Protein Kinase D Is Implicated in the Reversible Commitment to Differentiation in Primary Cultures of Mouse Keratinocytes

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Although commitment to epidermal differentiation is generally considered to be irreversible, differentiated keratinocytes (KCs) have been shown to maintain a regenerative potential and to reform skin epithelia when placed in a suitable environment. To obtain insights into the mechanism of reinitiation of this proliferative response in differentiated KCs, we examined the reversibility of commitment to Ca\textsuperscript{2+}-induced differentiation. Lowering Ca\textsuperscript{2+} concentration to micromolar levels triggered culture-wide morphological and biochemical changes, as indicated by derepression of cyclin D1, reinitiation of DNA synthesis, and acquisition of basal cell-like characteristics. These responses were inhibited by Goedecke 6976, an inhibitor of protein kinase D (PKD) and PKCs, but not with GF109203X, a general inhibitor of PKCs, suggesting PKD activation by a PKC-independent mechanism. PKD activation followed complex kinetics with a biphasic early transient phosphorylation within the first 6 h, followed by a sustained and progressive phosphorylation beginning at 24 h. The second phase of PKD activation was followed by prolonged ERK1/2 signaling and progression to DNA synthesis in response to the low Ca\textsuperscript{2+} switch. Specific knockdown of PKD-1 by RNA interference or expression of a dominant negative form of PKD-1 did not have a significant effect on normal KC proliferation and differentiation but did inhibit Ca\textsuperscript{2+}-mediated reinitiation of proliferation and reversion in differentiated cultures. The present study identifies PKD as a major regulator of a proliferative response in differentiated KCs, probably through sustained activation of the ERK-MAPK pathway, and provides new insights into the process of epidermal regeneration and wound healing.

Terminal differentiation defines cells that permanently exit the cell cycle in the process of acquiring specialized function. Epidermis is a continuously renewing stratified epithelium, which forms a protective barrier from the environment. In epidermis, proliferating KCs reside in the basal layer. As cells exit the proliferative compartment and begin their migration toward the skin surface, they withdraw from the cell cycle and commit to terminal differentiation in the spinous layer before completing their differentiation program in granular and cornified layers and sloughing from the skin surface (1). Under normal steady state conditions, proliferation is strictly restricted to the basal layer, although aberrant suprabasal proliferation is observed during wound healing and in some diseased states when normal epidermal proliferation and differentiation are perturbed (2, 3).

Regulation of epidermal cell growth and terminal differentiation has been extensively studied in primary cultures of mouse KCs through modulation of extracellular Ca\textsuperscript{2+} levels (4, 5). Cultivation of primary KCs in low Ca\textsuperscript{2+} conditions (0.05 mm) promotes expansion of a proliferative population of undifferentiated, basal-like cells. Raising extracellular Ca\textsuperscript{2+} levels (>1 mm; high Ca\textsuperscript{2+} conditions) in confluence cultures triggers culture-wide differentiation in a manner that closely resembles the in vivo event, including cell cycle withdrawal, cytoskeletal changes, stratification, and cornification (4). Growth arrest and morphological changes in these cultures are accompanied by induction of differentiation-related genes, such as keratins 1 and 10, involucrin (INV), transglutaminases, loricrin, and filaggrin (6). Although confluence in low Ca\textsuperscript{2+} conditions triggers withdrawal from the cell cycle and induces expression of early markers of epidermal differentiation, elevated extracellular Ca\textsuperscript{2+} is required for irreversible cell cycle arrest, stratification, and sustained up-regulation and stabilization of terminal differentiation markers and intercellular adhesion molecules (5, 7). Like other postmitotic terminally differentiated cells, however, irreversible growth arrest in terminally differentiated KCs can be overcome by suppression of cyclin-dependent kinase inhibitors, such as p21 (8, 9). Thus, theoretically, as long as KCs maintain the machinery needed for cell replication (i.e. until they lose their nuclei and form the cornified layers), they may be induced to re-enter the cell cycle and resume a proliferative program.

We have recently shown that when differentiated cultures of mouse KCs maintained for 3 days in high Ca\textsuperscript{2+} conditions were transplanted onto suitable sites in vivo, they not only re-entered MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; GF1, GF109203X; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; CaR, calcium-sensing receptor; shRNA, short hairpin RNA; BrdUrd, bromodeoxyuridine; WT, wild type; KD, kinase-dead; TPA, phorbol-12-myristate-13-acetate.

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the cell cycle but reformed a self-renewing, hair-bearing skin (10). Genetic labeling and lineage tracing in combination with an INV-driven Cre/lox reporter system confirmed the formation of skin epithelia by KCs that once expressed INV, a well characterized marker of epidermal differentiation (10, 11). This study suggested that signals from the microenvironment induced differentiated KCs to resume a less differentiated phenotype and to reinitiate a proliferative program. However, the nature of these signals or the underlying mechanism remains unknown. To enable such a study, we have transitioned from an in vitro-in vivo model to an all culture model.

In the present study, we first examined whether differentiated cultures of epidermis could be induced to reinitiate proliferation in culture, and then we used this model to delineate the mechanism(s) by which differentiated KCs resume a proliferative response. Using primary cultures of mouse KCs, we characterized the reversibility of commitment to differentiation in response to fluctuation of extracellular Ca²⁺ levels. Surprisingly, we found that when confluent cultures of normal mouse KCs maintained in media containing 1.2 mM Ca²⁺ were exposed to 1.2 mM Ca²⁺ at the time of the low Ca²⁺ switch. Inhibitors were added every other day when cultures were subconfluent, whereas differentiating cultures were reverted by replacing the media containing 1.2 mM Ca²⁺ with the same media containing 0.05 mM Ca²⁺. A significant number of KCs re-entered the cell cycle and reverted to a proliferative basal-like phenotype. We showed that this low Ca²⁺ switch in differentiated cultures of KCs induces a mitogenic response through PKD-dependent sustained activation of ERK-MAPK signaling.

EXPERIMENTAL PROCEDURES

Cell Culture—Epidermal cells were isolated from 1–2-day-old B6.cg-KitW-sh/HiNirJaeBsmj mice (Jackson Laboratories, Bar Harbor, ME) in accordance with institutional guidelines set forth by the State University of New York and plated as described previously (12). KCs were grown to confluence in KC serum-free media (Invitrogen) containing 0.05 mM Ca²⁺. Confluent cultures were exposed to 1.2 mM Ca²⁺ for at least 3 days to induce epidermal differentiation (high Ca²⁺ switch). Differentiated cultures were reverted by replacing the media containing 1.2 mM Ca²⁺ with the same media containing 0.05 mM Ca²⁺ (low Ca²⁺ switch). Proliferative KCs were treated with a selection of chemical inhibitors of signaling pathways 3 days after seeding, when cultures were subconfluent, whereas differentiated cultures were treated at the time of the low Ca²⁺ switch. In some experiments, inhibitors were added 1 h prior to the low Ca²⁺ switch. Inhibitors were added every other day when medium was changed.

Chemicals and Antibodies—TPA, rapamycin, Go6976, U0126, SB203580, LY294002, trypsin/EDTA, and 2 µg/ml PDGF were from LC laboratories (Woburn, MA). Y27632 was from Ascent Scientific (Princeton, NJ). Antibodies against p63 (SC-8431), PKD (SC-935), ERK1/2 (SC-135900), and β-actin (SC-1615) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); loricrin (PRB-145P) and involucrin (PRB-145P) and involucrin (PRB-145P) were from Covance (Berkeley, CA); and PKD (CS-2052), PKD-Ser⁷¹⁶ (CS-2051), PKD-Ser⁷³⁴⁻⁷⁴⁸ (CS-2054), phospho-ERK1/2 (CS-9101), PKCa (CS-2056), and phospho-PKCα (CS-9375) were from Cell Signaling Technologies (Danvers, MA). Antibody against PKD-Ser⁷³⁴⁻⁷⁴⁸ was purchased from Abcam (Cambridge, MA), and anti-cyclin D1 antibody was from BD Biosciences. Assay for DNA Synthesis—For the BrdUrd labeling index, cultures were pulsed with 10 µM BrdUrd for 6 h before immunostaining with anti-BrdUrd antibody (1:500; BD PharMingen), as described previously (10). The fraction of BrdUrd-positive cells in 1000 nuclei was determined by fluorescence microscopy. For assessing DNA synthesis by thymidine incorporation, cultures were grown in 24-well plates, pulsed with 2.5 µCi of [methyl-³H]thymidine/well for 16 h, and harvested using a semiautomatic harvester (Skatron Instruments, Lier, Norway). Thymidine uptake was measured with a scintillation counter and normalized for cell number.

Western Blot Analysis—Cells were lysed in lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM NaF, 1 mM Na3VO4, 5 mM sodium pyrophosphate) plus protease inhibitor mixture (Sigma). Samples (30 µg) were separated on 10% SDS-PAGE, transferred to nitrocellulose, and blocked using 5% nonfat dried milk in phosphate-buffered saline for 1 h. Membranes were incubated overnight at 4 °C with primary antibodies. Immunoreactive bands were detected using horseradish peroxidase-conjugated secondary antibodies, followed by a chemiluminescence detection system (Pierce ECL, Thermo Fisher Scientific). For developing, HyBlot CL (Denville Scientific, Metuchen, NJ) and Amersham Biosciences Hyperfilm were used. To detect multiple proteins, membranes were stripped and then reprobed. Quantification of immunoblots was done using the UN-SCAN-IT software (Silk Scientific Inc., Orem, UT). The total ERK activity was evaluated by adding the signals for phospho-ERK1 and phospho-ERK2 and then dividing by the signals of ERK1 and ERK2. The PKD activity was evaluated by dividing the signal from PKD-Ser⁷¹⁶ by the signal from PKD (SC-935).

mRNA Expression Analysis—Relative levels of mRNA expression in RNA isolated from epidermal cultures were determined by quantitative real-time PCR using a 7300 real time system (Applied Biosystems, Foster City, CA). Total RNA (1 µg) was first reverse transcribed, and the PCR reaction was run with SYBER Green Taq polymerase (Quantitect kit, Qiagen Sciences, Valencia, CA)) for 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. All samples were run in triplicate, and non-template controls were included in each run. The RNA levels of the target genes were normalized against Pgk-1 transcript levels, and the comparative C(T) (2⁻ΔΔC(T)) method was used for calculating relative cytokine mRNA expression. The PCR efficiencies, as determined by assaying serial dilutions of RNA, were approximately equal for the target genes and the housekeeping genes.

Retroviral Vectors and Transduction—Retroviral vectors encoding short hairpin RNA (shRNA) were constructed by subcloning a double-stranded hairpin oligonucleotide into pSUPERRetro.puro vector (13). The following two oligonucleotides, including 5'-ATGCTGTGGGGCTGTGTAC-3' (shRNA-1) and 5'-GAAGGAGATTTCATGAA-3' (shRNA-2), were designed to target murine PKD-1. The oligonucleotide 5'-GCGATGGAGTGTACGAGAGAAGAA-3' (C-shRNA) was used as a control. cDNAs encoding wild type PKD (PKDwt) and kinase-dead PKD (PKD(K612W)) fused at the C terminus to GFP (kindly provided by Dr. Angelika Hausser (University of Stuttgart, Stuttgart, Germany)) have been described previously.
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(14). The PKD cDNAs were subcloned into an LZRS-based retroviral vector.

High titer vesicular stomatitis virus G protein-pseudotyped recombinant retroviruses were generated as described previously (12). Mouse KCs were transduced at a multiplicity of infection of 2 at 3 or 4 days postseeding, when cultures were highly proliferative. Under these conditions, between 60 and 80% of cells were routinely transduced (12). Cultures transduced with viruses encoding shRNA were selected in 2 μg/ml puromycin starting at 36 h post-transduction.

To genetically label proliferating KCs in differentiated cultures, recombinant retroviruses encoding GFP (LZRS-GFP) were used to transduce differentiated epidermal cultures for 2 consecutive days starving at 36 h after the high Ca²⁺ switch. As controls, KCs grown in 0.05 mM Ca²⁺ were transduced at day 3 postseeding, grown to confluence, and induced to differentiate as described above. Both cultures were subsequently exposed to low Ca²⁺ conditions for an additional 7 days. The percentage of GFP-expressing KCs was determined by flow cytometry analysis of cultures either at 48 h post-transduction or at 7 days following the low Ca²⁺ switch.

Statistical Analyses—Differences among means were evaluated by one-way analysis of variance, and Tukey’s honestly significant difference post hoc comparison using SPSS 17 software (SPSS, Chicago, IL). Only values with p < 0.05 were accepted as significant.

RESULTS

Reversible Commitment to Epidermal Differentiation in Primary Cultures of Mouse KCs—Commitment to Ca²⁺⁺-induced differentiation in mouse epidermal cultures is generally thought to induce irreversible growth arrest (4, 5, 7). However, under some conditions, this process is reversed. For example, when differentiated KCs are transplanted onto a wound bed, they overcome differentiation-induced growth arrest and reform a multilinage tissue, or in culture the loss of p21 in differentiated KCs has been shown to allow reversal of differentiation upon a subsequent exposure to low Ca²⁺ containing media (8, 10). Because extracellular Ca²⁺ is a major regulator of KC differentiation, we investigated the response of differentiated KCs to a subsequent exposure to low Ca²⁺ conditions (0.05 mM) over a period of time. In order to restrict our analysis to KCs, epidermal cells were isolated from c-Kitw/sh mice, which are deficient in melanocytes (15). Confluent cultures of mouse KCs grown in low Ca²⁺ conditions were induced to differentiate by switching to high Ca²⁺ (i.e. exposure to 1.2 mM Ca²⁺ for 3 days) and subsequently switched to low Ca²⁺ conditions (i.e. 0.05 mM) for an additional 7 days (designated here as reverted cultures), as depicted in Fig. 1A. Proliferation rates and morphology were assessed at various time points. As expected, exposure of mouse KCs to high Ca²⁺ for 3 days (D3) induced striking changes in KC morphology from an angular to a flat and polygonal shape (Fig. 1B) and resulted in growth arrest of more than 97% of KCs (Fig. 1C). Interestingly, subsequent exposure of differentiated cultures to low Ca²⁺ conditions provoked heterogeneous morphological changes between 2 and 4 days with about 30% of cells detaching from culture, whereas the majority of cells underwent a morphological reversion of the differentiated phenotype toward a proliferative basal-like phenotype, suggesting reinitiation of the proliferative program (Fig. 1B, R2 and R4). Within a week (Fig. 1B, R7), the entire culture was repopulated by cells displaying basal cell-like morphology. Analysis of DNA synthesis by either BrdUrd labeling or [³H]thymidine incorporation indicated a gradual but significant increase in proliferation rate as early as 2 days after the low Ca²⁺ switch (R2). By day 7 (R7), the rate of proliferation was comparable with or even higher than that of proliferating cultures (P) before the induction of differentiation (Fig. 1C).

To determine if the duration of time spent in high Ca²⁺ altered the subsequent cellular response when switched to low Ca²⁺, cultures were maintained for various periods of time in high Ca²⁺ conditions before the subsequent low Ca²⁺ switch. Analysis of DNA synthesis at various times after Ca²⁺⁺-induced differentiation indicated a maximal growth arrest by 3 days (supplemental Fig. S1). When switched to low Ca²⁺ conditions, cultures maintained as long as 5 days in high Ca²⁺ conditions retained their ability to reinitiate DNA synthesis and to resume a proliferative phenotype although with slightly less efficiency (supplemental Fig. S1). These data indicated that although confluence and increased extracellular Ca²⁺ concentration induced striking morphological changes and culture-wide growth arrest within 3 days, the majority of KCs did not irreversibly commit to differentiation.

As expected, when KCs were exposed to high Ca²⁺ conditions, there was up-regulation of markers of epidermal differentiation, including keratin 10, INV, filaggrin, and loricrin, and down-regulation of p63, a KC proliferation marker (16) (Fig. 1, D and E). This pattern was generally reversed within 4 days following the low Ca²⁺ switch, when the morphological reversion from differentiated to replicative phenotype was evident. A notable exception was INV expression (at both transcript and protein levels), which increased upon Ca²⁺⁺-induced differentiation but, unlike other differentiation markers, remained elevated even in cultures when almost all cells displayed basal-like morphology and were highly proliferative (Fig. 1, D–E, R7). Reverted KCs maintained in low Ca²⁺ conditions for 7 days could be induced to differentiate when once again exposed to high Ca²⁺ conditions, as shown by the reappearance of the large, flat cells (Fig. 1B, R7D3), up-regulation of loricrin, and down-regulation of p63 (supplemental Fig. S2). Thus, although KCs in reverted cultures were not transcriptionally equivalent to the original proliferating KCs, their ability to respond to Ca²⁺⁺-induced differentiation was not altered.

Differentiation-resistant KCs Do Not Contribute to Repopulation of Differentiated Cultures—Although more than 97% of KCs exposed to high Ca²⁺ conditions exited the cell cycle (Fig. 1C) and expressed INV (10), a small population of KCs (2–3%) resisted Ca²⁺⁺-induced growth arrest and therefore could have potentially repopulated the culture upon the low Ca²⁺ switch. To test this possibility, we took advantage of a well established property of retroviruses to transduce only replicating cells to selectively label proliferating cells in differentiated cultures.
Differentiated cultures were transduced with a retroviral vector encoding GFP at a multiplicity of infection of 2 for 2 consecutive days starting 36 h after switching to high Ca\textsuperscript{2+}/H11001. Subsequent GFP expression was analyzed by flow cytometry either before or 7 days after switching to low Ca\textsuperscript{2+}/H11001. As a control to demonstrate high efficiency and persistent labeling of replicating cells, cultures of proliferating KCs were transduced before the induction of differentiation and the subsequent low Ca\textsuperscript{2+}/H11001 switch. Under these conditions, 91% of KCs in proliferating cultures (\textit{P}-GFP in Fig. 2) were labeled with GFP, whereas less than 3% of cells in differentiated cultures were labeled (\textit{D}-GFP). These data confirmed the presence of a small population of cells that continued to proliferate following Ca\textsuperscript{2+}-induced differentiation. Analysis of transduced cultures at 7 days following the low Ca\textsuperscript{2+} switch, however, indicated no change in the proportion of GFP-labeled cells in either cultures (Fig. 2A). The proportion of GFP\textsuperscript{+} KCs in R7 cultures remained below 3%, indicating a lack of a significant expansion of labeled differentiation-resistant cells following exposure to low Ca\textsuperscript{2+} conditions (\textit{D}-GFP-R7). These data indicated that a small population of proliferating cells could not account for the culture-wide proliferative response to the low Ca\textsuperscript{2+} switch.

The lack of contribution from differentiation-resistant KCs suggested reinitiation of proliferation in differentiated KCs. Cyclin D1 is a key regulator of cell proliferation, which acts as a sensor linking extracellular signaling to the cell cycle machinery (18). To examine the timing of cell cycle re-entry in differentiated cultures, cyclin D1 levels in response to Ca\textsuperscript{2+} fluctuations were measured.
Inhibition of Reinitiation of Proliferative Response in Differentiated Cultures by Go6976—PKD is a multifunctional signaling enzyme that is activated in vivo through a phosphorylation-dependent mechanism (22). Activation of PKD is mainly mediated by PKC-dependent phosphorylation of Ser<sup>744</sup> and Ser<sup>748</sup> in the activation loop, followed by autophosphorylation at Ser<sup>916</sup> in its C terminus, although PKD can also be activated independent of PKCs (23). To investigate PKD activity during reinitiation of proliferation, total and phosphorylated levels of PKD in response to Ca<sup>2+</sup> fluctuations in cultures were analyzed. Previous studies have reported down-modulation of PKD during mouse KC differentiation (24, 25). Surprisingly, Western blot analysis using two different anti-PKD antibodies recognizing a C-terminal epitope (CS-2052 and SC-935) indicated a significant up-regulation of total PKD levels in response to Ca<sup>2+</sup>-induced differentiation (Fig. 3D and supplemental Fig. S3). Immunostaining of mouse skin with SC-935 antibody confirmed higher PKD levels in suprabasal layers of epidermis consistent with cultured KCs. The specificity of SC-935 antibody was further verified by immunostaining of a skin graft expressing PKD-1 as a fusion protein with green fluorescent protein (PKD-GFP) (supplemental Fig. S3).

The higher levels of PKD in differentiated KCs persisted during the subsequent low Ca<sup>2+</sup> switch for at least 7 days (Fig. 3D). Despite a more than 6-fold increase in PKD levels, there was only a slight increase in PKD phosphorylation in response to Ca<sup>2+</sup>-induced differentiation, suggesting that PKD remains predominantly inactive in differentiated KCs (Fig. 3D). Exposure of differentiated cultures to low Ca<sup>2+</sup> conditions, however, induced a significant increase in autophosphorylation of PKD, which followed biphasic kinetics. After an initial drop in PKD phosphorylation, a 16-fold increase in PKD phosphorylation was observed by 3 h following the low Ca<sup>2+</sup> switch. These levels returned to basal levels by 12 h and increased again after 24 h. The magnitude of the latter phosphorylation increased progressively thereafter for at least 7 days, when cultures were highly proliferative (Figs. 3D and 4A). Further analysis of PKD autophosphorylation during a shorter time course indicated another wave of transient phosphorylation within the first 30 min after the low Ca<sup>2+</sup> switch (Fig. 3E), consistent with a recent report showing rapid activation of PKD in response to EDTA-mediated Ca<sup>2+</sup> depletion in HeLa Cells (26). Therefore, the low
The Ca\(^{2+}\) switch induced a biphasic transient PKD activation within the first 6 h, followed by a progressive and sustained activation beginning at 24 h. Contrary to PKD, PKC levels and activity remained unaffected during the first 2 days following the low Ca\(^{2+}\) switch and gradually declined to predifferentiation levels as KCs reverted to a more proliferative phenotype (Fig. 3D, lanes 4d and 7d).

Because PKD phosphorylation is mainly controlled by PKCs, the lack of inhibition of reinitiation of the proliferative response by GF1, which has been shown to inhibit PKC-dependent activation of PKD (22, 27, 28), was surprising (Fig. 3). The potency of GF1 to inhibit PKC-dependent PKD activation in mouse keratinocytes was confirmed by its ability to block PKD phosphorylation induced by phorbol esters (supplemental Fig. S4). To gain insight into the mechanism of PKD phosphorylation in response to the low Ca\(^{2+}\) switch, the phosphorylation state of two Ser residues in the kinase domain activation loop was determined. Although transphosphorylation by PKCs is a major mechanism targeting Ser\(^{744}\), recent studies have shown autophosphorylation as a predominant mechanism for Ser\(^{748}\) (29). Therefore, we used site-specific antibodies that detect the phosphorylation state of either Ser\(^{744}\) (CS-2501) or Ser\(^{748}\) (Abcam-17945) in the activation loop of PKD. As shown in Fig. 4A, although Ser\(^{748}\) in the activation loop of PKD was phosphorylated with the same kinetics as Ser\(^{916}\), we did not detect a prominent phosphorylation of Ser\(^{744}\) in response to the low Ca\(^{2+}\) switch (Fig. 4A). Stimulation of KCs with TPA (100 nM), a potent activator of the PKC-dependent PKD signaling pathway (22, 23), led to comparable levels of PKD phosphorylation on all three Ser residues (Fig. 4B), arguing against the differential affinity of these site-specific antibodies. The low signal observed for Ser\(^{744}\) is probably due to cross-reactivity of CS-2501 antibody with Ser\(^{748}\) that was included in the peptide used to

**FIGURE 3.** Involvement of PKD in reinitiation of a proliferative program in differentiated cultures. A, morphological changes in differentiated cultures treated with either DMSO (control), 1 \(\mu M\) Go6976, or 1 \(\mu M\) GF1 were analyzed by phase-contrast microscopy in cultures reverted for 5 days. Bar, 50 \(\mu M\). B and C, relative DNA synthesis measured by \(^{[3H]}\)thymidine incorporation in proliferative (P) or reverted cultures (RS) treated at the time of the low Ca\(^{2+}\) switch with increasing concentrations of either Go6976 (B) or GF1 (C). Similar results were obtained when cultures were pretreated for 1 h prior to the low Ca\(^{2+}\) switch. Bars, mean \(\pm\) S.D. (error bars) of three independent experiments, each in triplicate. *, \(p < 0.01\) when compared with untreated cultures. D, the kinetics of PKD activity in response to the low Ca\(^{2+}\) switch were analyzed in cell lysates prepared from proliferating or differentiated KCs exposed to low Ca\(^{2+}\) conditions for time indicated at the top by Western blotting using antibody against phosphorylated PKD-1 (pPKD) (Ser\(^{916}\)) or phospho-PKC\(\alpha\) (pPKC\(\alpha\)). The levels of total PKD-1 (using antibody CS-2052) or PKC\(\alpha\) were determined. Actin was used as a loading control. E, a shorter time course of PKD phosphorylation in response to the low Ca\(^{2+}\) switch showing a rapid and transient PKD auto-phosphorylation. Similar results were obtained in three independent experiments.
generate this antibody (29). The ineffectiveness of GF1 to inhibit DNA synthesis in reverted cultures and a lack of phosphorylation at Ser\(^{744}\) suggested PKC-independent activation of PKD during reinitiation of a proliferative response in differentiated cultures of KCs exposed to low Ca\(^{2+}\) conditions.

**PKD Is Required for Reinitiation of Proliferative Response in Differentiated Cultures of KCs**—To investigate, in a more direct fashion, the involvement of PKD in reinitiation of the proliferative response in differentiated cultures, RNA interference was used to deplete PKD1 from KCs. Primary cultures of KCs grown in low Ca\(^{2+}\) conditions were transduced with retroviruses encoding either an shRNA directed to PKD1 or a control shRNA. Following puromycin selection, transduced KCs were grown to confluence and induced to differentiate for 3 days. The knockdown efficiency of PKD1 in differentiated KCs using two different PKD1-directed shRNAs was found to be more than 80% (Fig. 5A). Despite depletion of PKD1, cultured KCs reached confluence at approximately the same time as transduced controls, indicating that depletion of PKD1 has no significant effects on KC growth under normal culture conditions (Fig. 5B). This was consistent with a lack of effect for Go6976 on KC proliferation (Fig. 3B). When exposed to low Ca\(^{2+}\) conditions, however, differentiated KCs expressing PKD-directed shRNAs failed to undergo morphological reversion (data not shown) or to reinitiate DNA synthesis (Fig. 5B). The observed effects were not attributed to RNA interference off-target effects because knockdown with two different PKD-directed shRNA gave similar results. Furthermore, differentiated KCs expressing a control shRNA responded to the low Ca\(^{2+}\) switch by reinitiating a proliferative program similar to that observed for non-transduced KCs (Fig. 5B).

Although there are three highly related PKD isoforms (PKD1, -2, and -3) in mammals that can be activated with the same stimuli, each member can have a distinct and non-redundant function (30). Despite the expression of all three PKD isoforms in mouse keratinocytes (supplemental Fig. S5) (24, 31), inhibition of reinitiation of proliferation in response to the low Ca\(^{2+}\) switch by PKD1-specific shRNA, which had no effect on PKD2 and -3 transcript levels (supplemental Fig. S5), indicated a specific and non-redundant role for PKD1.

To confirm that the effects of PKD1 depletion were specific to the loss of PKD activity, the ability of a dominant negative form of PKD (PKDKD) to block this process was assessed. The mutation in Lys\(^{612}\) in the catalytic domain of PKD has been shown to render this enzyme kinase-deficient and to act in a dominant negative fashion (14). Retroviral vectors encoding the wild type (PKD\(_{WT}\)-GFP) or the kinase-deficient (PKD\(_{KD}\)-GFP) variant of PKD-1 as a fusion with GFP were generated and used to transduce growing cultures of KCs. Analysis of DNA synthesis in proliferative cultures overexpressing PKD\(_{KD}\) showed comparable rates of proliferation with the control transduced cultures (Fig. 5D). Upon differentiation and subsequent switch to low Ca\(^{2+}\) conditions, however, KCs overexpressing a dominant negative form of PKD failed to undergo morphological reversion and showed a significant inhibition of reinitiation of DNA synthesis (Fig. 5, C and D). Interestingly, non-transduced cells in the same culture resumed a proliferative phenotype, indicating cell-autonomous effects of PKD inhibition (Fig. 5C, arrows in the insets). Thus, although PKD activity is not required for normal growth and differentiation of cultured mouse KCs, it plays an essential role in reinitiation of a proliferative response in differentiated KCs.

**PKD-mediated Activation of ERK Signaling in Response to the Low Ca\(^{2+}\) Switch**—The above results established a critical role for PKD in mediating a mitogenic response to the low Ca\(^{2+}\) switch. PKD has been shown to potentiate the mitogenic responses of Swiss 3T3 cells to G protein-coupled receptors by increasing the duration of ERK1/2 activation (32). The ERK-MAPK pathway is conserved in mammals and has been shown to be critical for epidermal homeostasis (33). ERK proteins are directly activated by phosphorylation by the dual specificity ERK kinase (or MEK). Our initial screen indicated that pharmacological inhibition of the ERK1/2 signaling cascade by U0126, a potent and specific inhibitor of MEK1/2 (34), at a concentration of 10 \(\mu M\) inhibited DNA synthesis in both proliferating and reverted cultures. These results are consistent with the critical role of this pathway in KC proliferation (33). Interestingly, however, a dose-response curve showed that at concentrations below 2 \(\mu M\), U0126 significantly attenuated DNA synthesis and morphological reversion of differentiated cultures stimulated by the low Ca\(^{2+}\) switch, while having little effect on DNA synthesis in proliferative cultures (Fig. 6, A and B), implying involvement of the MEK/ERK cascade in reinitiation of proliferation. The specific biological outcome of ERK activation (e.g. proliferation or differentiation) is dependent
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**FIGURE 5.** PKD plays a critical role in the reinitiation of a proliferative response in differentiated KCs. A, Western blot analysis of KCs showing efficient knockdown of PKD by RNA interference. Proliferative cultures of KCs were transduced with retroviral vectors encoding two different shRNAs against PKD1 (shRNA-1 or shRNA-2) or a control shRNA (C-shRNA). Transduced cells were induced to differentiate for 3 days and analyzed by immunoblotting for PKD1 and actin. B, the graph shows a significant inhibition of DNA synthesis as measured by [3H]thymidine incorporation in reverted (RS, gray bars) but not in proliferating (P, black bars) cultures expressing PKD1-specific shRNA. The bars represent the mean ± S.D. of four independent experiments, each performed in triplicates. *, p < 0.0001 comparing reverted cultures of PKD knockdown groups with controls. C, phase-contrast and GFP fluorescent images of cultures overexpressing either the wild type (PKDWT-GFP) or dominant negative (PKDKD-GFP) forms of PKD1 as a fusion with GFP at 5 days after the low Ca²⁺ switch. The arrow in the insets in C shows the expected morphological change in non-transduced KCs in the same culture. D, the graph shows the effect of overexpression of PKDWT or PKDKD on DNA synthesis in proliferating (P; black bars) or in reverted cultures (RS; gray bars). Results are expressed as means ± S.D. of three independent experiments, each performed in triplicates. *, p < 0.001 when comparing RS-PKDKD with RS-GFP; #, p = 0.042 when comparing RS-PKDWT with RS-GFP.

on the intensity and duration of its activity (35). Persistent ERK activation in quiescent fibroblasts has been shown to be associated with reinitiation of DNA synthesis, whereas transient ERK activation is not sufficient to induce proliferation (28, 36). To assess ERK activity in differentiated KCs in response to the low Ca²⁺ switch, the levels of active, phosphorylated ERK1/2 were analyzed at various time points. As shown in Fig. 6C, the low Ca²⁺ switch induced a biphasic pattern of ERK phosphorylation consistent with that of PKD. The low Ca²⁺ switch induced a rapid transient activation of ERK1/2 within the first 30 min, followed by a gradual, progressive, and sustained activation consistent with the mitogenic effects of ERK activation. The activation kinetics of ERK1 and ERK2, however, were distinct. Although both ERK1 and ERK2 were phosphorylated to the same extent during the transient ERK activation, ERK1 phosphorylation predominated during the sustained period of ERK activation (Fig. 6, C and D). This is consistent with differential regulation and the role of ERK1 and ERK2 in mouse epidermis (37).

To determine if ERK activation is a consequence of Ca²⁺-mediated PKD signaling, the levels of phosphorylated ERK1/2 were analyzed in differentiated cultures expressing either GFP as a control, PKDWT-GFP, or PKDKD-GFP following a 48-h exposure to low Ca²⁺ conditions. The level of ERK phosphorylation in cells was enhanced in cells expressing the wild type PKD and suppressed in those expressing the dominant negative forms of PKD (Fig. 6D) and directly correlated with the levels of DNA synthesis in these cultures (Fig. 5D). KCs depleted of PKD by RNA interference showed a significant suppression in total ERK levels and therefore could not be used for this analysis (data not shown). Collectively, these data suggest that reduction of extracellular Ca²⁺ transduces a mitogenic response in differentiated KCs through PKD-dependent sustained activation of ERK signaling.

**DISCUSSION**

Calcium is a major regulator of KC differentiation (6, 38). Using primary cultures of mouse KCs, we have shown here that Ca²⁺-induced epidermal differentiation is a reversible process and that this reversion is regulated, at least partially, through PKD-mediated activation of the MEK/ERK cascade. Under conditions used here to induce differentiation, more than 97% of KCs withdrew from the cell cycle and expressed markers of epidermal differentiation consistent with previous reports (4, 5, 10). Subsequent switch to low Ca²⁺ conditions, however, induced a proliferative program, as indicated by up-regulation of cyclin D1, reinitiation of DNA synthesis, and morphological reversion from differentiated to proliferative phenotype. Using pharmacological and genetic approaches to inhibit PKD, we showed that although PKD was not essential for normal proliferation and differentiation of cultured KCs, it played a critical role in this reversion from a differentiated to a proliferative phenotype. This is the first mechanistic approach to delineate the mechanism of reversion of commitment to epidermal differentiation in postmitotic, differentiated KCs.

Although initially, we suspected that a small population of Ca²⁺-resistant KCs was responsible for the robust reversion, our data did not support this hypothesis. Retroviral labeling of the Ca²⁺-resistant, proliferating cells in differentiated cultures did not show relative expansion of this labeled population in reverted cultures (Fig. 2A). If these labeled cells had been responsible for the robust reversion, we would have seen a significant increase in the percentage of labeled KCs after the low Ca²⁺ switch. Another source for reversion that we considered was from quiescent stem cells in the epidermal cultures. However, although stem cells are quiescent in vivo, they are highly proliferative in culture (39). In culture systems, quiescence is generally induced by confluence and is reversed upon subculture when contact inhibition is removed.
Our failed attempts to subculture cells from differentiated cultures under conditions where confluent cultures grown in low Ca\(^{2+}\)/H11001 conditions were readily subcultured (supplemental Fig. S6) argued against the presence of quiescent KCs in differentiated cultures. Thus, a more plausible explanation for repopulation of differentiated cultures with proliferating cells is reinitiation of a proliferative response in a large fraction of growth-arrested differentiated cells.

The divergent response of KCs to the low Ca\(^{2+}\) switch with detachment and loss of one subpopulation and stimulated growth of another resembled that of proliferative epidermal cultures following treatment with phorbol esters. This response to phorbol esters is attributed to differences in maturation potential of basal KCs in vivo and in vitro (41). We suggest that the responses of KCs to the high Ca\(^{2+}\)/H11001 switch can be explained in a similar manner. When proliferating cultures of KCs were switched to elevated Ca\(^{2+}\) levels, almost all cells initiated differentiation, as indicated by growth arrest and expression of early markers of differentiation (i.e. expressed INV), but not all of these cells completed the differentiation program (i.e. expressed loricrin) (10). Using a similar approach, one can argue that KCs responded differently at the time of the low Ca\(^{2+}\) switch, depending on their state of differentiation, with cells in the later stages of terminal differentiation desquamating whereas those in the earlier stages of differentiation responded by re-entering the cell cycle. This is consistent with our in vivo studies in which analysis of early events during skin regeneration by differentiated KCs showed a contribution from INV-expressing KCs but not from cells in the later stages of differentiation (10). Therefore, commitment to differentiation may be reversed during the earlier stages of differentiation (i.e. in spinous layers) before KCs start to express markers of granular layers, as speculated previously (42). It is noteworthy that, although the expression of several markers of epidermal proliferation and differentiation like p63, cyclin D1, keratin 10, loricrin, and filagrin returned to predifferentiation levels, INV and PKD levels remained elevated in reverted cultures. Although the reason for this continued expression of INV and PKD is currently unclear, it is another indication that the source of proliferating cells in

**FIGURE 6. Involvement of ERK-MAPK pathway in reinitiation of the proliferative response in differentiated KCs.**
A, relative DNA synthesis was measured by \(^{3}H\)thymidine incorporation in proliferative (P; black bars) or reverted cultures (R5; gray bars) treated with increasing concentrations of U0126 as described under “Experimental Procedures.” Values shown represent means ± S.D. of the results of three separate experiments, each performed in duplicate. B, phase-contrast images showing the morphology of R5 cultures treated at the time of the low Ca\(^{2+}\) switch with 4 \(\mu\)M U0126 or DMSO (control). C, time course of ERK1/2 phosphorylation in response to the low Ca\(^{2+}\) switch. Protein lysates of differentiated KCs at the indicated time points following exposure to 0.05 mM Ca\(^{2+}\) were analyzed by immunoblotting for phosphorylated ERK1/2, total ERK1/2, and actin. The relative levels of total activated ERK are indicated at the bottom. D, the ratio of phospho-ERK1 to phospho-ERK2 was determined by densitometric scanning of the corresponding bands. The results shown are the mean ± S.D. from three blots. E, differentiated KCs expressing wild type (PKD\(_{WT}\)-GFP) or dominant negative (PKD\(_{DN}\)-GFP) forms of PKD were exposed to low Ca\(^{2+}\) conditions for 48 h, followed by Western blot analysis for the indicated antibodies. PKD-GFP shows comparable expression of the exogenous PKD. The relative levels of total activated ERK are indicated at the bottom. This experiment was repeated twice, yielding a similar pattern.
Keratinocyte Dedifferentiation

reverted cultures are KCs that had already committed to
differentiation.

Although a proproliferative and antidifferentiative role for
PKD in epidermis has been suggested (21), the role of PKD in
epidermis remains ill defined. Using both biochemical and
genetic approaches to inhibit PKD activity, we showed a critical
role for PKD1 in reinitiation of a proliferative program in dif-
ferentiated cultures but not in replication and differentiation of
proliferative KCs. We showed that PKD was predominantly
expressed in the differentiated mouse KCs both in vitro and in vivo. Despite its higher expression levels, PKD remained in a
state of low activity in differentiated KCs (Fig. 3D). Kinetic anal-
ysis of PKD activation following a low Ca²⁺ switch showed a
biphasic pattern of autophosphorylation of PKD during the first
few h, followed by a progressive and persistent activation start-
ing at 24 h as cells gradually acquired a basal cell phenotype.
Only following a subsequent Ca²⁺-induced differentiation was
PKD activity down-regulated (supplemental Fig. S2). PKD has
been shown to mediate a diverse array of biological activities in
various cell types (43). The observed kinetics of PKD phosphor-
ylation may reflect either the response of different subpopula-
tions of KCs or the dynamic changes in temporal activation of
different signaling pathways regulating PKD activity. Although
the initial transient activation of PKD in response to the low
Ca²⁺ switch is probably driven by receptor-mediated signaling,
the sustained and progressive activation of PKD after 24 h may
reflect the higher base-line activity of PKD in proliferating KCs
(Figs. 3D and 4A). It is worth noting that, contrary to differen-
tiated KCs, PKD appears to be in a state of high activity in
proliferating KCs, as indicated by a 4-fold higher phosphor-
ylated/total PKD ratio. Clearly, further studies are necessary to
delineate the mechanism of regulation of PKD levels and activ-
ity during normal growth and differentiation of KCs.

Our data indicated that mitogenic responses induced by the
low Ca²⁺ switch in differentiated KCs were mediated through
PKC-independent activation of PKD. Treatment of differenti-
ated KCs with GF1, which potently inhibits PKC-dependent
activation of PKD (22, 27, 28), did not impair the proliferative
response of differentiated KCs to the low Ca²⁺ switch, although
similar treatment effectively inhibited TPA-induced PKD activ-
ation (supplemental Fig. S4) (24). Consistent with this obser-
vation was the low level of phosphorylation of Ser⁷⁴⁴ but
enhanced phosphorylation of Ser⁷⁴⁸ in the activation domain
of PKD (Fig. 4). Although Ser⁷⁴⁴ is a direct target of PKCs, phos-
phorylation of Ser⁷⁴⁸ has been shown to be mediated predom-
inantly by autophosphorylation (29). The mitogenic response to
Gq-coupled receptor agonists in Swiss 3T3 cells has also been
owned to be mediated by sustained PKD activation via a PKC-
-independent pathway (28). Activation of PKD via either PKC-
dependent or -independent pathways appears to be involved in
the release of the autoregulatory pleckstrin homology domain
(23). This could be achieved either by phosphorylation on Ser
or Tyr residues in the regulatory or the catalytic domain of PKD
or by direct interactions between regulatory proteins, such as
the Gβγ subunits and the pleckstrin homology domain or by
proteolytic cleavage (44–48).

Although the mechanism of PKD activation by Ca²⁺ deple-
tion in KCs needs to be explored in depth, a recent study has
shown that EDTA-mediated disruption of cadherins in HeLa
cells leads to NFκB activation and cell survival through a rapid
and transient activation of PKD via the RhoA/ROCK/novel
PKC pathway (26). However, in our model, treatment of differ-
entiated KCs with the ROCK inhibitor Y27632, at doses that
were shown to inhibit EDTA-induced PKD activation (26), did
not impair reinitiation of proliferation but rather resulted in a
dose-dependent enhancement of DNA synthesis in reverted
cultures (supplemental Fig. S7). It is worth noting that despite
the growth-promoting role of NFκB in many cell types, in KC
activation of NFκB inhibits cell cycle progression (49). On the
other hand, disruption of adherence junctions in epidermis has
been shown to stimulate a mitogenic response through activa-
tion of Ras/ERK MAPK signaling (50). Involvement of PKD in
this process has not been studied; however, in other models,
PKD has been shown to directly up-regulate Ras/Raf/MEK/
ERK signaling by phosphorylating Ras-binding protein RIN1
(23). It is noteworthy that the calcium-sensing receptor (CaR), a
GPCR that is activated in response to fluctuations in extracel-
lar Ca²⁺, has been shown to be required for Ca²⁺-induced
activation of E-cadherin signaling and epidermal differentia-
tion (51). Contrary to KCs, elevated extracellular Ca²⁺ levels in
several other cell types induce cell proliferation via the CaR-
EGFR-ERK pathway (52, 53). Rapid and transient phosphor-
lyation of PKD and ERK1/2 during the first 30 min of the low
Ca²⁺ switch is consistent with CaR-mediated regulation of the
MAPK signaling pathway (54). The activation of CaR, howev-
ner, has often been studied in response to elevating levels of
extracellular Ca²⁺, whether CaR is activated in response to deple-
tion of extracellular Ca²⁺ needs to be explored.

In summary, the results presented here demonstrate that
commitment to Ca²⁺-induced differentiation in primary cul-
tures of KCs is a reversible process. Lowering extracellular
Ca²⁺ levels induces PKC-independent activation of PKD result-
ing in prolonged ERK1/2 activity, reinitiation of DNA
synthesis, and a gradual morphological reversion to a basal-like
phenotype. Our data imply a critical role for PKD in conditions
such as wound healing or tumor progression, where the normal
differentiation process may be reversed (55–57).

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