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Protein Kinase Cδ-mediated Phosphorylation of α6β4 Is Associated with Reduced Integrin Localization to the Hemidesmosome and Decreased Keratinocyte Attachment[1]

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ABSTRACT

In mammalian epidermis, expression of the α6β4 integrin is restricted to the hemidesmosome complexes, which connect the proliferative basal cell layer with the underlying basement membrane. Keratinocyte differentiation is associated with down-regulation of α6β4 expression and detachment of keratinocytes from the basement membrane. Neoplastic keratinocytes delay maturation, proliferate suprabasally, and retain the expression of the α6β4 integrin in suprabasal cells disassociated from the hemidesmosomes. We now show that the α6β4 integrin is a substrate for serine phosphorylation by protein kinase C in keratinocytes. Furthermore, protein kinase C-mediated phosphorylation of α6β4 is associated with redistribution of this integrin from the hemidesmosome to the cytosol. Specifically, *in vitro* kinase assays identified the protein kinase Cδ as the primary isoform phosphorylating α6 and β4 integrin subunits. Using recombinant protein kinase C adenoviruses, overexpression of protein kinase Cδ but not protein kinase Cα in primary keratinocytes increased β4 serine phosphorylation, decreased α6β4 localization to the hemidesmosome complexes, and reduced keratinocyte attachment. Taken together, these results establish a link between protein kinase Cδ-mediated serine phosphorylation of α6β4 integrin and its effects on α6β4 subcellular localization and keratinocyte attachment to the laminin underlying matrix.

INTRODUCTION

The interaction of basal and suprabasal cells of the epidermis with the extracellular matrix via specific cell surface integrins provides an important level of control for both proliferation and cell maturation (1). This relationship became apparent through studies of genetically or pharmacologically modified integrins of cultured keratinocytes and transgenic mice with epidermal integrin modifications (2–7). Several integrin complexes are expressed in skin, including the α6β4- and β1-associated integrins, α3β1 and α2β1 (8–10). In normal epidermis, the α6β4 complex is expressed exclusively on the basal surface of the basal layer apposing the basement membrane, where it is localized to the hemidesmosomes (10–14). Commitment to keratinocyte maturation and the detachment of basal cells from the basement membrane initiates a cascade of biochemical events including the early down-regulation of α6β4, leaving adhesion complexes such as the β1-associated integrins and cadherins to maintain cell-cell contacts (9, 10, 15). When cultured keratinocytes are induced to differentiate by raising extracellular calcium, early loss of α6β4 reduces cell attachment to laminins selectively (10). As maturation proceeds, both β1 integrins and attachment to all matrix proteins are diminished coincident with loss of viability and production of cornified squames. The down-regulation of α6β4 integrin is associated first with proteolytic cleavage of both the α6 and β4 integrin subunits and later with repression of integrin gene expression, suggesting that posttranscriptional regulation of α6β4 is a primary consequence of commitment to differentiation (10). In support of this concept, neoplastic keratinocytes delay maturation, proliferate suprabasally, and retain the expression of the α6β4 integrin in suprabasal strata (16, 17). Although alterations in α6β4 integrin expression and function are well documented in keratinocytes, the biochemical signals that control these changes are not defined. Growth factor-mediated regulation of integrin function has been linked to protein phosphorylation of integrin subunits or phosphorylation of docking molecules associated with integrin complexes (18). Modifications on both tyrosine and serine/threonine moieties have been documented (13, 19, 20). Stimulation and phosphorylation by the serine/threonine PKC[4] family has been linked to integrin activation (21–23). PKC is a known regulator of keratinocyte differentiation, cell adhesion, and migration (24–27). In mammalian skin, PKC α, δ, η, ϵ, and ζ have been localized in the epidermis, and PKC activation modulates expression of maturation-associated genes and is essential for terminal differentiation (24, 25, 28, 29). In several studies, specific isoforms of PKC have been implicated in particular functions associated with keratinocyte maturation (25, 26, 30, 31).

To evaluate PKC activation as a modifier of integrin interaction with basement membrane ligands, we have studied the ability of several PKC activators to regulate the α6β4 integrin in cultured primary murine keratinocytes. We demonstrate specific changes in α6β4 phosphorylation state and alterations of function as a result of PKC activation. These changes in α6β4 phosphorylation appear to occur rather specifically through activation of PKCδ. Taken together, our results suggest that PKCδ regulation of α6β4 function via phosphorylation constitutes an important pathway associated with keratinocyte homeostasis.

MATERIALS AND METHODS

Antibodies and Extracellular Matrix Molecules. The α6 rat antimouse 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). Polyclonal antibody to PKCδ or PKCα was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rat mAb to phosphotyrosine was purchased from Sigma Chemical Co. (St. Louis, MO), and rabbit anti-phosphoserine was purchased from Zymed (San Francisco, CA). Laminin 5 was a gift from Dr. R. Burgeson (Harvard U. Cambridge, MA). Calf intestine alkaline phosphatase was purchased from Worthington (Worthington, NJ).

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[4] The abbreviations used are: PKC, protein kinase C; mAb, monoclonal antibody; TPA, phorbol 12-myristate 13-acetate; EMEM, Eagle's Minimal Essential Medium; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride.

Isolation and Culture of Murine Keratinocytes. Primary keratinocytes were isolated from newborn BALB/c mice. Keratinocytes were cultured in EMEM containing 8% Chelex (Chelex-100; Bio-Rad)-treated fetal bovine serum. To maintain a proliferative basal cell phenotype, the final Ca^{2+} concentration was adjusted to 0.05 mM as described (32). Experiments were performed 5–7 days after plating.

Attachment Assays. Twenty-four-well Petri plates (Greiner) were coated with laminin 5 as described (32). After incubation, plates were washed and incubated with 0.1% BSA for 30 min at room temperature to block nonspecific binding. Keratinocyte cultures were trypsinized briefly, and after detachment, cells were resuspended; Keratinocytes ($1 \times l0^6$) were added to the coated wells and incubated for 5 min at 37°C. Nonadherent cells were removed, the wells were rinsed two times with PBS, and cells were extracted in 1 M NaOH. Cell count was determined by protein concentrations using a modified Lowry assay (Bio-Rad DC Protein Assay kit; Bio-Rad Laboratories). Results were calculated by percentage relative to untreated controls.

Immunofluorescence. Primary keratinocytes were plated on laminin 5-coated glass slides. Two-day-old keratinocytes were infected either with PKCδ, PKCα, or control adenoviruses for 1 h, washed two times with PBS, and maintained in culture in low Ca^{2+} EMEM. For PKC isoform staining analysis, 24-h-postinfection keratinocytes were fixed in methanol, rinsed with PBS, and incubated overnight at 4°C with PKCδ- or PKCα-specific antibodies diluted in 1% BSA in PBS. After incubation, slides were washed twice for 10 min with PBS and incubated with biotinylated secondary antirabbit antibody for 20 min, washed two times in PBS, and incubated with Streptavidin-FITC for 20 min. For α6β4 staining analysis, keratinocytes were fixed in 4% paraformaldehyde for 30 min, followed by permeabilization with 0.2% Triton for 5 min. The slides were incubated with anti-α6 antibody overnight, followed by incubation with biotinylated secondary antirat antibody, respectively, for 20 min, washed two times in PBS, and incubated with Strepavidin-FITC for 20 min. After two washes in PBS, slides were mounted with glycerol buffer containing 1% *p*-phenylenediamine (Sigma Chemical Co.), and fluorescence was examined by laser scanning confocal imaging microscopy (MRC1024; Bio-Rad, Hemel Hampstead, United Kingdom).

SDS-PAGE and Western Blot Analysis. For crude membrane fractions, lysates were prepared by scraping cells into PBS containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin (Boehringer Mannheim, Mannheim, Germany), 1 mM PMSF, 10 mM EDTA (Digene, Silver Spring, MD), 200 μM $NaVO_4$, and 10 mM NaF. After four cycles of freeze/thaw, cells were centrifuged at 4°C at 16,000 × *g*. The supernatant containing the soluble protein fraction was transferred to another tube. The pellet was resuspended in 250 μl of PBS containing 1% Triton X-100 with proteinase and phosphatase inhibitors. Cells were incubated for 30 min on ice and centrifuged at 16,000 × *g* at 4°C. The supernatant was transferred to a fresh tube. The remaining pellet contains cytoskeletal proteins. Protein concentrations were measured using a modified Lowry assay (Bio-Rad DC Protein Assay kit). Twenty μg of protein were loaded onto SDS-PAGE gel in reducing (for PKC analysis) or nonreducing (for integrin analysis) conditions, and separated proteins were transferred to a nylon membrane. Specific protein bands were detected by incubating the protein blots with specific antibodies to the different integrin subunits. Bands were visualized by enhanced chemiluminescence using the Renaissance kit (DuPont, Boston, MA).

Cell Fractionation. Keratinocytes were extracted for 5 min on ice in lysis buffer containing 0.2% Triton X-100, 150 mM NaCl, and 50 mM Tris (pH 7.5) with protease and phosphatase inhibitors. After centrifugation at 14,000 rpm for 30 min, supernatants were transferred to other tubes, the insoluble cell extracts were washed two times in lysis buffer, and 500 μl of lysis buffer containing 1% Triton X-100 were added to each sample. The soluble and insoluble lysates were used for immunoprecipitation as described.

PKC Immunokinase Assay. Purified and standardized PKC isozymes were kindly supplied by Dr. P. Blumberg (National Cancer Institute, NIH, Bethesda, MD) and Dr. Marcello G. Kazanietz (University of Pennsylvania, School of Medicine, Philadelphia, PA). Primary keratinocytes were harvested in 500 μl of 1% Triton lysis buffer (1% Triton X-100, 10 μg/ml aprotinin and leupeptin, 2 μg/ml pepstatin, 1 mM PMSF, 1 mM EDTA, 200 μM Na2VO4, and 10 mM NaF in 1× PBS). Lysates were incubated at 4°C for 30 min and spun at 16,000 × *g* for 30 min at 4°C. Supernatants were transferred to a fresh tube. Immunoprecipitation of cell lysates was carried out overnight at 4°C with 5 μg/sample anti-α6/GoH3 (PharMingen) and 30 μl/sample of protein A/G-Plus agarose slurry (Santa Cruz Biotechnology). Beads were washed once with RIPA buffer and twice with 50 mM Tris/HCl (pH 7.5). Thirty-five μl of reaction buffer (1 mM $CaCl_2$, 20 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.5) were added to each assay. To each assay, 5.5 μl/assay of a suspension of phospholipid vesicles containing either DMSO or 10 mM TPA was added to the slurry together with a standardized amount of specific PKC isozyme. The reaction was initiated by adding 10 μl/assay 125 mM ATP (1.25 μCi/assay [γ-^{32}P]ATP; Amersham) and allowed to continue for 10 min at 30°C. The beads were then washed two times with RIPA buffer. Thirty μl/sample protein loading dye (3× Laemmli, 5% SDS) were added, and the samples were boiled for 5 min in a water bath. Proteins were separated on 8.5% SDS-PAGE gel, transferred onto Protran membranes (Schleicher & Schuell), and visualized by autoradiography. Phosphorylation of histones and phosphorylation of PKC substrate peptide were used as controls for PKC activity.

P_i Labeling and Immunoprecipitation. Primary keratinocytes were incubated overnight in 75% phosphate-free MEM and 25% MEM, followed by labeling with 400 μCi/100-mm dish $HP_3O_4{}^{32}P$ (DuPont/NEN) for 3 h. For the last 30 min of incubation (or as detailed otherwise in the experimental procedure), labeled keratinocytes were treated with bryostatin 1 (ICN, Costa Mesa, CA) or TPA at given concentrations. In selected cultures, PKC activity was inhibited by pretreatment for 30 min with 5 μM GF-109203X (ICN) prior to stimulation by TPA or bryostatin 1. Cells were harvested in 500 μl of 1% Triton lysis buffer, left at 4°C for 30 min, and spun at 16,000 × *g* for 30 min at 4°C. Supernatants were transferred to a fresh tube. Immunoprecipitation of cell lysates was carried out overnight at 4°C with 5 μg/sample anti-α6/GoH3 (PharMingen) and 30 μl/sample protein A/G-Plus agarose slurry (Santa Cruz Biotechnology). Beads were washed three times with RIPA buffer. Thirty μl/sample protein loading dye (3× Laemmli, 5% SDS) were added, and the samples were boiled for 5 min in a water bath. Proteins were separated on 8.5% SDS-PAGE, transferred onto Protran membranes (Schleicher & Schuell), and visualized by autoradiography.

Two-Dimensional Phosphoamino Acid Analysis. For two-dimensional phosphoamino acid analysis, ^{32}P-labeled proteins were purified and separated as above but transferred onto a PVDF membrane (Immobilon membranes; Millipore). The membrane was washed three times in deionized H_2O, and labeled protein bands were excised. Membrane fragments containing protein bands were incubated in 200 μl of 6N HCl at 110°C for 90 min. Samples were spun briefly in a benchtop centrifuge, and the HCl extract was transferred to a fresh tube. Samples were spun in a SpeedVac to dry. Samples were washed three times in 200 μl of deionized H_2O and dried after each wash by spinning in a SpeedVac. Samples were combined with 1 μl each of 1 mg/ml stock solution of nonradioactive phosphoserine, phosphothreonine, and phosphotyrosine/sample and spotted onto a TLC plate (Merck 5577). Samples were electrophoresed in pH 1.9 buffer (15% acetic acid and 5% formic acid) at 500 V for 3 h, and the plate was allowed to air dry. The buffer was changed in the apparatus to pH 3.5 (5% acetic acid and 0.5% pyridine). Blotters were wet thoroughly in the buffer, and plates were rotated 90° counterclockwise. For the second dimension, electrophoreses was carried out at pH 3.5, 1.6 kV for 16 min. The locations of nonradioactive phosphoamino acid standards were visualized by spraying with 0.2% ninhydrin in ethanol (Sigma Chemical Co.), and radioactive amino acids were detected by autoradiography.

Overexpression of PKC Isoforms. PKC adenovirus constructs were constructed as described previously (33, 34). Five-day-old keratinocytes were infected with distinct PKC adenoviruses for 1 h. Cultures were then washed twice with PBS and refed with 0.05 mM Ca^{2+} containing EMEM. β-Galactosidase adenovirus was used as a control virus in all experiments. Twenty-four h after infection, cells were extracted for further analysis, as described.

Alkaline Phosphatase Digestion of Phosphoproteins. Primary keratinocytes were either infected with control adenovirus vector or with recombinant PKCδ adenovirus for 1 h. Twenty-four h after infection, cultures were harvested in 500 μl of 1% Triton lysis buffer. Immunoprecipitation of cell lysates was carried out overnight at 4°C with 5 μg/sample anti-α6 (PharMingen) and 30 μl/sample of protein A/G-Plus agarose slurry (Santa Cruz Biotechnology). Beads were washed twice with PBS and once in Tris/$MgCl_2$ buffer, pH 7.5 (50 mM Tris-Cl and 1 mM $MgCl_2$). After centrifugation, beads were resuspended in 20 μl of Tris/$MgCl_2$ buffer and incubated for 10 min at 30°C. Twenty units of calf intestine alkaline phosphatase (Worthington, Lakewood, NJ) were added to the reaction tubes, and samples were incubated for 15 min at 30°C. Adding an equal volume of 2× SDS-PAGE sample buffer terminated dephosphoryl-

ation reactions. Samples were boiled for 5 min in a water bath, and proteins were separated by 7.5% SDS-PAGE gel and transferred onto membrane filters (Bio-Rad). Specific protein bands were detected by immunoblotting using specific antibodies and visualized by enhanced chemiluminescence.

RESULTS

Our previous studies have identified two pharmacological activators of PKC that differ in their isoform specificity and in their effect on keratinocyte differentiation. TPA is a powerful activator of PKCα, PKCδ, PKCϵ, and PKCη and induces terminal differentiation and cornification of skin keratinocytes. Bryostatin 1 activates PKC transiently but fails to induce differentiation in primary keratinocytes and inhibits TPA-induced differentiation. Bryostatin 1 down-modulates PKCα, PKCϵ, and PKCη in a dose-dependent manner but protects PKCδ from activation and down-modulation by TPA (35, 36).

To test the hypothesis of a potential link between the intracellular responses elicited by PKC activation and the regulation and function of the $\alpha6\beta4$ integrin, we first examined the ability of PKC activation to induce PKC-mediated phosphorylation of the $\alpha6\beta4$ integrin complex. The phosphorylation of the $\alpha6\beta4$ integrin complex was examined in primary keratinocytes labeled with ^{32}P-P$_i$ and exposed to TPA or bryostatin 1 for 30 min. As seen in Fig. 1, a M_r 200,000 protein corresponding to the $\beta4$ chain was constitutively phosphorylated in untreated control keratinocytes, whereas no phosphorylation was detected in the $\alpha6$ chain. TPA and bryostatin 1 increased phosphorylation of both $\beta4$ and $\alpha6$ integrin subunits (Fig. 1*A*). As expected of a PKC-mediated event, treatment with GF-109203X, a specific inhibitor of PKC isoforms in keratinocytes, reduced significantly the phosphorylation on the $\beta4$ subunit and prevented the phosphorylation of the $\alpha6$ subunit induced by the PKC activators (Fig. 1*B*). Phosphorylated bands corresponding with the $\alpha6$ and $\beta4$ subunits were transferred to a PVDF membrane, and extracted proteins were subjected to phosphoamino acid analysis (Fig. 2*A*). Under control conditions, serine phosphorylation was detected in both the $\alpha6$ and the $\beta4$ chains. Phosphorylated tyrosine residues were detected to a lesser extent but only on the $\beta4$ subunit. Treatment of cells with bryostatin 1 increased serine phosphorylation in both subunits (Fig. 2*A*). In parallel experiments, $\alpha6\beta4$ immunoprecipitates were analyzed by anti-phosphotyrosine and anti-phosphoserine antibodies on Western blots (Fig. 2*B*), confirming that both TPA and bryostatin 1 increased serine phosphorylation of the $\beta4$ and $\alpha6$ subunits. However, an increase in tyrosine phosphorylation of the $\beta4$ subunit could not be detected by this method. Altogether, these results suggest a direct link between PKC activation and serine kinase-mediated phosphorylation of the $\alpha6\beta4$ integrin subunits.

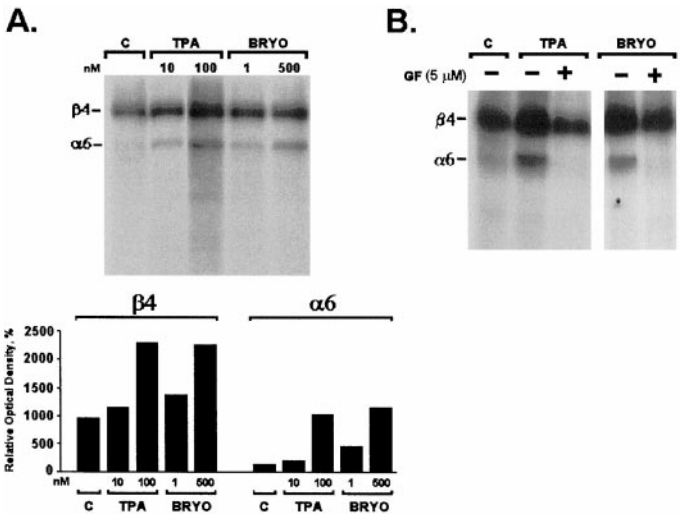

Fig. 1. Phosphorylation of $\beta4$ and $\alpha6$ integrin subunits by PKC activation. *A*, PKC activators phosphorylate $\beta4$ and $\alpha6$ integrin subunits in a dose-dependent manner. Primary mouse keratinocytes were isolated and plated in EMEM, 8% FCS, and 0.05 mM Ca^{2+} as described in "Materials and Methods." After 5 days in culture, cells were transferred to low phosphate-containing medium overnight, metabolically labeled with ^{32}P-P$_i$ for 4 h, and stimulated with TPA (10 or 100 nM) or bryostatin 1 (1 or 500 nM) for 30 min. Cells were extracted in RIPA buffer, and equal amounts of extracted proteins were subjected to immunoprecipitation with anti-$\alpha6$ antibody. Immunoprecipitates were analyzed by SDS-PAGE gels and transferred proteins exposed to X-ray film. The autoradiograph is representative of five different experiments. *Bottom panel*, relative absorbance of $\alpha6$ and $\beta4$ phosphorylation relative to the expression of the integrin subunits as determined by Western blot analysis of the same blots. *B*, GF-109203X, a specific inhibitor of PKC, prevents phosphorylation of $\beta4$ and $\alpha6$ by TPA and bryostatin 1. The 5-day-old primary keratinocytes were metabolically labeled with ^{32}P-P$_i$ for 4 h as described in Fig. 1*A*. At 3.5 h, 5 μM GF was added to the culture dishes, followed by 30-min stimulation with TPA (100 nM) or bryostatin 1 (500 nM). Cells were harvested in radioimmunoprecipitation assay buffer, and protein extracts were subjected to immunoprecipitation with anti-$\alpha6$ antibody. Immunoprecipitates were run on SDS-PAGE gels, and transferred proteins were exposed to X-ray film and analyzed by autoradiography. The autoradiograph is representative of three different experiments.

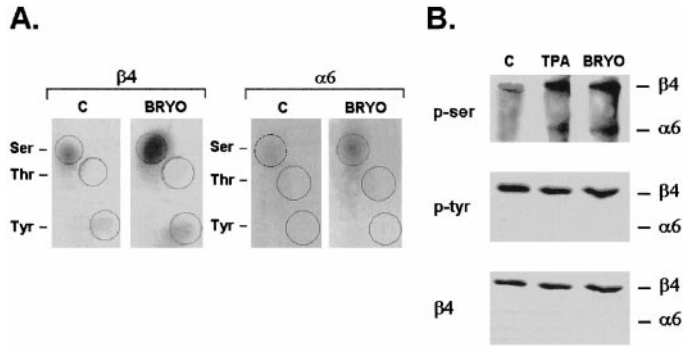

Fig. 2. PKC-mediated phosphorylation of the $\alpha6\beta4$ protein. *A*, phosphoamino analysis of $\alpha6$ and $\beta4$ proteins phosphorylated by bryostatin 1 stimulation. Primary mouse keratinocytes were metabolically labeled with ^{32}P-P$_i$ for 4 h as described in Fig. 1. After 30-min incubation with 500 nM bryostatin 1, cells were extracted and subjected to immunoprecipitation with anti-$\alpha6$ antibody. Immunoprecipitates were run on SDS-PAGE gels and transferred to PVDF membranes. Metabolically labeled bands associated with the $\alpha6$ and $\beta4$ proteins were excised and subjected to phosphoamino analysis as described in "Materials and Methods." The results presented in the autoradiograph are representative of four repeated experiments. *Marked circles* represent the locations of nonradioactive phosphoamino acid standards, as visualized by spraying with ninhydrin. *B*, immunoblotting of $\alpha6$ and $\beta4$ proteins stimulated with bryostatin 1 or TPA. Primary mouse keratinocytes were either untreated or stimulated with TPA (100 nM) or bryostatin 1 (500 nM) for 30 min. Samples were immunoprecipitated with anti-$\alpha6$ antibody, run on SDS-PAGE gels, and immunoblotted with polyclonal anti-phosphoserine, monoclonal anti-phosphotyrosine, and monoclonal anti-$\beta4$ antibodies. The blots are representative of eight different experiments.

To determine whether PKC activation and consequent phosphorylation modifies the association of the $\alpha6\beta4$ integrin with the cytoskeleton and the assembly of the hemidesmosomes, we examined the Triton X-100 solubility of $\alpha6\beta4$ extracted from bryostatin 1- and TPA-treated primary keratinocytes (Fig. 3*A*). The $\alpha6\beta4$ protein was distributed evenly between the soluble and the cytoskeletal fractions in untreated keratinocytes. Treatment of cells with TPA or bryostatin 1 caused a shift of the $\beta4$ subunit to the soluble fraction, which was apparent in cells 30 min after treatment with TPA and bryostatin 1 (Fig. 3*A*). These results suggest that PKC-mediated phosphorylation events could determine the subcellular distribution of $\alpha6\beta4$ and the composition of hemidesmosomes. Indeed, by immunofluorescent analysis, localization of $\alpha6\beta4$ to the hemidesmosomes was significantly reduced after TPA or bryostatin 1 treatment as compared with $\alpha6\beta4$ localization to hemidesmosomal densities in Triton X-100-extracted cells of control keratinocytes (Fig. 3*B*).

To determine whether $\alpha6\beta4$ is a specific substrate for a particular PKC isoform expressed in keratinocytes, the ability of specific PKCs to phosphorylate $\alpha6\beta4$ was tested in an *in vitro* immunokinase assay (Fig. 4). The $\alpha6$ and $\beta4$ subunits from membrane immunoprecipitates

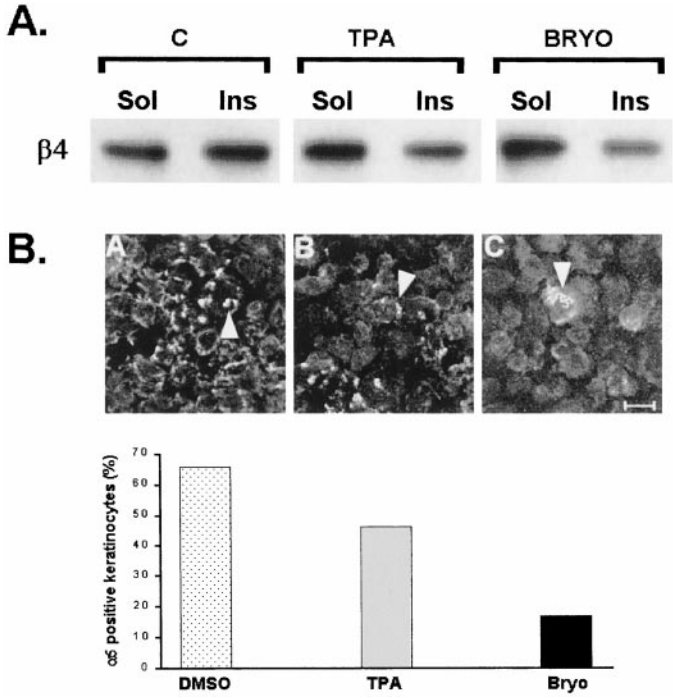

Fig. 3. PKC activation regulates α6β4 integrin association with the cytoskeleton. *A*, localization of β4 integrin subunit in subcellular fractions. Primary mouse keratinocytes were stimulated with bryostatin 1 or TPA for 30 min and differentially extracted in 0.02% (*Sol*) or 1% Triton X-100 (*Ins*) as described in "Materials and Methods." One hundred μg of protein were immunoprecipitated with anti-α6 antibody, and precipitates were run on SDS-PAGE gels and immunoblotted with anti-β4 antibody. The blot is representative of an experiment repeated seven times. *B*, immunolocalization of α6 integrin subunit to the hemidesmosome. Primary keratinocytes were plated on laminin 5-coated glass slides, and keratinocyte cultures were maintained in low Ca^{+2} EMEM for 48 h. Cultured keratinocytes were left untreated (*A*) or stimulated with TPA (100 nM; *B*) or bryostatin 1 (500 nM; *C*) for 30 min. After fixation and extraction with 0.2% Triton-X-100, slides were subjected to immunofluorescent analysis with anti-α6 antibodies as described in "Materials and Methods." *Arrowheads*, hemidesmosomal structures. *Bar*, 50 μm. *Bottom panel*, analysis of α6-positive keratinocytes as a percentage of the total cell population. α6-positive staining was designated as staining of α6 protein localized to the hemidesmosomes. Results were calculated by counting the α6-positive keratinocytes in at least five distinct fields in each experiment and represent data obtained from three independent experiments.

were used as phosphate acceptors in PKC assays using baculovirus recombinant PKC isoforms in the presence of [γ-^{32}P]ATP. A marked specific phosphorylation of α6 and β4 subunits was observed with PKCδ, even in the absence of TPA stimulation, but not with α, ζ, ϵ, and η. In contrast, all isoforms phosphorylated histone H6 *in vitro* (Fig. 4*B*). To directly assess the ability of PKCδ isoform to modify α6β4 protein *in vivo*, PKCδ and PKCα were overexpressed in primary keratinocytes using recombinant PKC adenoviruses (Fig. 5). Eighteen h after infection, exogenous PKC protein could be efficiently detected by immunofluorescent analysis in primary keratinocytes (Fig. 5). No change in expression levels of all other PKC isoforms was observed (data not shown). Similar to PKC activation by TPA and bryostatin 1, overexpression of PKCδ also interfered with the incorporation of α6β4 integrin into the hemidesmosomes, as determined by immunofluorescent analysis and shown in Fig. 5. PKCδ-overexpressing cells displayed reduced α6β4 staining associated with the hemidesmosomes after extraction with Triton X-100 (Fig. 5). In contrast, overexpression of PKCα induced hemidesmosomal localization of the α6β4 subunit (Fig. 5). Although both PKCδ and PKCα were efficiently expressed in primary keratinocytes (Fig. 6*A*) only in PKCδ-overexpressing keratinocytes, serine phosphorylation of the β4 subunit was constitutively elevated (Fig. 6*B*). This suggests that PKCδ but not PKCα is a kinase involved in phosphorylating β4 *in vivo* (Fig. 6*B*). The specificity of induction and detection of serine phosphorylation by the anti-phosphoserine antibody was further confirmed by alkaline phosphatase treatment, which specifically reduced detection of β4 serine phosphorylation (Fig. 7). Overexpression of PKCδ did not change the tyrosine phosphorylation of the β4 subunit (Fig. 7). To examine whether PKC-mediated phosphorylation could modify α6β4 integrin-dependent cell adhesion, keratinocytes overexpressing β-galactosidase, PKCα, PKCδ, or their kinase-inactive mutants were subjected to adhesion assay on laminin 5-coated dishes (Fig. 8). PKCδ-overexpressing keratinocytes significantly prevented adhesion to laminin 5. In contrast, PKCα overexpression resulted in increased attachment to laminin 5 (Fig. 8). The specific affects of distinct PKC isoform overexpression on keratinocyte adhesion was confirmed by using dominant-negative mutants of PKCα and PKCδ isoforms. Overexpression of the mutant isoforms abolished the affects on adhesion to laminin 5 obtained by overexpressing the wild-type PKC isoforms (Fig. 8). These results suggest that PKCδ and PKCα have distinct and contrasting effects on the hemidesmosomal localization of α6β4 and its effects on keratinocyte adhesion. Furthermore, PKCδ but not PKCα induces serine phosphorylation of the α6β4 integrin, which in turn reduced α6β4 localization to the hemidesmosomes and influenced keratinocyte adhesion to the underlying matrix.

DISCUSSION

Studies defining the biochemical pathways mediating intracellular signaling associated with integrin-ligand interactions are in progress in several laboratories. Protein phosphorylation is one of the earliest events detected in response to integrin stimulation in many cell types (37, 38). Modification of certain integrins by phosphorylation can induce conformational changes that enhance the affinity of the integrin for its ligand, inducing ligand binding and cytoskeletal interactions (20, 39). Epidermal basal cells interact with the basement membrane via the α6β4 integrin located within the hemidesmosome, a multiprotein receptor complex (40). In addition to adhesion, the hemidesmosome may function to transduce signals between the laminin-rich extracellular matrix and the cell interior (13, 19). Although

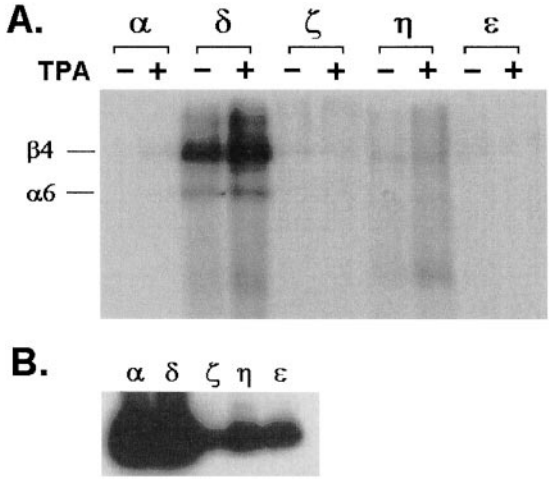

Fig. 4. Specific recombinant PKC isoforms are able to phosphorylate the α6β4 protein *in vitro*. *A*, 5-day-old primary keratinocytes were extracted in RIPA buffer, and α6β4 proteins were immunoprecipitated using anti-α6 antibodies. Precipitates were subjected to *in vitro* kinase assays using [γ-^{32}P]ATP and recombinant baculovirus-purified PKC isoforms α, δ, η, ϵ, and ζ as described in "Materials and Methods." After completion of the 10-min kinase reaction, beads containing the α6β4 complex were washed in RIPA buffer and separated on SDS-PAGE gels, and transferred proteins were exposed to X-ray film. Associated bands were visualized by autoradiography. The autoradiograph is representative of three separate experiments. *B*, recombinant PKC isoforms catalyzed the phosphorylation of histone H6 in *in vitro* kinase assays as described in *A* and in "Materials and Methods."

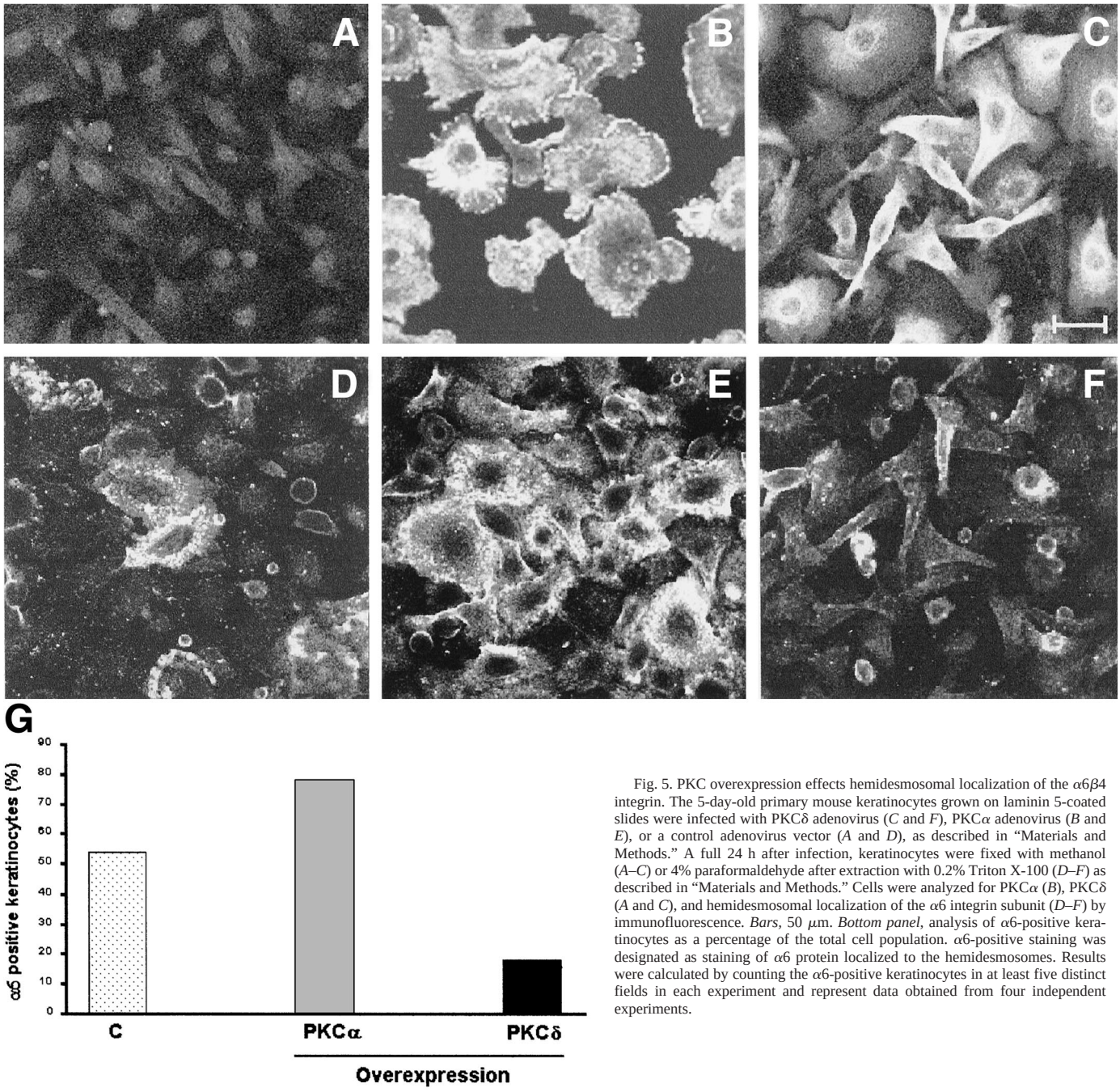

Fig. 5. PKC overexpression effects hemidesmosomal localization of the α6β4 integrin. The 5-day-old primary mouse keratinocytes grown on laminin 5-coated slides were infected with PKCδ adenovirus (*C* and *F*), PKCα adenovirus (*B* and *E*), or a control adenovirus vector (*A* and *D*), as described in "Materials and Methods." A full 24 h after infection, keratinocytes were fixed with methanol (*A–C*) or 4% paraformaldehyde after extraction with 0.2% Triton X-100 (*D–F*) as described in "Materials and Methods." Cells were analyzed for PKCα (*B*), PKCδ (*A* and *C*), and hemidesmosomal localization of the α6 integrin subunit (*D–F*) by immunofluorescence. *Bars*, 50 μm. *Bottom panel*, analysis of α6-positive keratinocytes as a percentage of the total cell population. α6-positive staining was designated as staining of α6 protein localized to the hemidesmosomes. Results were calculated by counting the α6-positive keratinocytes in at least five distinct fields in each experiment and represent data obtained from four independent experiments.

the molecular nature of the putative signaling events is not defined, serine/threonine kinases, mitogen-activated protein kinases, and the small GTPase family of proteins such as c-ras, R-ras, and Rho A have all been implicated (41–44). The contribution of PKC to inside-out signaling via integrins was suggested because treatment with PKC activators "activated" integrin binding to specific ligands, enhancing cell attachment and spreading (22, 23, 45). These effects could be blocked by specific PKC inhibitors. PKC activation also was shown to enhance cell adhesion through the formation of focal adhesions and regulation of integrin-cytoskeletal interactions (46, 47). In addition, PKC indirect stimulation by phospholipid metabolism including the generation of diacylglycerol and inositol-3-phosphate as well as activation of phosphatidylinositol 3-kinase in response to integrin stimulation have all been implicated in both integrin inside-out as well as outside-in signaling (22, 48). Finally, recently it has been shown that overexpression of distinct PKC isoforms regulated integrin-associated signaling (21). Specifically, regarding the α6β4 integrin, studies have shown that α6β4 ligation can cause activation of the ras as well as phosphatidylinositol 3-kinase-mediated pathways (19, 49, 50). However, although PKC activation is functionally linked to integrin action, previous studies in K562 cells failed to find a significant influence of PKC-mediated serine phosphorylation of the α6 integrin on ligand binding. Furthermore, serine mutations introduced into the cytoplasmic domain of the α6 subunit did not reduce adhesion to laminins (51, 52). However, in α6β1-expressing cells, the integrin localizes to focal contacts that differ significantly from the hemidesmosomes where α6β4 integrin is localized in basal keratinocytes. In agreement with this concept, in previous studies in A431 carcinoma cells expressing the α6β4 integrin, constitutive and PKC-induced serine phosphorylation of the β4 subunit was demonstrated (53, 54). Furthermore,

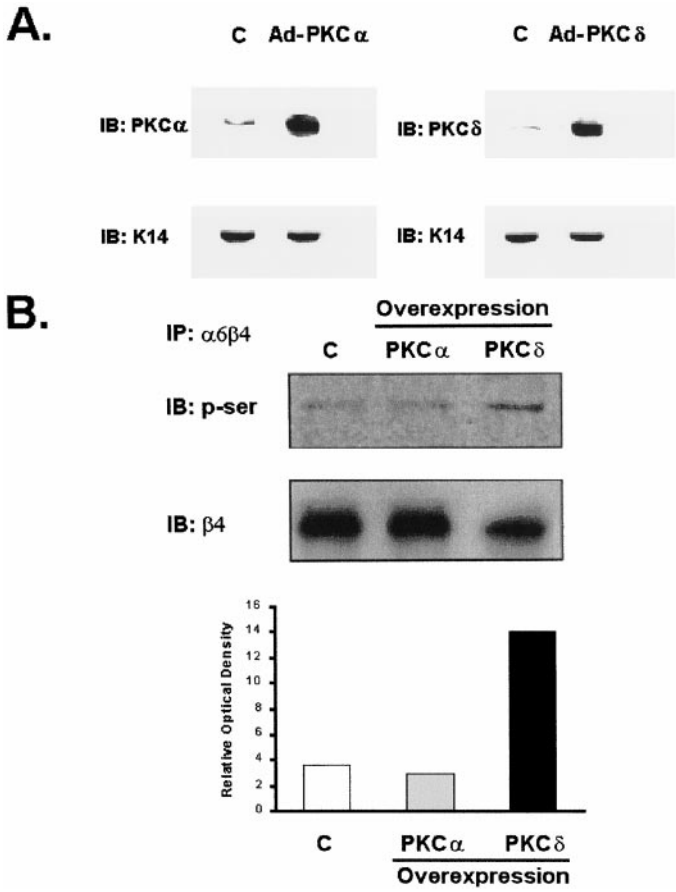

Fig. 6. Overexpression of PKCδ regulates α6β4 integrin phosphorylation. Primary mouse keratinocytes were either infected with control adenovirus vector or with PKCδ or PKCα adenoviruses as described in "Materials and Methods." A full 24 h after infection, cultures were extracted. *A*, 20 μg of total protein extracts were subjected to SDS-PAGE electrophoresis, and PKC expression was analyzed by immunoblotting (*IB*) with PKC-specific antibodies in control (*C*), PKCα (*Ad-PKCα*), or PKCδ (*Ad-PKCδ*) infected keratinocytes. Equal loading was verified by immunoblotting with anti-keratin 14 antibodies. The blot is representative of five distinct experiments. *B*, 300-μg protein samples were immunoprecipitated (*IP*) with anti-α6 antibody. Thereafter, samples were separated on SDS-PAGE gels and immunoblotted (*IB*) with polyclonal antiphosphoserine (*p-ser*) and monoclonal anti-β4 antibodies. The blot is representative of three distinct experiments. The increase in serine phosphorylation of the β4 subunit is presented as absorbance relative to the levels of β4 protein expression on the same blots. *Lane C*, control.

similar to our results, using primary keratinocytes, PKC-induced integrin phosphorylation was associated with decreased localization of α6β4 to the hemidesmosome. In contrast to our data, serine phosphorylation of the α6β4 integrin in A431 cells was associated with PKCα activation, and there was no effect on laminin attachment. Nonetheless, in their study, Rabinovitz *et al.* did not evaluate basal expression and activation of PKCα or other isoforms expressed in A431 cells (50). Furthermore, the ability of PKCα to specifically serine phosphorylate the α6β4 integrin *in vivo* or *in vitro* was not corroborated. Therefore, PKCα overexpression could have altered the distribution and activation of other PKC isoforms as PKCδ, which was shown to be expressed in A431 cells (55). Finally, because A431 cells represent an invasive carcinoma model cell system and as PKCα is known to be activated in epithelial cancer, the results in A431 cells could apply to the changing role of the PKCα isoform during cancer development. Therefore, the interpretation of results associated with PKC activation should be carefully evaluated because of the diverse roles of distinct PKC isoforms in different systems. As shown in this study, overexpression of PKCδ-mediated serine phsophorylation of the α6β4 integrin reduced its hemidesmosomal localization and decreased keratinocyte attachment to laminin. In contrast, PKCα overexpression did not increase α6β4 serine phosphorylation, stabilized α6β4 expression to the hemidesmosomes, and increased adhesion to laminin. These results are in agreement with studies where activation or inhibition of PKC produced differing or even contradictory results, depending on the model system or end points under study (27, 45, 48). For example in keratinocytes, inhibition of PKCα expression restores differentiation of neoplastic keratinocytes, whereas overexpression of PKCη induces keratinocyte differentiation (31, 33). PKCδ was shown to participate in apoptosis (56, 57), proliferation and differentiation (58, 59), and cell cycle arrest (60, 61) in specific cell types. Specific substrates have been associated with PKCδ phosphorylation *in vitro* including elongation factor (62), adducin (63), and a high affinity immunoglobulin E receptor (64). Another specific feature of PKCδ is its ability to be phosphorylated on tyrosine residues, in response to cytokines such as platelet-derived growth factor (65), neurotransmitters (58), epidermal growth factor (66), and by the oncogenic form of c-Ha-ras (67) and v-Src (68). Thus, PKCδ could be involved in complex cross-talk between tyrosine kinases and other signaling components, a property of PKCδ that could be relevant in modification of

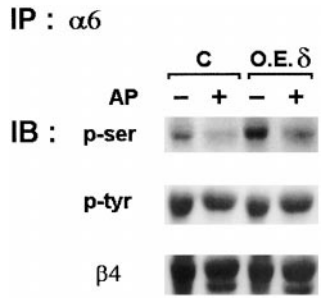

Fig. 7. β4 integrin subunit phosphorylation by PKCδ. Primary mouse keratinocytes were either infected with control adenovirus vector or with PKCδ adenovirus, as described in "Materials and Methods." A full 24 h after infection, cultures were extracted, and 300-μg protein samples were immunoprecipitated (*IP*) with anti-α6 antibody. Immunoprecipitates were either treated (+) or untreated (−) with alkaline phosphatase for 15 min, as described in "Materials and Methods." Thereafter, samples were separated on SDS-PAGE gels and immunoblotted (*IB*) with polyclonal anti-phosphoserine (*p-ser*), monoclonal anti-phosphotyrosine (*p-tyr*), or monoclonal anti-β4 antibodies. The blot is representative of three distinct experiments. *AP*, alkaline phosphatase; *C*, control; *O.E.δ*, overexpression of PKCδ isoform.

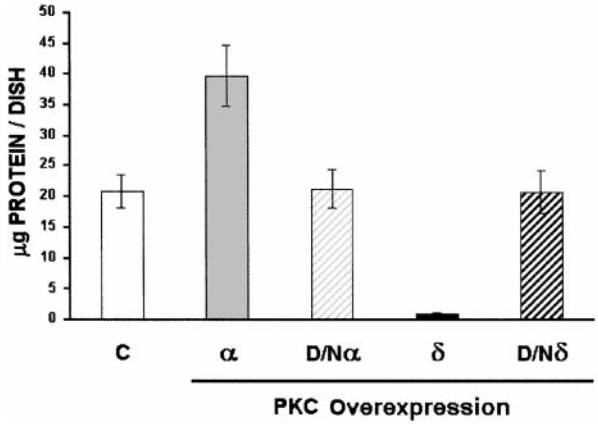

Fig. 8. Overexpression of PKCδ affects keratinocyte attachment to laminin 5. Primary mouse keratinocytes were either untreated or infected with control adenovirus, PKCδ, PKCα adenovirus, or their dominant-negative, kinase-inactive mutants (*D/N*), as described in "Materials and Methods." A full 24 h after infection, keratinocytes were trypsinized briefly, resuspended in 0.1% BSA in 0.05 mM Ca^{+2} EMEM medium without FCS, and reattached to laminin 5-coated dishes. After 5 min incubation at 37°C, unattached cells were removed, and plates were rinsed twice with PBS. Cell count was quantitated using a microprotein assay, as described in "Materials and Methods." Results are presented as μg protein/dish, calculated as a means of four wells in each experiment, which was repeated three times; *bars*, SD. *C*, control.

integrin function, as seen in the current study. Interestingly, in A431 cells, constitutive phosphorylation of the β4 subunit was also predominantly on serine residues, and tyrosine phosphorylation of the β4 subunit was induced only upon stimulation with epidermal growth factor (54). Therefore, although growth factor receptors and integrins often mediate tyrosine phosphorylation of integrin receptor subunits and associated proteins via similar downstream pathways (37, 54, 69), a multifunctional serine kinase such as PKCδ that interacts with tyrosine kinase pathways could have dual regulatory functions in basal keratinocytes. This could be achieved by PKC induction of phosphorylation of serine sites directly and tyrosine sites indirectly. The specificity of PKC action in keratinocytes could also be related to the integrin cytoplasmic domains. It is well known that the 1000 amino acid cytoplasmic tail of the β4 subunit regulates specialized features of the α6β4 integrin (13). Recent studies have identified several distinct domains on the connecting segment and cytoplasmic tail of the α6 and β4 proteins. Distinct functions have been associated with specific domains including: regulation of integrin heterodimerization, localization to the hemidesmosome, hemidesmosomal assembly, adhesion to laminin substrates, and association with signaling molecules (70–75). Interestingly, in these studies ligand binding was not necessarily linked to hemidesmosomal localization of the α6β4 integrin or to hemidesmosome assembly (72, 73, 76). Therefore, phosphorylation of serine sites in the specialized domains could indeed be influencing hemidesmosomal localization of the α6β4 integrin and hemidesmosomal assembly. Our results suggest that phosphorylation on serine sites of α6β4 integrin could implicate a direct action of PKCδ in regulating the interaction of α6β4 with the hemidesmosomes of skin keratinocytes. This specificity of action could also contribute to the regulation of PKC-mediated keratinocyte cell detachment during differentiation. Of interest are the results of Ohba *et al.* (33) suggesting a definitive role for PKCδ and PKCη in keratinocyte maturation. Because PKCη is most abundant in granular cells that lack hemidesmosomes, PKCδ in the basal cells may be the major isoform responsible for initiating the loss of cell attachment to the basement membrane in the early stages of keratinocyte differentiation. Therefore, studies designed to assess the contribution of activation of specific PKC isoforms to the signaling mechanism involving the α6β4 integrin should reveal important mechanisms designed to maintain the integrity of the epidermis.

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