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Differential regulation of α6β4 integrin by PKC isoforms in murine skin keratinocytes☆

Addy Alt,[a] Marina Gartsbein,[a] Motoi Ohba,[b] Toshio Kuroki,[c] and Tamar Tennenbaum[a,*]

[a] *Faculty of Life Sciences, Bar Ilan University, Ramat Gan 52900, Israel*
[b] *Institute of Molecular Oncology, Showa University, Tokyo 142-8555, Japan*
[c] *Gifu University, Gifu 501-1193, Japan*

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Abstract

In mammalian epidermis, α6β4 integrin is expressed exclusively on the basal layer localized to the hemidesmosomes, where it interacts extracellularly with the laminin-5 ligand. During differentiation, loss of α6β4 is associated with keratinocyte detachment from the basement membrane and upward migration. The protein kinase C (PKC) family of isoforms participates in regulation of integrin function and is linked to skin differentiation. Exposure of primary murine keratinocytes to PKC activators specifically downregulates α6β4 expression. Utilizing recombinant adenoviruses, we selectively overexpressed skin PKC isoforms in primary keratinocytes. PKCδ and PKCζ induced downregulation of α6β4 protein expression, leading to reduced keratinocyte attachment to laminin-5 and enhanced gradual detachment from the underlying matrix. In contrast, PKCα upregulated α6β4 protein expression, leading to increased keratinocyte attachment to laminin-5 and to the underlying matrix. Altogether, these results suggest distinct roles for specific PKC isoforms in α6β4 functional regulation during the early stages of skin differentiation.

Keywords: Skin; Keratinocytes; α6β4 integrin; Hemidesmosome; Protein kinase C

Skin consists of stratified squamous epithelium in which cells undergoing growth and differentiation are strictly compartmentalized. In the physiologic state, proliferation is confined to the basal cells, which adhere to the basement membrane. Differentiation is associated with the ability of the basal cells to detach and migrate away from their basement membrane [1,2]. In previous studies, we have shown that suprabasal migration in the early stages of cell differentiation is associated with a selective loss of the α6β4 integrin complex [3]. The α6β4 integrin is a laminin receptor associated with the hemidesmosomes localized to the basement membrane [4–6]. Several observations have supported a critical role for α6β4 in mediation of cellular attachment to the basement membrane via the hemidesmosome complex. In addition to its role in cell adhesion and hemidesmosome assembly, α6β4 has also been implicated in signal transduction of cell proliferation, differentiation, and apoptosis [7–9].

One of the important regulators of both skin keratinocyte differentiation and adhesive characteristics is the protein kinase C (PKC) family of serine/threonine kinases. PKC plays an important regulatory role in a variety of biological phenomena [10–12]. The family is composed of at least 11 individual isoforms, which belong to 3 distinct categories, depending on their structural characteristics and cofactor requirements [13–15]. The type of isoform and pattern of distribution vary among different tissues [15,16]. In skin, 5 PKC isoforms have been identified including PKCs α, δ, η, ε, and ζ. We and others have previously reported the involvement of specific PKC isoforms in regulation of proliferation, migration, and differentiation processes in skin [16–22]. Several lines of evidence indicate that PKC also has an important role in integrin-mediated adhesion and signaling events. Stimulation with pharmacological

☆ *Abbreviations:* PKC, protein kinase C; TPA, phorbol 12-myristate 13-acetate; EMEM, Eagle's minimal essential medium; PMSF, phenylmethylsulfonyl fluoride.

* Corresponding author. Fax: +972-3-531-1598.
E-mail address: tennet@mail.biu.ac.il (T. Tennenbaum).

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activators of PKC such as TPA causes increased adhesion, spreading, and migration, which could be specifically blocked by the use of PKC inhibitors [23,24]. Furthermore, several PKC isoforms have been shown to be activated following integrin activation [25–27]. Finally, integrin function is associated with activation of downstream elements, including increased phospholipase C and diacylglycerol levels, which are known to be involved in PKC activation [23,28].

In order to identify the specific PKC isoform, which could regulate α6β4 expression and function we have used pharmacological and genetic approaches to study their distinct effects on integrin expression in skin keratinocytes. We show here that while PKC activation specifically regulates α6β4 expression, the various PKC isoforms differentially regulate α6β4 integrin expression and function, and specifically modulate the adhesive characteristics of skin keratinocytes.

Materials and methods

Reagents and antibodies. Tissue culture media and serum were purchased from Biological Industries (Beit HaEmek, Israel). The PKC pharmacological activators bryostatin 1 and TPA were purchased from ICN (ICN, CA, USA). The specific PKC inhibitor GF-109203X was purchased from ICN (ICN, CA, USA). Laminin-5 was a gift from Dr. R. Burgesson (Harvard U. Cambridge, MA). The α6 rat anti-mouse mAb (GoH3) was purchased from PharMingen (San Diego, CA). The rat mAb directed against the extracellular domain of mouse β4 (346-11A) was a gift from Dr. S. J. Kennel (Oak Ridge National laboratory, Oak Ridge, TN). The α3 rabbit anti-mouse antibody and the β1 rat anti-mouse antibody were purchased from Chemicon (Temecula, CA). Polyclonal antibodies to specific PKC isoforms were purchased from Santa-Cruz (Santa-Cruz, CA). HRP-anti-rabbit IgG was purchased from Bio-Rad (Bio-Rad Laboratories, Bio-Rad, Israel), HRP-anti-rat IgG and HRP-anti-mouse IgG were purchased from Jackson Laboratories (PA, USA).

Isolation and culture of murine keratinocytes. Primary keratinocytes were isolated from newborn BALB/c mice. Keratinocytes were cultured in Eagle's minimal essential medium (EMEM) containing 8% Chelex (Chelex–100)-treated fetal bovine serum (Bio-Rad). To maintain a proliferative basal cell phenotype, the final Ca^{2+} concentration was adjusted to 0.05 mM as described [29].

Cell fractionation and Western blot analysis. For membrane protein fraction, cultures were scraped and extracted on ice into 500 μl PBS buffer containing 150 mM NaCl; 50 mM Tris, pH 7.5; 10 mM EDTA; 10 mM NaF; 10 μg/ml aprotinin and leupeptin; 2 μg/ml pepstatin; 1 mM PMSF; and 200 μM NaVO4 (Sigma, St. Louis, MO). Following four freeze/thaw cycles, lysates were centrifuged at 4 °C at 13,000 rpm for 25 min and the pellet was re-suspended with 250 μl PBS buffer containing 1% Triton X-100 with proteinase and phosphatase inhibitors. Lysates were homogenized twice during 30 min incubation on ice and centrifuged at 13,000 rpm at 4 °C for 30 min. Protein concentrations of the supernatant membrane fraction were measured using a modified Lowry assay (Bio-Rad DC Protein Assay Kit). For integrin analysis under non-reducing conditions, Laemmli sample buffer without 2-β-mercaptoethanol was added to 20 μg of membrane fractions. For whole cell lysates, cells were scraped on ice into 500 μl lysis buffer containing 5% SDS, 20% 2-β-mercaptoethanol, and 0.5 M Tris, pH 6.8, and homogenized. Protein samples were boiled before loading onto SDS–PAGE gel and separated proteins were transferred to a nitrocellulose membrane (Bio-Rad). Specific protein bands were detected by immunoblotting using specific antibodies against different PKC isoforms or integrin subunits. Bands were visualized by enhanced chemiluminescence utilizing Pierce SuperSignal kit (Rockford, Illinois).

Overexpression of PKC isoforms in keratinocytes. The β-galactosidase (β-Gal) and the distinct PKC recombinant adenoviruses were constructed as described previously [22,30]. Cultured keratinocytes were incubated with either each one of the distinct viral supernatants for 1 h, washed twice with PBS, and re-fed with 0.05 mM Ca^{2+} containing EMEM. Twenty-four hours following infection, cultures were extracted for further analysis, as described.

Cell adherence assay. Primary skin keratinocytes were either uninfected or infected with either each one of the recombinant adenoviruses. Twenty-four hours following infection, the fraction of cells attached to, or detached from, the culture matrix, was analyzed. In order to quantitate the detached cells from the culture dish, growth medium from the different cultures was transferred to conic tubes and centrifuged at 1000 rpm for 10 min at 4 °C. Cell pellets were rinsed twice with PBS and extracted in 1 M NaOH. To quantitate the remaining attached cells on the culture dish, following the medium removal, cultures were rinsed twice with PBS and cells were extracted in 1 M NaOH. Cell counts were determined by protein concentrations (μg protein/dish) using a modified Lowry assay.

Cell Attachment assay. Twenty-four well Petri plates (Greiner labortechnic, Germany) were coated either with laminin-5 or 20 μg/ml fibronectin, for 1 h at 37 °C. Following incubation, plates were washed and incubated at 37 °C with 0.1% BSA in 0.05 mM Ca^{2+} EMEM for 30 min, to block non-specific binding. Keratinocyte cultures were either stimulated with TPA (100 nM) or bryostatin 1 (500 nM) for 30 min, or infected for 1 h with distinct recombinant adenoviruses. Thirty minutes following PKC pharmacological stimulation, or 24 following infection, cultures were rinsed twice with PBS and trypsinized briefly in 0.25% trypsin/0.02% EDTA solution, while incubated at 37 °C. Upon detachment, cells were resuspended in 0.05 mM Ca^{2+} EMEM with FCS and centrifuged at 1000 rpm for 10 min at 4 °C. Pellets were resuspended in 1% BSA in 0.05 mM Ca^{2+} EMEM without FCS. Keratinocytes ($1 \times l0^6$) were added to the coated wells and allowed to adhere to the laminin-5 or fibronectin matrices for 5 min or 1 h, respectively, while incubated at 37 °C. Non-adherent cells were removed, the wells were rinsed twice with PBS and cells were extracted in 1 M NaOH. Cell counts were determined by protein concentrations using a modified Lowry assay. Non-specific keratinocyte attachment to uncoated dishes was measured as blank measurements and reduced from the obtained results of keratinocyte attachment to laminin-5 and fibronectin coated dishes. Laminin-5 enriched matrix was prepared as described previously [29].

Results

PKC activation downregulates α6β4 integrin expression

PKC activation is a major signaling pathway involved in skin differentiation. Our previous results suggest that downregulation of the α6β4 integrin is one of the earliest events in keratinocyte differentiation leading to cell detachment [3]. In order to determine whether PKC activation is implicated in the regulation of α6β4, we utilized the pharmacological PKC activators TPA and bryostatin 1 and followed their effect on α6β4 expression. As seen in Fig. 1A, treating primary mouse keratinocytes with bryostatin 1 or TPA led specifically to downregulation of the α6 integrin subunit in a dose and time dependent manner. Downregulation of α6

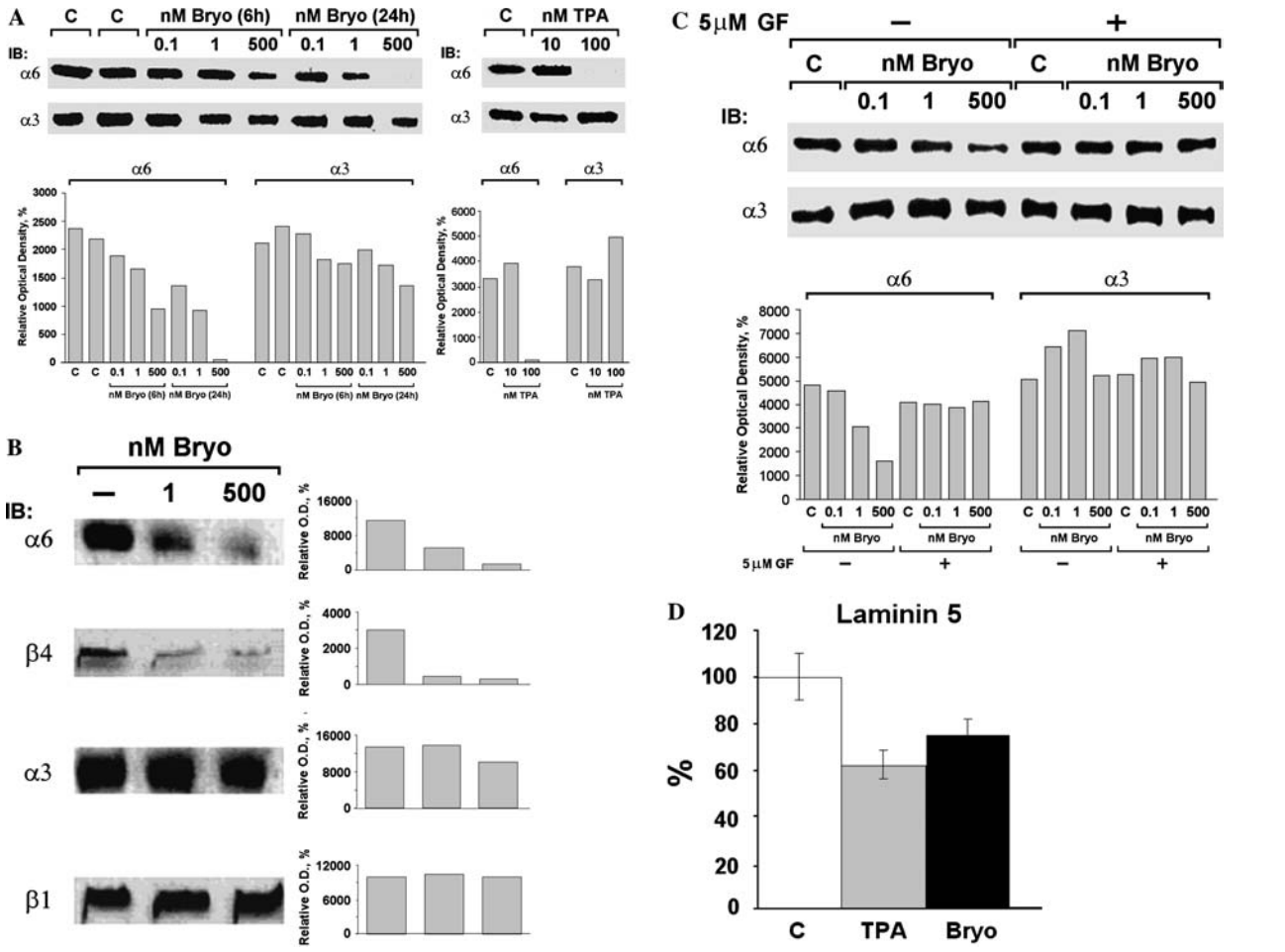

Fig. 1. PKC activation mediates α6β4 downregulation and cell adhesion to laminin-5. (A) Upper panel; Keratinocytes were either untreated (C) or stimulated with bryostatin 1 (0.1, 1, and 500 nM Bryo) or TPA (10, 100 nM), for various time periods (6, 24 h). Twenty micrograms samples of membrane proteins were immunoblotted with anti-α6 or anti-α3 antibodies and analyzed by ECL. The blots are representative of three different experiments. (Lower panel) Scanning densitometry of the relative intensity of the presented immunoblots. (B) Left panel; Keratinocytes were either untreated (−) or treated with bryostatin 1 (1, 500 nM Bryo) for 30 min. 20 μg samples of membrane proteins were immunoblotted with anti-α6, anti-β4, anti-α3 or anti-β1 antibodies and analyzed by ECL. The blots are representative of five different experiments. Right panel; Scanning densitometry of the relative intensity of the presented immunoblots. (C) Upper panel; Keratinocytes were either untreated (C) or treated with bryostatin 1 (0.1, 1, and 500 nM Bryo) in the absence (−) or presence (+) of 5 μM GF 109203X for 30 min. Membrane proteins samples (20 μg) were immunoblotted with anti-α6 or anti-α3 antibodies and analyzed by ECL. The blots are representative of three different experiments. Lower panel; Scanning densitometry of the relative intensity of the presented immunoblots. (D) Keratinocytes were either untreated (C) or stimulated with TPA (100 nM) or bryostatin (500 nM Bryo) for 30 min. Cells were then trypsinized briefly and replated onto laminin-5 coated dishes for 5 min at 37 °C and the attached cells were quantitated, as described in "Materials and methods." Results are presented as the percentage of attached cells of cultures treated with PKC activators relative to untreated control cultures (100%). Results were calculated as means ± SD of four wells in each experiment, repeated four times. $p < 0.01$ for TPA and bryostatin 1 treatment vs control untreated cultures.

integrin subunit by stimulation with PKC activators was associated with downregulation of the β4 integrin subunit, while neither α3 nor β1 integrin subunits were affected (Fig. 1B). As expected from a PKC-mediated event, treating the cells with the PKC inhibitor GF109203X, which inhibits all PKC isoforms in keratinocytes, abrogated downregulation of α6 following bryostatin 1 stimulation (Fig. 1C). Next, we examine whether PKC activation could modify α6β4 integrin dependent cell adhesion. Primary keratinocytes were exposed to TPA or bryostatin 1 and replated onto laminin-5 coated dishes. Indeed, as shown in Fig. 1D, both TPA and bryostatin 1 stimulation resulted in decreased keratinocyte attachment to laminin-5 (by 40% and 30%, respectively), in comparison to untreated cultures.

Effects of PKC isoform overexpression on integrins

In order to explore the involvement of specific PKC isoforms in α6β4 downregulation, we overexpressed distinct PKC isoforms in primary keratinocyte cultures, utilizing the recombinant adenovirus delivery system. As can be seen in the immunoblots presented in Fig. 2, 24 h

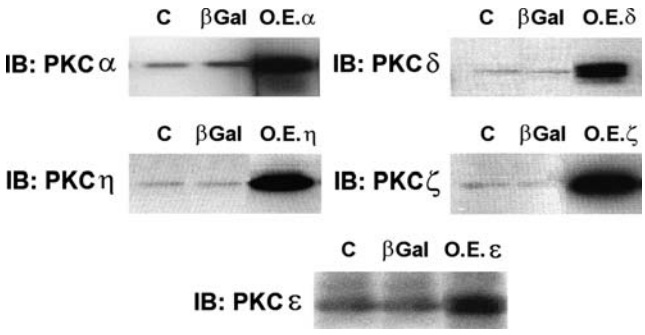

Fig. 2. Overexpression of PKC isoforms utilizing recombinant adenoviruses. Keratinocytes were either uninfected (C) or infected with either each one of the control β-Gal, PKCα, PKCδ, PKCη, PKCζ or PKCε recombinant adenoviruses. Twenty-four hours later, cultures were extracted and protein lysates from equal cell amounts were immunoblotted with isoform specific anti-PKC antibodies and analyzed by ECL. The blots are representative of eight different experiments.

following infection of mouse keratinocyte cultures, a significant increase in specific PKC protein expression could be observed, in comparison to the endogenous levels of these isoforms in the uninfected or control β-galactosidase adenovirus infected cultures. The significant elevation in the PKC expression levels was specific to the distinct overexpressed isoforms (data not shown).

Next we explored the relationships between distinct PKC isoforms and the α6β4 integrin. Initially, we studied the effects of specific PKC isoforms on α6β4 protein expression. As can be seen in the immunoblots presented in Fig. 3, only PKCδ and PKCζ isoforms were able to downregulate α6β4 expression, in comparison to α6β4 expression in control β-galactosidase adenovirus infected cells. Overexpression of either PKCη or PKCε isoforms did not significantly affect α6β4 expression levels. In contrast, overexpression of the PKCα isoform resulted in a significant increase in α6β4 expression. At the same time, neither α3 nor β1 integrin subunit expression levels were reduced. Nevertheless, the changes in α6β4 expression were associated with distinct effects on cell–matrix associations. Twenty-four hours following infection, PKCδ overexpression, and to a lesser extent PKCζ overexpression, was associated with enhanced cell detachment (up to 50% of the PKCδ overexpressing keratinocytes) from the culture matrix (Fig. 4A). No significant cell detachment was observed in keratinocytes overexpressing the PKCα, PKCη or PKCε isoforms (Fig. 4A). Finally, in order to investigate the link between α6β4 expression and keratinocyte adhesion characteristics, we conducted adhesion assays to the specific α6β4 ligand laminin-5 and to fibornectin matrix proteins. As can be seen in Fig. 4B, in comparison to control β-galactosidase infected keratinocytes, overexpression of PKCα isoform increased keratinocyte attachment to laminin-5, while overexpression of PKCδ and PKCζ reduced cell attachment to this specific ligand. At the same time, keratinocyte adhesion to fibronectin was not altered.

Overall, these results suggest specific involvement of PKCδ and PKCζ isoforms in regulation of α6β4 expression and keratinocyte attachment to laminin-5.

Discussion

The α6β4 integrin is localized to a specific cellular location, the hemidesmosome complex, which mediates keratinocyte adhesion to the underlying basement membrane [31–33]. The importance of α6β4 integrin

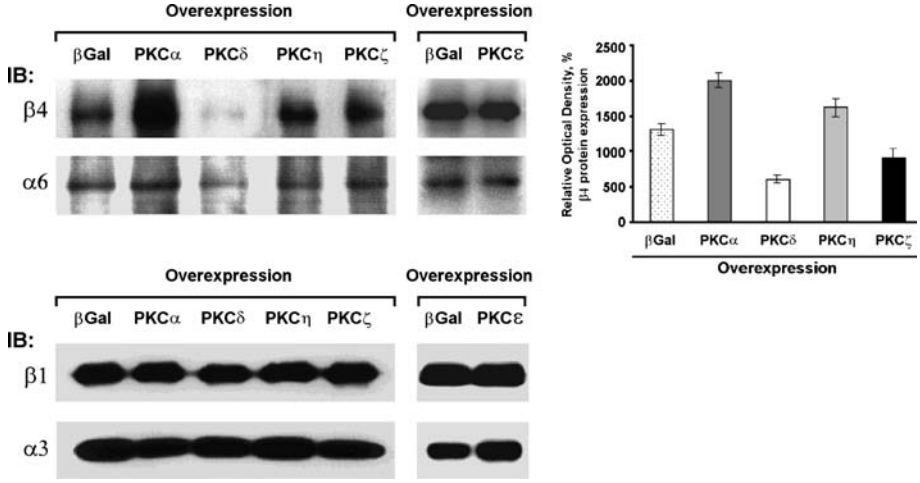

Fig. 3. Overexpression of PKC isoforms specifically regulates α6β4 integrin expression. Keratinocytes were infected with either each one of the control β-Gal, PKCα, PKCδ, PKCη, PKCζ or PKCε recombinant adenoviruses. Twenty-four hours later, cells were extracted and 20 μg samples of membrane proteins were immunoblotted with anti-β4, anti-α6, anti-β1 or anti-α3 antibodies and analyzed by ECL. The blots are representative of five different experiments. Right panel; analysis of the average scanning densitometry of the relative intensity of the β4 integrin subunit bands, calculated as means ± SD of β4 immunoblots obtained in five different experiments.

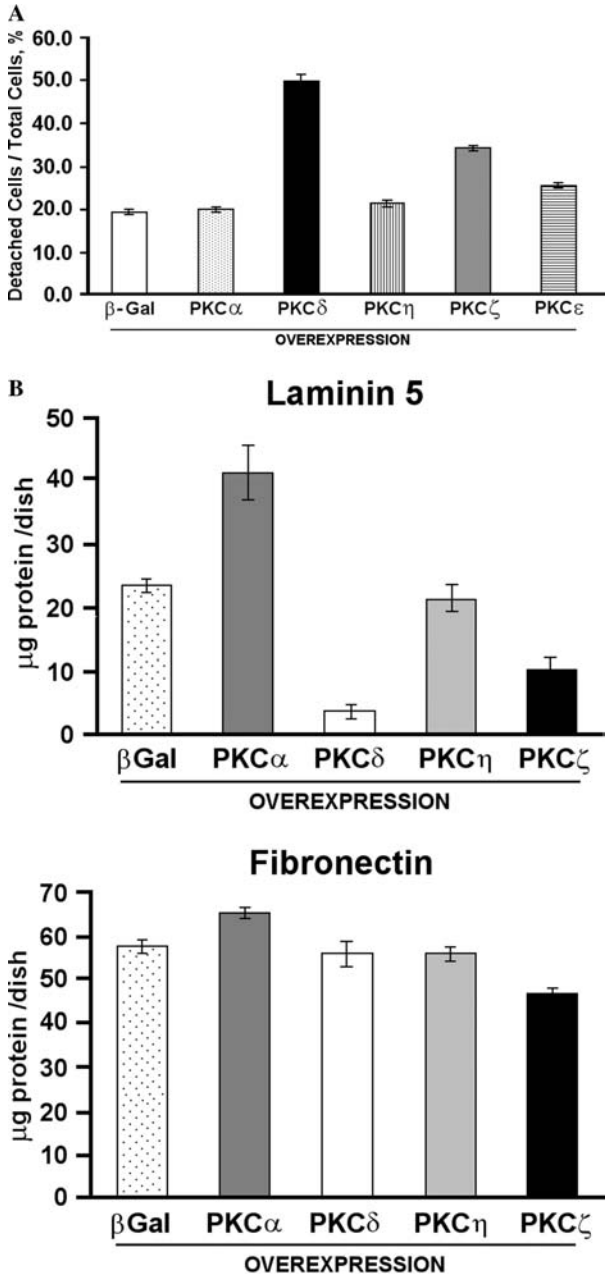

Fig. 4. Overexpression of PKCs α, δ, and ζ distinctively effects keratinocyte attachment to the underlying matrix. (A) Keratinocytes were infected with either each one of the control β-Gal, PKCα, PKCδ, PKCη, PKCζ or PKCε recombinant adenoviruses. The amount of cells attached or detached from the culture matrix was analyzed 24 h later, as described in "Materials and methods." Results are presented as detached cells/total cells (%), as measured by protein concentrations, calculated as the means ± SD of four wells in each experiment, repeated four times. $p < 0.05$ for PKCζ and $p < 0.01$ for PKCδ vs control β-Gal infected cells. (B) Keratinocytes were infected with either each one of the recombinant adenoviruses. Twenty-four hours following infection, cultures were trypsinized briefly and replated onto laminin-5 or fibronectin coated dishes for 5 min or 1 h, respectively. Attached cells were quantitated as described in "Materials and methods." Results are presented as microgram protein/dish, calculated as means ± SD of four wells in each experiment repeated four times. $p < 0.001$ for PKCδ and $p < 0.01$ for PKCα and ζ vs control β-Gal infected cells, in the attachment to laminin-5.

expression in epidermal integrity was corroborated in both animal models and in human patients, where ablation of the α6 or β4 subunits interferes with the formation of normal hemidesmosomes and results in epidermal shedding [34,35].

In both physiological settings, as well as in pathological conditions, α6β4 expression is dynamically regulated and is intimately associated with the adhesive and migratory characteristics of cells. Specifically, in skin, the expression of α6β4 is linked to the proliferative basal layer compartment of the epidermis, and upon differentiation, the loss of α6β4 is associated with detachment of keratinocytes from the underlying basement membrane followed by suprabasal migration [1,3]. In pathological settings in vitro and in vivo, increased suprabasal expression of α6β4 is localized away from the hemidesmosome structures and is associated with the migrating epithelia in wound healing and skin cancer [36–43].

It is clear that the activation of specific protein kinases can alter integrin function in cell adhesion and migration. While progress has been made in elucidating the biochemical pathways associated with integrin–ligand interactions, details are still elusive regarding the regulation of integrin expression via the interplay between the various adaptor proteins and kinases. The linkage between the PKC family of isoforms and integrin-mediated signaling events was established in previous studies, where it was shown that exposure of keratinocytes to PKC activators resulted in integrin activation, leading to integrin binding to specific ligands, enhancing cell attachment, and spreading on distinct matrices. These effects could be blocked by pre-exposure of cultures to specific PKC inhibitors [23–25]. Moreover, PKC activation was shown to enhance cell adhesion to distinct matrices through the formation of focal adhesions and regulation of integrin–cytoskeleton interactions [44–48]. In addition, indirect activation of PKC by phospholipid metabolism, including the generation of physiological activators as diacylglycerol (DAG) and inositol triphosphates (IP3), as well as PI3-K activation in response to integrin stimulation, has all been implicated in both integrin inside-out as well as outside-in signaling [23,28]. Finally, previous studies have suggested the involvement of different overexpressed PKC isoforms in regulation of integrin-mediated signaling pathways [27]. Much of the information on the cellular and physiological functions of the PKC isoforms has been gathered by utilizing pharmacological activators as TPA and bryostatin 1, as also was done in this study. In primary murine keratinocytes, PKC activation by pharmacological stimulation induced specific downregulation of the α6β4 complex with no effect on expression of other β1 integrins expressed in skin, such as α3β1. Furthermore, reduced α6β4 protein expression led to functional changes in keratinocyte adhesion properties.

These results could also account for the downregulation of α6β4 during keratinocyte differentiation in vivo and explain the dramatic sloughing of primary keratinocytes observed following TPA stimulation.

The existence of multiple PKC isoforms, which are distinct in their tissue distribution, cellular localization, and substrate specificity, suggests specific roles for each one of these isoforms in PKC-mediated events. Therefore, we next studied the contribution of distinct PKC isoform activation to the regulation of α6β4 expression and the resulting biological consequences. Utilizing recombinant adenovirus delivery system, we could achieve efficient overexpression of distinct PKC isoforms, which resulted in 5- to 10-fold increase in specific protein expression above endogenous basal levels. Moreover, each of the PKC isoforms differentially and specifically affected α6β4 protein expression and cellular distribution. Overexpression of PKCδ and PKCζ isoforms reduced α6β4 protein expression, whereas in contrast, overexpression of PKCα markedly increased the expression of the α6β4 integrin complex.

The expression levels of α6β4 correlated with the adhesive characteristics of the cells to the underlying matrix. Moreover, as shown, PKCδ and PKCζ overexpression resulted in gradual detachment from the underlying matrix and specifically reduced cell adhesion to laminin-5. In contrast, PKCα overexpression was associated with cell attachment to the underlying matrix and increased adhesion to laminin-5. These results suggest the activation of PKCδ and PKCζ as the possible trigger, which could initiate the loss of basal cell attachment to the basement membrane in the early stages of keratinocyte differentiation. Indeed, these results are in agreement with the previously described roles of distinct PKC isoforms in skin proliferation and differentiation [26,28], and with the studies suggesting a role for PKC activation in regulation of α6β4 integrin expression and function [9,49]. Interestingly, our previous results linked the downregulation of α6β4 integrin and loss of cell attachment to laminin-5 to PKCδ-mediated serine phosphorylation of the α6β4 [17]. However, our current study supports a role for both PKCδ and PKCζ in downregulation of α6β4 integrin expression, and in contrast a role for PKCα in upregulation of α6β4 expression, suggesting both phosphorylation dependent as well as phosphorylation independent pathways in regulation of α6β4 integrin.

Overall these results suggest that PKC isoforms could play a role in initiating the loss of basal cell attachment to the basement membrane, via α6β4 integrin regulation in the early stages of keratinocyte differentiation. Therefore, future studies will be aimed at assessing the distinct signaling elements which contribute to each of the pathways leading to keratinocyte differentiation and to the complex interplay between specific PKC isoforms and the α6β4 integrin. These studies should reveal important mechanisms designed to maintain the integrity of the normal epidermis and to contribute to our understanding of the changes, which occur during normal differentiation and the pathology of skin cancer.

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