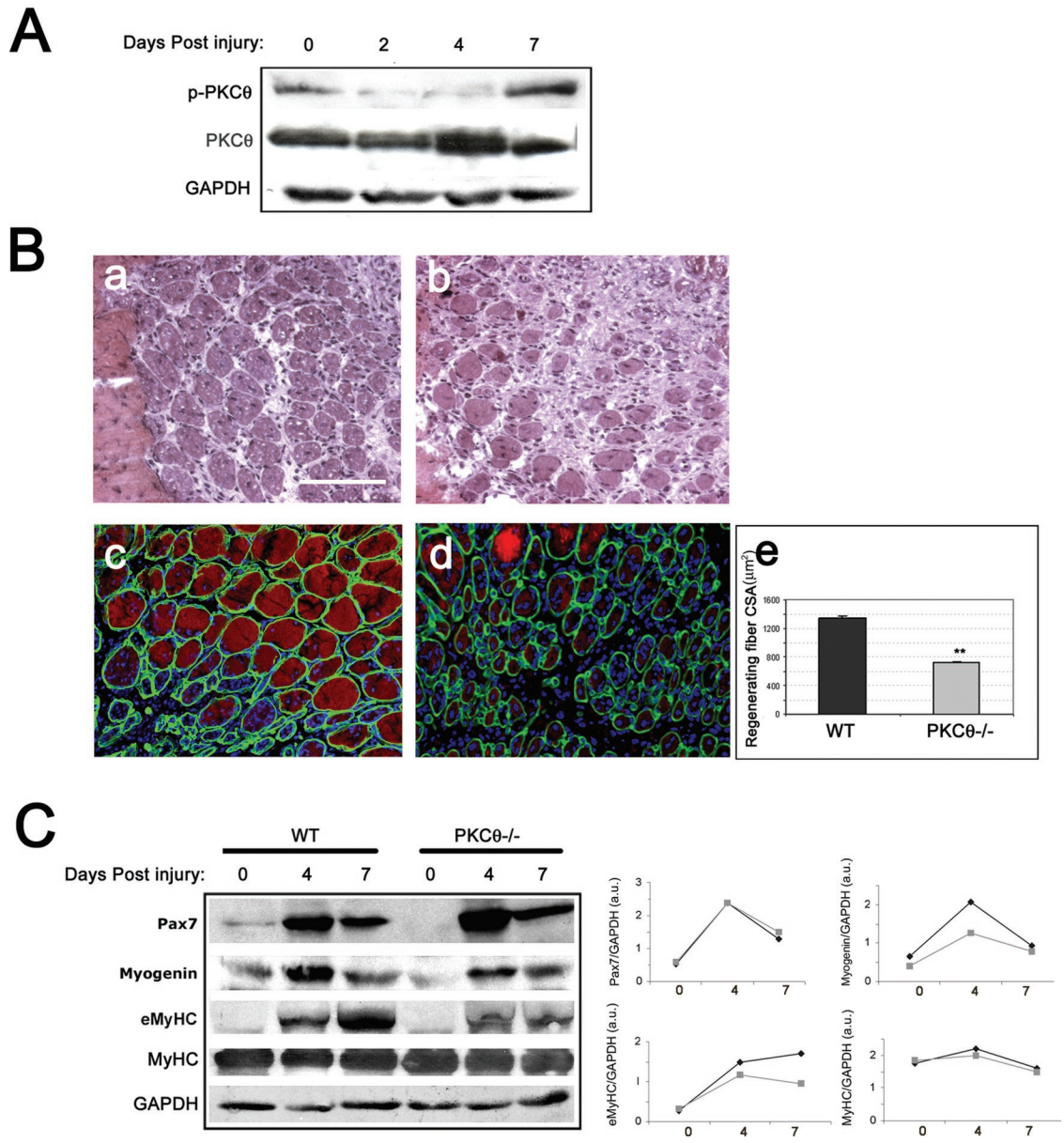

FIGURE 2: Lack of PKCθ impairs muscle regeneration in vivo. (A) Western blot analysis of total protein fractions from TA muscle at different periods of time after freeze injury in WT mice; the blot was incubated with the anti–phospho^{Thr538} PKCθ (p-PKCθ) and with the anti-PKCθ antibodies, as indicated; GAPDH level of expression was used for normalization. Representative experiment is shown (n = 3 per genotype). (B) Representative images of H&E staining (a and b) of TA cryosections obtained from 2-mo-old WT (a) and PKCθ$^{-/-}$ (b) mice at day 7 after freeze injury (n ≥ 3 per genotype). Double immunofluorescence analysis (c and d) of TA muscle cryosections at day 7 after freeze injury for laminin (green) and eMyHC (red) expression in WT (c) and PKCθ$^{-/-}$ (d) (bar = 100 μm). Mean CSA of eMyHC-expressing (regenerating) myofibers in WT (black bar) and PKCθ$^{-/-}$ 7 d after freeze injury (n = 3 per genotype; $^{***}p < 0.001$ vs. WT). (C) Western blot analysis of total protein fractions from WT and PKCθ$^{-/-}$ regenerating TA muscle 2, 4, and 7 d after freeze injury, as indicated. Representative blot from three independent experiments is shown (n = 3 per genotype at each time point); the blot was incubated with the α-Pax7, or -myogenin, or -eMyHC or -sarcomeric-MyHC (MyHC) antibodies, as indicated. GAPDH level of expression was used for normalization. Densitometric analysis is shown at the right.

evaluated by immunofluorescence analysis in freshly isolated cell populations obtained from WT and PKCθ$^{-/-}$ muscle. As shown in Figure 4, the majority of the cells coexpressed all the markers analyzed, and no differences were detectable between the two genotypes. Neither were the observed defects the result of differences in cell survival, because terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis revealed that the number of TUNEL-positive cells grown in growth medium (GM), among PKCθ$^{-/-}$ myoblasts, was comparable to that of WT cells (Figure 4A). Similar results were obtained when the medium was replaced with DM to induce differentiation (unpublished data).

To verify whether the observed defects were, instead, the result of differences in growth rate, low-density cultures were assessed to obtain single-cell–derived clones. At first, the number of clones obtained per plated cell was evaluated, and the results were similar in both WT and PKCθ-null myoblasts (Figure 4B). Growth rate,

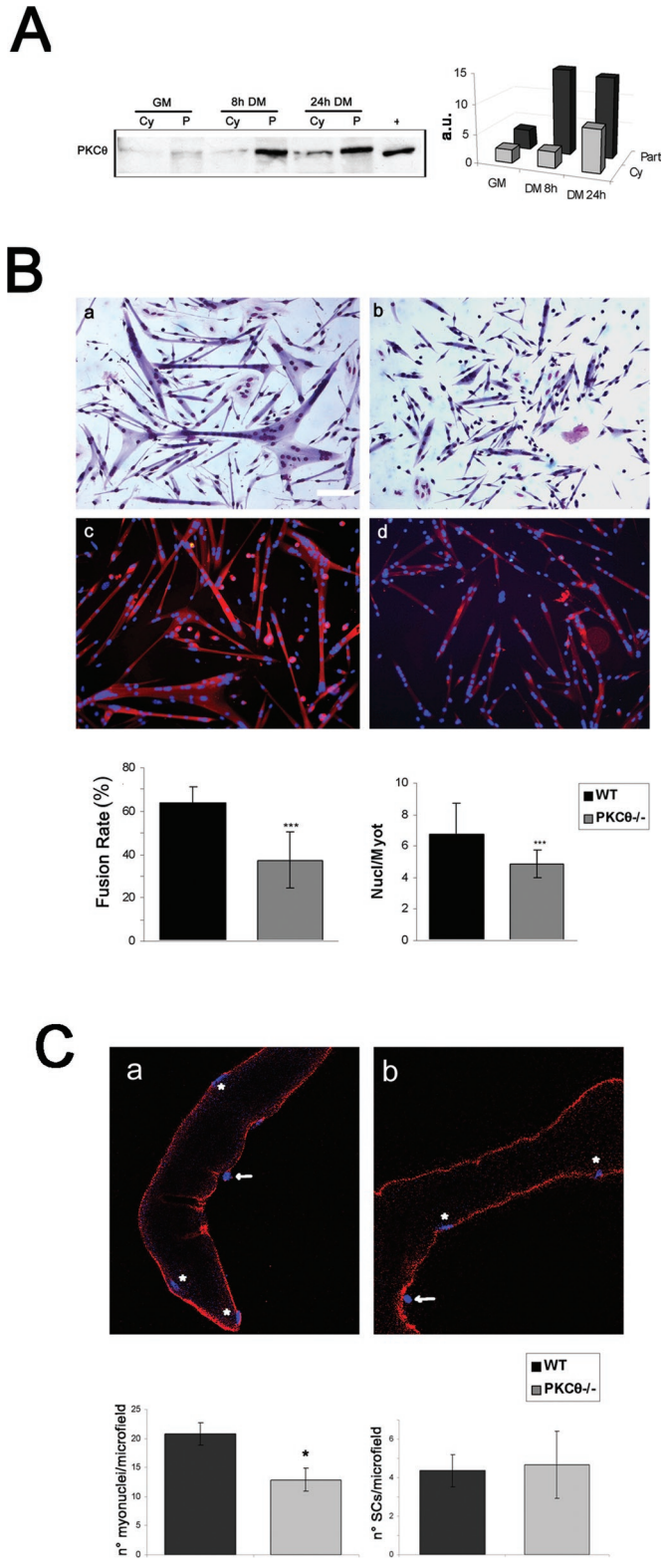

FIGURE 3: Lack of PKCθ inhibits myotube growth in vitro. (A) Western blot analysis of both cytosolic (Cy) and particulate (P) subcellular protein fractions of primary SCs derived from WT muscle cultured in GM or in DM for the indicated periods of time; the blot was incubated with the α-PKCθ antibody. Representative experiment is shown (n = 3 per genotype). Whole muscle protein lysate was used as positive control (+). Densitometric analysis is shown at the right. (B) Representative Wright staining (a and b) and immunofluorescence analysis of MyHC expression (c and d) of WT (a and c)- and PKCθ$^{-/-}$ (b and d)-derived muscle SCs, cultured in DM for 48 h. Cells in c and d were counterstained with Hoechst; bar = 100 μm. Bottom panel: fusion rate is shown at the left, determined as the percentage of nuclei included in myotubes (containing ≥ 3 nuclei), with respect to the total number of nuclei; the mean number of nuclei contained within each myotube is also shown at the right (WT = black bar; PKCθ$^{-/-}$ = gray bar). The data were collected from at least five independent experiments (***$p < 0.001$ vs. WT). (C) Representative z section images of immunofluorescence analysis of myofibers isolated from WT (a) or PKCθ$^{-/-}$ (b) EDL muscle, immunostained with the α-caveolin-3 antibody (red, used as sarcolemma marker). Nuclei were counterstained with TO-PRO-3; asterisks indicate myonuclei included within sarcolemma, arrows indicate nuclei of associated SCs, outside the sarcolemma. At least 10 myofibers per mouse were analyzed (n = 3 per genotype). Each myofiber was analyzed under confocal microscopy, and the number of myonuclei in each microfield was counted for the entire thickness of the myofiber, taking pictures every 5 μm z section (shown in Supplemental Figure S1). The mean number of nuclei and of SCs is shown at the bottom (WT, black bars; PKCθ$^{-/-}$, gray bars).

evaluated in randomly selected clones (20 clones per genotype) as the number of cells per clone counted every 24 h during a 5-d culturing time period, also showed similar results in both genotypes. Most of the clones derived from WT myoblasts, however, had formed elongated myotubes containing a large number of nuclei; in contrast, clones derived from PKCθ-null cells exhibited minimal fusion, similar to the nonclonally derived primary cultures (Figure 4B).

Lack of PKCθ prevents β1D integrin and caveolin-3 up-regulation during myoblast differentiation

On the basis of these results, we thus analyzed the expression of factors regulating myogenesis, during in vitro differentiation of PKCθ-null myoblasts, as compared with WT. Similar to what was observed during regeneration in vivo, Western blot analysis revealed that the expression of Pax7 was high in both PKCθ$^{-/-}$ and WT cells cultured in GM, as well as after 1 d in DM; after 2 d in DM, the expression of Pax7 was almost undetectable in both PKCθ$^{-/-}$ and WT cells (Figure 5A). In contrast, the expression of myogenin, following similar time-course kinetics in both genotypes, was lower in mutant cells with respect to WT cells, at all the time points considered (Figure 5A). Surprisingly, the expression level of muscle-specific genes, such as sarcomeric myosin, was similar in PKCθ$^{-/-}$ and WT differentiated cells (Figure 5A). In contrast, the expected up-regulation of both β1D integrin and caveolin-3 expression, recently shown to be essential for myoblast fusion (Quach *et al.*, 2009), observed in WT myoblasts after 1 d in DM, was significantly reduced in PKCθ$^{-/-}$ myoblasts To confirm the requirement of PKCθ for myoblast fusion using a different approach, primary myoblasts were prepared from a different mouse model, the mPKCθK/R mouse (Serra *et al.*, 2003), in which the expression of a kinase dead mutant form of PKCθ cDNA is driven by the muscle-specific enhancer of the desmin promoter; thus the activity of PKCθ is selectively inhibited in differentiating myoblasts. mPKCθK/R myoblasts behaved similarly to the PKCθ$^{-/-}$ ones at the morphological level, giving rise to thin, oligonucleated myotubes in culture (unpublished data). Accordingly, the expression of β1D integrin and caveolin-3 was prevented at day 1 in DM, as compared with WT myoblasts (Figure 5B). At day 2 in DM, the expression level of β1D integrin and caveolin-3 in both PKCθ mutant cells was still reduced, although to a lesser extent, with respect to WT cells.

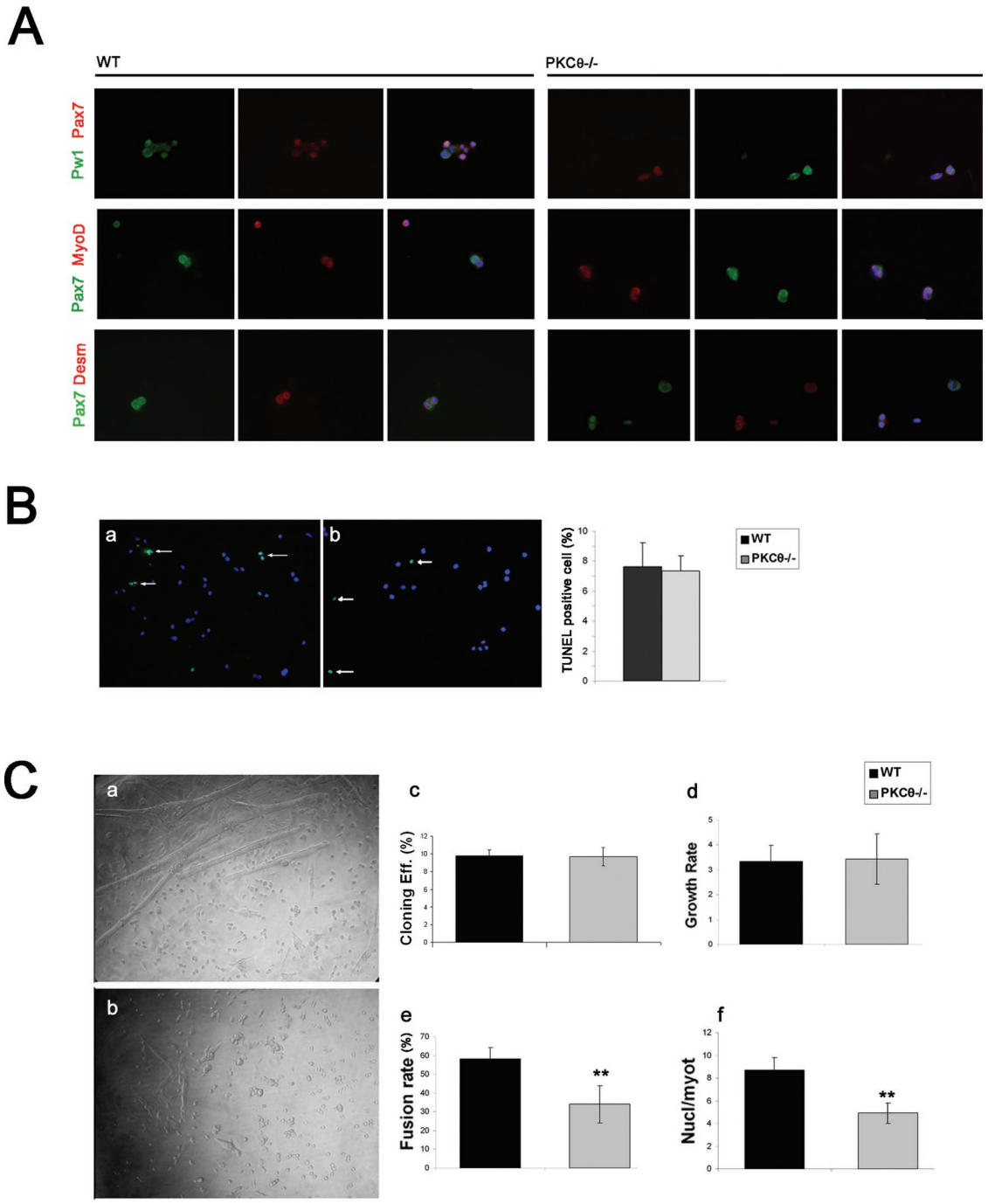

FIGURE 4: Lack of PKCθ inhibits myoblast fusion. (A) Immunofluorescence analysis of freshly isolated cells derived from WT or PKCθ$^{-/-}$ hind limb muscle, as indicated. The cells were immunolabeled with the α-Pax7, -PW1, -MyoD, or -desmin antibodies, as indicated. (B) Representative microimages of TUNEL analysis in WT (a) and PKCθ$^{-/-}$ (b) muscle-derived cells cultured in GM. The number of TUNEL-positive nuclei divided by the total number of nuclei in WT (black bar) or PKCθ$^{-/-}$ (gray bar) is shown at the right (20 microfields per genotype, from three independent experiments). (C) Representative phase contrast images of clonally cultured muscle-derived SCs from WT (a) and PKCθ$^{-/-}$ (b) after 6 d in DM; c: cloning efficiency, expressed as the percentage of clones obtained divided by the total number of plated cells; d: growth rate, expressed as the mean fold increase in cell number within a single clone (15 clones per genotype); e: fusion index, expressed as the percentage of nuclei included in myotubes (containing ≥ 3 nuclei), with respect to the total number of nuclei, within a single clone cultured for 6 d in DM (20 clones per genotype); f: mean number of nuclei included in each myotube within a single clone cultured for 6 d in DM (20 clones per genotype). WT = black bar; PKCθ$^{-/-}$ = gray bar; $^{**}p < 0.01$ vs. WT.

PKCθ expression/activity is required for FAK phosphorylation

We then analyzed the expression and activation/phosphorylation level of FAK, an important nonreceptor protein tyrosine kinase involved in integrin signaling, recently proposed as a key factor in myoblast fusion through its ability to regulate β1D integrin and caveolin-3 expression (Quach *et al.*, 2009). As shown in Figure 6A, Western blot and immunofluorescence analyses revealed that lack

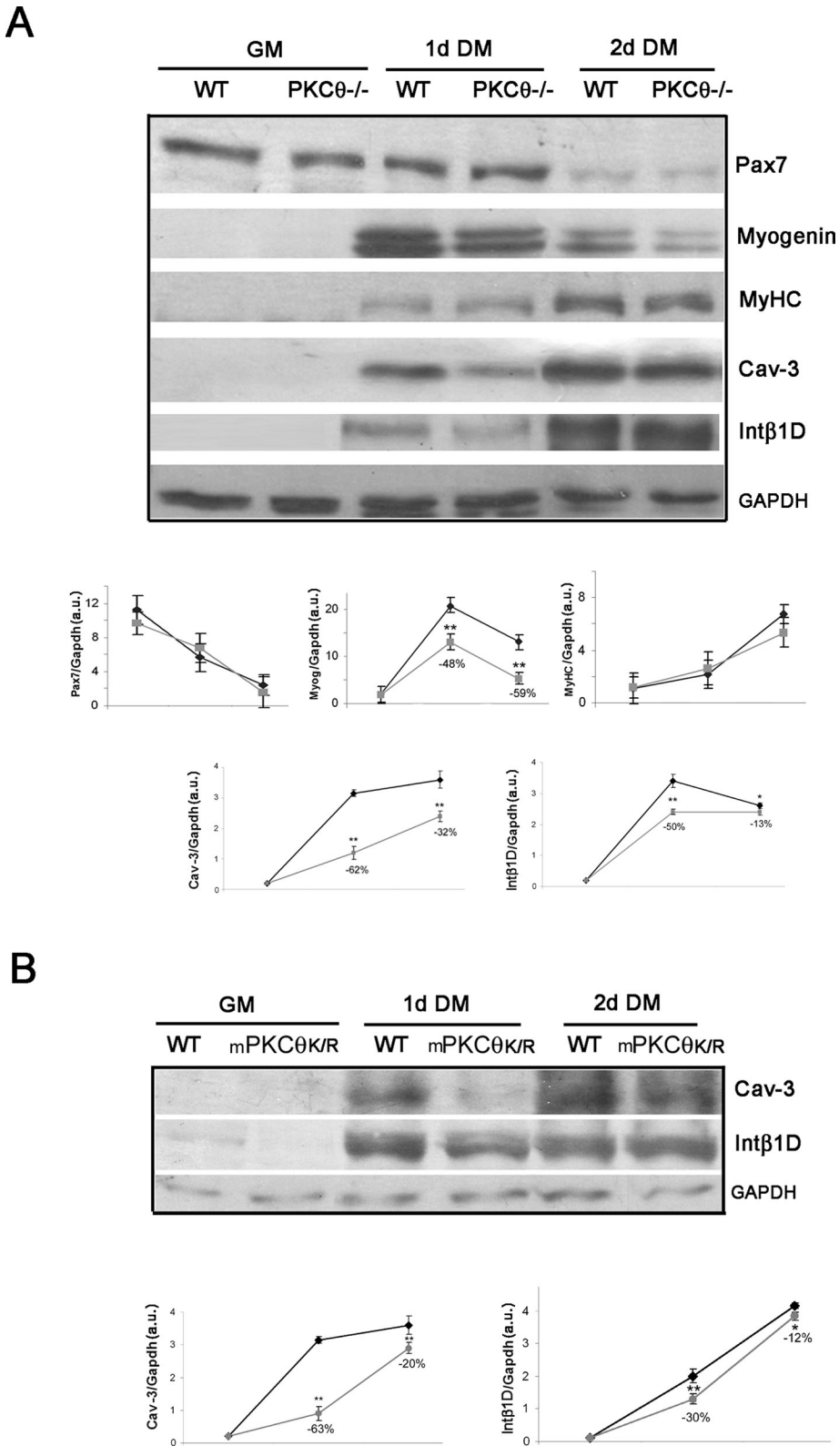

FIGURE 5: Lack of PKCθ expression or activity prevents up-regulation of caveolin-3 and β1D-integrin expression. (A) Western blot analysis of WT and PKCθ$^{-/-}$ muscle-derived SCs, cultured in GM, or for different periods of time in DM, as indicated. Representative experiment is shown of three independent experiments. The blot was incubated with the α-Pax7, α-myogenin, α-sarcomeric myosin heavy chain, α-caveolin-3, and α-β1D integrin antibodies. The level of expression of each protein in both genotypes, determined by densitometric analysis using the α-GAPDH antibody for normalization, is shown at the bottom; WT (♦), PKCθ$^{-/-}$ (■). (B) Western blot analysis of WT and mPKCθK/R muscle-derived cells, cultured in GM, or for different periods of time in DM, as indicated. The blot was incubated with the α-caveolin-3 and α-β1D integrin antibodies. Densitometric analysis is shown at the bottom, WT (♦) and mPKCθK/R (■), using the α-GAPDH antibody for normalization as in A (**p < 0.01, *p < 0.05 vs. WT). When significant, reduction of expression in PKCθ$^{-/-}$ or in mPKCθK/R cells, expressed as percentage vs. WT (assumed as 100%) is also shown, resulting from at least three independent experiments.

of expression/activity of PKCθ significantly prevented FAK activation/phosphorylation during myoblast differentiation in vitro, as compared with WT myoblasts. To verify whether PKCθ signaling actually leads to FAK phosphorylation, a constitutively active mutant form of PKCθ (PKCθA/E; D'Andrea *et al.*, 2006) was transfected in C2C12 cells (a mouse muscle cell line) cultured in GM. Parallel cultures were mock transfected. The ability of the exogenously expressed PKCθ mutant form to drive FAK phosphorylation was evaluated 16 h after transfection, by double immunofluorescence using the anti–phospho-FAK and the anti-PKCθ antibodies. As shown in Figure 6B, a–d, whereas no p-FAK-positive or PKCθ-positive cells were detectable in mock-transfected cells, all the PKCθ-expressing cells, in PKCθA/E-transfected cultures, coexpressed p-FAK. In parallel, another group of plates was processed for immunoprecipitation using the anti-FAK antibody. The immunoprecipitate was then analyzed by Western blot for p-FAK and for total FAK content. As shown in Figure 6B, increased FAK phosphorylation was observed in PKCθA/E-transfected cultures compared with mock-transfected cultures. To verify whether the observed decrease in FAK phosphorylation is the main event driving the phenotype, WT and PKCθ$^{-/-}$ primary muscle cells were cultured in the presence of the FAK inhibitor 14 (Beierle *et al.*, 2010), and both fusion rate and the number of nuclei per myotube were evaluated. As shown in Figure 6C, full FAK inhibition, by means of the inhibitor, strongly reduced fusion rate and number of nuclei per myotube in both WT and PKCθ$^{-/-}$ cultures to a similar extent. Moreover up-regulation of both β1D integrin and caveolin-3 was similarly prevented in both WT and PKCθ$^{-/-}$ treated cells (Figure 6D).

DISCUSSION

In this article we demonstrate that the expression/activity of the θ isoform of PKCs is required for initial myoblast fusion events, but not for the induction of terminal

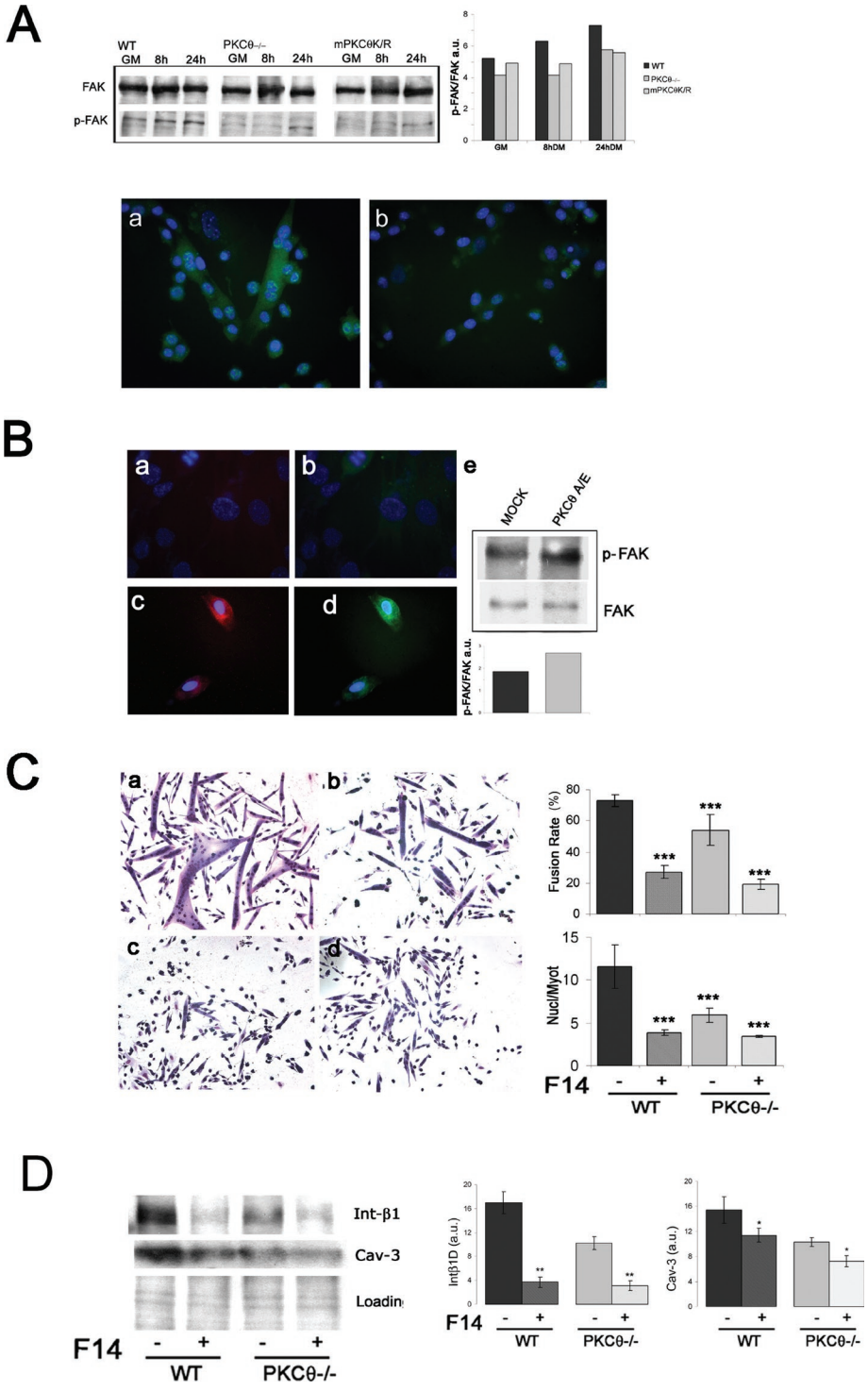

FIGURE 6: PKCθ expression/activity is required for FAK phosphorylation. (A) Western blot analysis of WT, PKCθ$^{-/-}$, and mPKCθK/R muscle-derived SCs, cultured in GM or for different periods of time in DM, as indicated. The blot was incubated with the α-FAK or with the α-phospho FAK antibodies. Representative experiment is shown of three independent experiments. Level of activation, determined by densitometric analysis as the phospho/total FAK ratio, is shown at the right. Representative immunofluorescence analysis of WT (a) or PKCθ$^{-/-}$ (b) muscle-derived cells cultured for 24 h in DM and incubated with the α-phospho FAK antibody is shown at the bottom. (B) Double immunofluorescence analysis of mock-transfected (a and b) or PKCθA/E-transfected (c and d) C2C12 cells using the α-PKCθ (red) and the α-pFAK (green) antibodies; e: Western blot analysis of mock-transfected or PKCθA/E-transfected C2C12 cells immunoprecipitated with the α-FAK antibody and incubated with the α-pFAK or the α-FAK antibodies. Representative experiment is shown of three independent experiments. Level of activation was determined by densitometric analysis, as the phospho/total FAK ratio. (C) Representative Wright staining of WT (a and c) or PKCθ$^{-/-}$ (b and d) muscle-derived primary cells cultured for 24 h in DM, in the absence (a and b) or presence (c and d) of the FAK inhibitor 14 (F14, 5 μM). Fusion rate, evaluated in each condition, is shown at the right, determined as the percentage of nuclei included in myotubes (containing ≥ 3 nuclei), with respect to the total number of nuclei; the mean number of nuclei contained within each myotube is also shown (WT = black bars; PKCθ$^{-/-}$ = gray bars). (D) Western blot analysis of the expression of caveolin-3 and β1D integrin in cells cultured as in C. Densitometric analysis is shown at the right; Red Ponceau staining of the membrane is shown to ensure equal loading. Representative experiment is shown of two independent experiments.

differentiation events. As first, lack of PKCθ in vivo resulted in reduced mice weight during the first weeks of postnatal life. The observed reduction was associated with reduction in both muscle mass and myofiber CSA. By 8–12 wk of postnatal life, both body weight and myofiber CSA in PKCθ$^{-/-}$ mice were similar to those in WT mice, suggesting that lack of PKCθ delays the initial growth and building of the muscle mass. Indeed a reduction in lean muscle has previously been reported in PKCθ$^{-/-}$ mice with respect to WT (Gao *et al.*, 2007). The fact that PKCθ is required for muscle growth is further supported by the observed delay in muscle regeneration. Experimentally induced muscle regeneration is a valuable model to recapitulate myofiber differentiation and growth. We show in this article that PKCθ expression was strongly up-regulated 4 d after injury, just during the phase of muscle rebuilding. This observation is consistent with a previously published study that showed that PKCθ activity increased significantly during the final phases of muscle regeneration in rat (Moraczewski *et al.*, 2002). Indeed we show that lack of PKCθ delayed muscle rebuilding after injury. The observed delay can be accounted for by impairment of the late phases of the growth/regeneration process. In fact, whereas no alteration in the expression of early markers of activated SCs, such as Pax7, was observed, full up-regulation of myogenin expression, an "executor" of myogenesis, was prevented, as well as the expression of the terminal differentiation marker of regenerating myofibers, eMyHC. More important, the size of regenerating myofibers, identified as eMyHC-positive myofibers, was significantly smaller in PKC$^{-/-}$ regenerating muscle than in time-matching regenerating WT muscle. Taken together, these observations demonstrate that,

although SC activation and differentiation are not altered in PKCq$^{-/-}$, further addition of fusing cells to regenerating myofibers (a process known as "secondary fusion") is, instead, prevented. Thus PKCθ expression/activity is required for the "resolution" of the SCs differentiation program during the late phases of regeneration, when myoblast fusion is required.

Accordingly, we show that PKCθ expression and activity were strongly induced in cultured muscle-derived cells within a few hours in DM, and inhibition of its expression (in PKCθ$^{-/-}$ muscle-derived cells) or activity (in mPKCθK/R muscle-derived cells) significantly reduced fusion index and myonuclei content, as compared with WT cells. This defect was actually dependent on a defect in the fusion process itself, because no differences in the nature of the cell populations obtained from the different genotypes were observed, and, when cells were clonally cultured, no differences in cell proliferation or in cell death were observed. Indeed single clones from PKCθ$^{-/-}$ muscle-derived cells still gave rise to thinner, oligonucleated myotubes, as compared with clones derived from WT cells. Taken together, these results demonstrate that PKCθ plays a critical role in myoblast fusion and myofiber growth, which can be referred to as "secondary fusion." This conclusion is further strengthened by the observation that the number of nuclei in PKCθ$^{-/-}$ freshly isolated myofibers was lower than that in WT ones, suggesting that similar alterations are occurring in vivo as well. Many factors have been shown to be implicated in myoblast fusion, including components of the extracellular matrix remodeling, such as matrix metalloproteinase (MMP)-2 and MMP-9 (Lluri and Jaworski, 2005; Lluri *et al.*, 2008); cell surface molecules, such as NCAM, N- and M-cadherins, a disintegrin and metalloproteinase 12, β1 integrins, and caveolin-3 (Galbiati *et al.*, 1999; Abmayr *et al.*, 2003; Gullberg, 2003; Horsley and Pavlath, 2004); or even cytokines, such as interleukin 4 (IL-4; Horsley *et al.*, 2003). Whereas the activity of MMP-2 and -9 was not altered by ablation of PKCθ expression/activity (our unpublished observations), we show in this article that the up-regulation of β1D integrin and of caveolin-3 upon differentiation was significantly prevented. Thus PKCθ activity appears to be involved in signaling pathways regulating the expression of molecules involved in myoblast fusion. To date, a clear picture of these pathways is not depicted yet; however, full activation of caveolin-3 and β1D integrin expression has been recently shown to be required for "secondary fusion" and dependent on FAK activation (Quach *et al.*, 2009). Indeed full FAK phosphorylation was prevented when PKCθ expression/activity was ablated in differentiating myoblasts, by either knocking the gene out or by expressing a dominant-negative mutant form. Accordingly, when a constitutively active PKCθ mutant form was exogenously expressed in cultured muscle cells, FAK phosphorylation was induced, demonstrating that PKCθ, either directly or indirectly, is involved in FAK activation. Several studies have indicated that PKC activation is required for FAK phosphorylation in cell-to-extracellular-matrix adhesion events and that they colocalize at focal adhesion sites (Vuori and Ruoslahti, 1993; Haimovich *et al.*, 1996; Disatnik and Rando, 1999). The precise functional relationship between these two kinases, however, is not completely understood yet. It has been previously shown that, during muscle cell adhesion and spreading, integrin engagement leads to FAK phosphorylation via a PKC-dependent signaling pathway (Disatnik and Rando, 1999); among the PKC isoforms analyzed, sequential activation of PKCε, -α, and -δ has been shown to be necessary to promote muscle cell spreading (Disatnik *et al.*, 2002). It is worth noting that in those studies no PKCθ expression was detected; thus its involvement in those events was ruled out. Indeed those studies were carried out using proliferating muscle cells, because cell adhesion and spreading were analyzed. As we show here, PKCθ is poorly expressed in proliferating myoblasts, but its expression is strongly up-regulated upon differentiation. Altogether, these observations suggest that, although FAK activation is a common feature, different PKC isoforms can be involved in cell to extracellular matrix and in cell-to-cell interactions, where different integrins are involved, and we show in the present study that activation of the θ isoform is indeed involved in FAK-mediated signaling leading to myoblast fusion. The fact that pharmacologically mediated full inhibition of FAK activity leads to similar fusion defects in both WT and PKCθ-null myoblasts further supports the hypothesis that the decreased FAK activation observed in PKCθ-null cells is sufficient to cause the phenotype. However, since no full FAK inhibition is observed in PKCθ-null myoblasts, other pathways might also contribute to FAK activation.

Different from what was observed when FAK activity was inhibited (Quach *et al.*, 2009), lack of PKCθ also prevented the full activation of myogenin. Indeed other pathways have been shown to be involved in "secondary fusion," such as calcineurin-dependent NFATc2 activation, leading to the production of IL-4 (Pavlath and Horsley, 2003), proposed as a profusion cytokine. It is worth noting that we and others have shown that PKCθ is involved in many calcineurin-dependent signaling pathways, in both lymphocytes and myoblasts (Villunger *et al.*, 1999; Pfeifhofer *et al.*, 2003; D'Andrea *et al.*, 2006), suggesting that it may be involved in multiple signaling pathways which ultimately lead to complete differentiation/fusion events.

In conclusion, although the involvement of PKCθ in other pathways in regulating myoblast differentiation/fusion cannot be ruled out, we show in this article that it is indeed required for myoblast fusion, regulating FAK activation and, in turn, the expression of the profusion genes caveolin-3 and β1D integrin.

MATERIALS AND METHODS

Animal models

PKCθ$^{-/-}$ mice were provided by Dan Littman (New York University, New York). In these mice, the gene encoding PKCθ was inactivated in all cells of the body, as previously described (Sun *et al.*, 2000).

mPKCθ-K/R transgenic mice express a PKCθ kinase dead mutant form, which acts as dominant negative, specifically in muscle, as previously described (Serra *et al.*, 2003). The animals were housed in the Histology Department–accredited animal facility. All the procedures were approved by the Italian Ministry for Health and were conducted according to the U.S. National Institutes of Health (NIH) guidelines.

Experimentally induced muscle regeneration

To induce freeze injury, a steel probe precooled in dry ice was applied to the TA muscle belly of anesthetized adult (8–12 wk old) male mice for 10 s. TA muscles were isolated at different time points after the injury, as specifically indicated. Uninjured, age-matched animals were used as controls.

Antibodies and reagents

The following primary antibodies were used: anti-PKCθ and anti-phospho^{Thr538} PKCθ rabbit polyclonal antibodies, anti–caveolin-3 and anti-MyoD mouse monoclonal antibodies (mAbs; BD Biosciences, San Diego, CA); anti–desmin mouse mAb (Dako, Glostrup, Denmark); anti–myosin heavy chain MF20, anti–eMyHC F1.652, anti-Pax7, and anti–myogenin F5D mouse mAbs (Developmental Studies Hybridoma Bank, Iowa City, IA); rabbit polyclonal anti-PW1 (provided by D.A. Sassoon; Mitchell *et al.*, 2010); anti-β1D integrin (provided by G. Tarone; Belkin *et al.*, 1996); and anti-FAK and anti–phospho^{Tyr397} FAK antibodies (Santa Cruz Biotechnology, Santa

Cruz, CA). To pharmacologically inhibit FAK, FAK inhibitor 14 (Santa Cruz Biotechnology) was used.

Muscle cell culture and cell transfections

Primary cultures were prepared from total limb muscles of WT, PKCθ$^{-/-}$, or mPKC-K/R mice, as previously described (Castaldi *et al.*, 2007). Muscle-derived cells were grown on collagen-coated dishes, in GM (DMEM containing 20% horse serum, HS, 3% chick embryo extract, EE, all from Invitrogen, Carlsbad, CA) in a humidified 5% CO_2 atmosphere at 37°C. Differentiation was induced by replacing the medium with medium containing lower serum and EE concentration, DM (DMEM containing 5% HS, 0.75% EE). C2C12 cells, a mouse SC-derived cell line (D'Andrea *et al.*, 2006), were grown in DMEM supplemented with 10% fetal calf serum. For transient transfection assays, 10^5 C2C12 cells were plated on 35-mm tissue culture dishes. After 24 h, proliferating myoblasts were transfected with a total of 1.5 μg of plasmid DNA/dish, using the lipid-based Lipofectamine reagent (Invitrogen), according to the manufacturer's instructions. After an additional 16 h, the cells were either fixed for immunofluorescence analysis or disrupted for immunoprecipitation and Western blot analysis. The expression plasmids encoding the constitutively active mutant form of PKCθ cDNA, PKCθ A/E (provided by G. Baier) (Villunger *et al.*, 1999), was used for transfections. The expression plasmid lacking cDNA was used for mock transfections.

Myofiber isolation

Two-month-old male WT and PKCθ$^{-/-}$ mice were killed by cervical dislocation, and the EDL muscles were carefully dissected. Muscles were digested in 0.2% collagenase type 1/DMEM (Sigma, St. Louis, MO); individual myofibers were dissociated by gently passing through Pasteur pipettes with different size apertures and then abundantly washed, as described (Aulino *et al.*, 2010; White *et al.*, 2010). Intact myofibers with tapered/sculptured ends were selected for fixation in 4% paraformaldehyde/phosphate-buffered saline (Sigma) for 6–10 min and processed for immunofluorescence analysis.

Histological and immunofluorescence analyses

Muscle cryosections were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) on ice, and cultured cells were fixed in ethanol/acetone (1:1 [vol/vol] ratio) at –20°C for 20 min. For histological analysis, muscle cryosections were stained with hematoxylin and eosin (H&E) solution (Sigma-Aldrich), and cultured cells with Wright's solution (Fluka, Milwaukee, WI). The muscle fiber mean CSA was determined by measuring the CSA of all fibers in the entire section, using Scion Image 4.0.3.2 software (NIH, Bethesda, MD). Immunofluorescence analysis of cells, cryosections, or isolated myofibers was performed as previously described (Castaldi *et al.*, 2007; Aulino *et al.*, 2010). Nuclei were counterstained with Hoechst 33342 (Fluka) or with TO-PRO-3 (Invitrogen), and the samples were analyzed under an epifluorescence Zeiss Axioskop 2 Plus microscope (Carl Zeiss, Oberkochen, Germany) or a Leica Leitz DMRB microscope fitted with a DFC300FX camera (Leica, Wetzlar, Germany).

Fusion assay

After different periods of time in DM, cells were either stained with Wright's solution or immunostained with the anti-myosin heavy chain antibody MF20. Myotubes were defined as cells containing three or more nuclei. The fusion rate was determined as the percentage of nuclei in myotubes compared with the total number of nuclei in the field. The mean number of nuclei contained within each myotube was also determined. Approximately 100 myotubes were counted per dish.

Cell death assay

Cell apoptosis was determined by TUNEL reaction (Roche Applied Science, Indianapolis, IN). At the indicated time intervals, cells were fixed, incubated for 1 h at 37°C with the TUNEL mixture, and processed following the manufacturer's instructions. Nuclei were counterstained with Hoechst 33342 (Fluka). Positive nuclei were detected under an epifluorescence Zeiss Axioskop 2 Plus microscope.

Western blot analysis and immunoprecipitation

For the total protein extract preparation, tissue samples or cell pellets were homogenized in ice-cold buffer (H-buffer) containing 20 mM Tris (pH 7.5), 2 mM EDTA, 2 mM EGTA, 250 mM sucrose, 5 mM dithiothreitol, leupeptin at 200 mg/ml, Aprotinin at 10 mg/ml, 1 mM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100 (all from Sigma-Aldrich, St. Louis, MO), as previously described (Zappelli *et al.*, 1996). The obtained homogenate was disrupted by sonication, incubated for 30 min on ice with repeated vortexing, and then centrifuged at 15,000 × *g* for 15 min. The pellet was discarded, and the supernatant was used for Western blot analysis. For the preparation of subcellular protein fractions, the cell pellet was homogenized in H-buffer lacking Triton X-100, and incubated 30 min on ice. Samples were then spun at 100,000 × *g* for 30 min at 4°C. The supernatant was saved as cytosolic fraction, and the remaining pellet was suspended in H-buffer containing 0.1% Triton X-100, and incubated for 30 min on ice. At the end, the samples were spun at 100,000 × *g* for 30 min at 4°C, and the remaining supernatant was saved as the particulate fraction. An equal amount of protein from each sample was loaded onto 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membranes were then incubated with the appropriate primary antibodies. Alkaline phosphatase (ALP)-conjugated goat anti–mouse IgG (Roche Applied Science) or ALP-conjugated goat anti–rabbit IgG (Zymed Laboratories, South San Francisco, CA) were used as secondary antibodies, and immunoreactive bands were detected using CDP-STAR solution (Roche Applied Science), according to the manufacturer's instructions. Densitometric analysis was performed using Aida 2.1 Image software (Raytest, Straubenhardt, Germany).

For immunoprecipitation, cell lysate was incubated with the anti-FAK antibody (1 μg/100 μl of lysate) overnight at 4°C. At the end of incubation, 20 μl of protein-A agarose (Santa Cruz Biotechnology) was added and incubated for an additional 3 h at 4°C. The immunoprecipitate was then collected by centrifugation and washed three times with H-buffer, and the final pellet was used for Western blot analysis.

Statistical analysis

Quantitative data are presented as means ± SD of at least three experiments. Statistical analysis to determine significance was performed using paired Student's *t* tests. Differences were considered to be statistically significant at the $p < 0.05$ level.

ACKNOWLEDGMENTS

We thank D.A. Littman, New York University, New York, for providing the mutant mice; G. Tarone, University of Turin, Italy, for the α-β1D integrin antibody; D.A. Sassoon, Myology Group, Université Paris VI/Pierre et Marie Curie, Paris, France, for the α-PW1 antibody; and G. Baier, University of Innsbruck, Innsbruck, Austria, for the

PKCθA/E expression plasmid. We also thank M. Immacolata Senni for her continuous technical support and Carla Ramina for confocal microscopy technical support. This work was supported by the Italian Ministry for University and Research (2006064322_004), by Sapienza University of Rome (C26F07MY89 and C26A08EY99), by Association Françoise contre les Myopathies (AFM) (12668–2007 and 13951–2009), and by Agenzia Spaziale Italiana (ASI).

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