In this study we have investigated hyaluronan (HA)-CD44 interaction with protein kinase N-γ (PKNγ), a small GTPase (Rac1)-activated serine/threonine kinase in human keratinocytes. By using a variety of biochemical and molecular biological techniques, we have determined that CD44 and PKNγ kinase (molecular mass ~120 kDa) are physically linked in vivo. The binding of HA to keratinocytes promotes PKNγ kinase recruitment into a complex with CD44 and subsequently stimulates Rac1-mediated PKNγ kinase activity. The Rac1-activated PKNγ in turn increases threonine (but not serine) phosphorylation of phospholipase C (PLC) γ1 and up-regulates PLCγ1 activity leading to the onset of intracellular Ca2+ mobilization. HA/CDC44-activated Rac1-PKNγ also phosphorylates the cytoskeletal protein, cortactin, at serine/threonine residues. The phosphorylation of cortactin by Rac1-PKNγ attenuates its ability to cross-link filamentous actin in vitro. Further analyses indicate that the N-terminal antiparallel coiled-coil (ACC) domains of PKNγ interact directly with Rac1 in a GTP-dependent manner. The binding of HA to CD44 induces PKNγ association with endogenous Rac1 and its activity in keratinocytes. Transfection of keratinocytes with PKNγ-ACCcDNA reduces HA-mediated recruitment of endogenous Rac1 to PKNγ and blocks PKNγ activity. These findings suggest that the PKNγ-ACC fragment acts as a potent competitive inhibitor of endogenous Rac1 binding to PKNγ in vivo. Most important, the PKNγ-ACC fragment functions as a strong dominant-negative mutant that effectively inhibits HA/CDC44-mediated PKNγ phosphorylation of PLCγ1 and cortactin as well as keratinocyte signaling (e.g., Ca2+ mobilization and cortactin-actin binding) and cellular functioning (e.g. cell-cell adhesion and differentiation). Taken together, these findings strongly suggest that hyaluronan-CD44 interaction with Rac1-PKNγ plays a pivotal role in PLCγ1-regulated Ca2+ signaling and cortactin-cytoskeleton function required for keratinocyte cell-cell adhesion and differentiation.

In the epidermis, extracellular matrix (ECM) components form an integral part of hemidesmosomes and mediate keratinocyte attachment to the underlying basement membrane. Hyaluronan (HA) is the major glycosaminoglycan in the extracellular matrix (ECM) of most mammalian tissues including epidermis and dermis (1–3) and HA has been implicated in skin epidermal function (1–3). However, the cellular and molecular mechanism by which keratinocytes respond to HA is not understood.

The predominant receptor for HA on the cell surface of keratinocytes is CD44 (1–12). CD44 is encoded by a single gene that contains 19 exons (5). The most common form, CD44s (CD44 standard form), contains exons 1–5 (N-terminal 150 amino acids), exons 15 and 16 (membrane proximal 85 amino acids), exon 17 (transmembrane domain), and a portion of exons 17 and 19 (cytoplasmic tail, 70 amino acids) (5). Of the 19 exons, 12 exons can be alternatively spliced (5). Most often, the alternative splicing occurs between exons 5 and 15 leading to an insertion in tandem of one or more variant exons (exon 6–exon 14:v1–v10) within the membrane-proximal region of the extracellular domain (5). For example, keratinocytes contain additional exons v3–v10, which are inserted into the CD44s transcripts (6–12). This isoform has been designated as CD44v3–10 (or Epican) (6–12). Various skin cancer cells and tissues express different CD44 variant (CD44v) isoforms (e.g., CD44v3 and CD44v10) in addition to CD44s and CD44v3–10 (Epican) (6–12). These CD44 isoforms have the same amino acid sequences at the two ends of the molecule but differ within the CD44 membrane-proximal region located at the external side of the membrane (5–12). Different CD44 isoforms are also further modified by extensive N- and O-glycosylations and glycosaminoglycan additions (13–15). Consequently, both post-translational modifications and/or alternative splicing within the CD44 molecule appear to determine the functional outcome of this important molecule.

The binding of HA to CD44 causes cells to adhere to extracellular matrix (ECM) components and has also been implicated in the stimulation of several different biological activities (16–19). The intracellular domain of CD44 binds to signaling proteins, such as RhoA- and Rac1-specific guanine nucleotide exchange factors (20–23), and cytoskeletal proteins including...
Ankyrin (16–19) and the ERM proteins (ezrin, radixin, and moesin) (24). In addition, the cytoplasmic domain of CD44 is known to be tightly coupled with c-Src kinase, which promotes tyrosine phosphorylation of cortactin (a filamentous actin (F-actin)-associated protein) and cytoskeleton function resulting in specific structural changes in the plasma membrane and cell migration (18, 19, 61). These findings strongly suggest that the CD44 molecule provides a direct linkage between the ECM and the cytoskeleton.

RhoGTPases (small molecular weight GTPases (e.g., RhoA, Rac1, and Cdc42)) act as molecular switches that alternate between GTP- and GDP-bound states. The “activated” GTP-bound enzymes preferentially interact with downstream effectors to modulate the activities of the effectors (25).

Several enzymes have been identified as possible downstream targets for Rac1 signaling. One such enzyme is protein kinase N-γ (PKNγ) (also called PRK2), which belongs to a family of serine-threonine kinases known to interact with Rac1 in a GTP-dependent manner, and it shares a great deal of sequence homology with protein kinase C in the C-terminal region (29–31). The N-terminal region of PKN contains three homologous stretches of ∼70 amino acids (relatively rich in charged residues) and forms an antiparallel coiled-coil fold (ACC domain) (29). This ACC domain has been shown to interact with RhoGTPases such as RhoA and Rac1 (and to a lesser extent with Cdc42) (29–31). The C-terminal region contains the C2-like region, which functions as an auto-inhibitory domain (32). The ACC and the C2-like domains, together with the catalytic domain, are conserved among the PKN family members (29–32). In keratinocytes, RhoA-activated PKNγ has been found to be involved in Fyn/Src kinase-regulated cell-cell adhesion during Ca2+-induced differentiation (33). The question of whether HA/CD44-induced Rac1 activation and PKNγ-targeted downstream effectors are involved in regulating keratinocyte cell-cell adhesion and differentiation has not been clearly addressed.

HA binding to CD44-expressing cells also stimulates intracellular Ca2+-mobilization, which is a prerequisite for the onset of a variety of biological activities (34–36). Although the cellular and molecular mechanisms involved in HA/CD44-mediated Ca2+-signaling are currently not well understood, one of the likely pathways involves the phosphatidylinositol cascade that leads to Ca2+-release from intracellular stores (37). In response to different stimuli (including HA), phosphoinositide-specific phospholipase Cs (PLCs) hydrolyze phosphatidyl-4,5-bisphosphate into diacylglycerol and inositol 1,4,5-trisphosphate (IP3) (38). IP3, as a second messenger, binds to the IP3 receptor and induces the release of Ca2+ from intracellular stores (34–37). At least four major PLC families exist, PLC-β, PLC-γ, PLC-δ, and PLC-ε, and each family type has a number of subtypes (38, 39). One of the best characterized PLCs in keratinocytes is PLC-γ1. This enzyme is involved in inositol lipid signaling and keratinocyte differentiation (40, 41). Specifically, Xie and Bikle (40) have shown that blockage of PLC-γ1 expression with antisense PLC-γ1 cDNA or down-regulation of PLC-γ1 activity with a specific inhibitor (41) can effectively impair Ca2+-induced keratinocyte differentiation. These findings suggest that intracellular PLC-γ1-mediated Ca2+-mobilization plays a critical role in regulating keratinocyte differentiation.

Although RacGTPase has been closely associated with remodeling of cortical actin (in particular, cortactin-cytoskeleton interaction) (28, 33, 42, 43), Ca2+ signaling (44), and cell-cell adhesion (35), Rac-specific effectors and their roles in regulating HA/CD44-mediated keratinocyte functions have not yet been characterized. In this study, we have investigated HA/CD44-mediated Rac1-PKNγ kinase signaling and its downstream effector function (PLC-γ1-mediated Ca2+ mobilization and cortactin-actin interaction) during human keratinocyte cell-cell adhesion and differentiation.

MATERIALS AND METHODS

Cell Culture Model Systems—Normal human keratinocytes were isolated from neonatal human foreskins and grown in serum-free keratinocyte growth medium (KGM, Clonetics, San Diego, CA) as described previously (45). Briefly, keratinocytes were isolated from newborn human foreskins by trypsinization (0.25% trypsin, 4°C, overnight), and primary cultures were established in KGM containing 0.07 mM calcium. After the first and second passages, keratinocytes were incubated with KGM containing 0.03 mM calcium and used in the experiments described below.

Antibodies and Reagents—Monoclonal anti-human CD44 antibody (clone, 020; iso type, IgG1) obtained from CMB-TECH, Inc., San Francisco (used in this study recognizes a common determinant of the CD44 class of glycoproteins including CD44s (the standard form), CD44 variant isoforms, and CD44s,α (Epic). Rabbit anti-phosphothreonine antibody and rabbit anti-phosphoserine antibody were obtained from Zymed Laboratories Inc. Polyclonal mouse anti-involucrin and polyclonal mouse anti-transglutaminase were purchased from Covance Inc. (Princeton, NJ) and Neomarkers (Fremont, CA), respectively. Rabbit anti-PKNγ, rabbit anti-PLC-γ1 mouse anti-RhoA, and mouse anti-Rac1 were obtained from Santa Cruz Biotechnology. Mouse anti-His and mouse anti-cortactin antibody (clone 4F11) were purchased from Invitrogen and Upstate Biotechnology, Inc. (Lake Placid, NY), respectively. Several other reagents including GST-tagged RhoA, GST-tagged Rac1, and inhibitors (e.g. U73122, Xestospongin C, and BAPTA) were obtained from Calbiochem. Rooster comb hyaluronic (HA) and cytochalasin D were purchased from Sigma. High molecular weight HA polymers (∼106 daltons) were purified by gel filtration column chromatography using Sephacryl S1000 column. The purity of high molecular weight HA polymers used in our experiments was further verified by anion exchange high performance liquid chromatography. No small HA fragments were detected in these preparations.

Method for Preparing His-tagged Dominant-negative Form (Contain ACC Domain) of PKNγ-kinesin—The cDNA fragment encoding the ACC domain of PKNγ (amino acids 98–228) was subcloned into pCRII vector using the T-tailed PCR using two specific primers (5′-GGTGCACGAGAATTTGGATGA-3′ and 5′-CTTGGACGTTGCACTTGTTGGTG-3′). The PCR product digested with EcoRI and HindIII was purified with QiAquick PCR purification kit (Qiagen). The PKNγ-ACC fragment cDNA was subsequently cloned into pCDNA3.1/HisC vector that contains Xpress epitope to create His-tagged PKNγ-ACC/cDNA. The inserted ACC domain sequence was confirmed by nucleotide sequencing analyses. This His-tagged PKNγ-ACC cDNA was then used for transient expression in keratinocytes as described below.

Cell Transfection—To establish a transient expression system, keratinocytes were transfected with various plasmid DNAs (e.g. His-tagged PKNγ-ACC domain/pcDNA vector alone) using TransIT, Keratinocyte Transfection Reagent (Mirus, Madison, WI) according to those procedures described previously (46). Briefly, keratinocytes were plated at a density of 2 × 106 cells per 100-mm dish and transfected with 25 μg/dish plasmid cDNA using TransIT-Keratinocyte Transfection Reagent. Transfected cells were grown in the culture medium containing 0.01 mM CaCl2 for at least 3 days. Various transfectants were then analyzed for their protein expression (e.g. PKNγ-ACC-related proteins) by immunoblot, PKNγ activity, and keratinocyte functional assays as described below.

Measurement of Rac1 Activation—Keratinocytes (∼5 × 106 cells) were resuspended in a buffer containing 118 mM KCl, 5 mM HEPES, 0.4 mM CaCl2, 1 mM EGTA, 1.2 mM magnesium acetate, 1.2 mM KHPO4, 25 mM Tris-HCl (pH 7.4), 20 mg/ml bovine serum albumin. An aliquot of the cell suspension was added to the electrophoresis cuvette and incubated at 4°C for 5–10 min followed by addition of [32p]GTP-S (12.5 μCi). Subsequently, cells were electrophoresed at 25 microamper and 2.0
Kv/cm followed by incubating with 50 \mu g/ml HA (in the presence or absence of rat anti-CD44 antibody (50 \mu g/ml) or without any HA treatment at 37°C for 10 min. Subsequently, \[^{35}S\]GTP\(_S\)-labeled cells were washed in PBS (pH 7.4) and solubilized in 1% Nonidet P-40 with 1 m\(M\) GTP, 25 m\(M\) magnesium acetate, and protease inhibitors in PBS (pH 7.4). Nonidet P-40-solubilized cells were then incubated with mouse anti-Rac1 IgG (5 \mu g/ml) plus goat anti-mouse conjugated beads. The amount of \[^{35}S\]GTP\(_S\)-Rac1 associated with anti-Rac1-conjugated immunobeads was measured using a gamma counter. The values expressed represent an average of triplicate determinations of five experiments with a standard deviation of less than 5%.

**Immunoblotting and Immunoprecipitation Procedures**—Keratinocytes (untreated transfected cells, PKN\(\gamma\)-ACCcDNA-transfected cells, or vector-transfected cells) grown in 0.03 m\(M\) Ca\(^{2+}\) were treated with no HA or with HA (50 \mu g/ml) or pretreated with anti-CD44 antibody followed by adding HA (50 \mu g/ml) at 37°C for various time intervals (5, 10, 15, and 30 min and 2, 24, 36, and 48 h). These cells were then solubilized in 50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM MgCl\(_2\), 0.2 mM Na\(_2\)VO\(_4\), 0.2 mM phenylmethylsulfonyl fluoride, 10 \mu g/ml leupeptin, and 5 \mu g/ml aprotinin. The sample was then centrifuged at 14,927 \(\times\) g for 15 min, and the supernatant was analyzed by SDS-PAGE in a 5 or 7.5% polyacrylamide gel. Separated polyepitope were then transferred onto nitrocellulose filters. After blocking nonspecific sites with 2% bovine serum albumin, the nitrocellulose filters were incubated with each of the specific immunoreagents (e.g. rat anti-CD44 IgG (5 \mu g/ml), mouse anti-involucrin (5 \mu g/ml), mouse anti-transglutaminase (5 \mu g/ml), rabbit anti-PKN\(\gamma\) (5 \mu g/ml), rabbit anti-PLC\(_1\) (5 \mu g/ml), mouse anti-cortactin (5 \mu g/ml), mouse anti-His antibody (5 \mu g/ml)) followed by incubating with horseradish peroxidase-labeled goat anti-rat IgG, horseradish peroxidase-labeled goat anti-mouse IgG, or horse- radish peroxidase-labeled goat anti- mouse IgG. The blots were then developed by the ECL\(^{\text{TM}}\) system (Amersham Biosciences). For analyzing the recruitment of endogenous PKN\(\gamma\) into CD44 complex, keratinocytes (either treated with HA (50 \mu g/ml) or without any HA treatment) were solubilized by 1% Nonidet P-40 followed by anti-PKN\(\gamma\)-mediated immunoblotting.

Untransfected keratinocytes (untreated or pretreated with the various inhibitors U73122 (1 \mu M), Xestospongin C (1 \mu M), BAPTA/AM (1 \mu M), cytochalasin D (20 \mu g/ml), and colchicine (1 \times 10\(^{-5}\) M) for 1 h at 37°C) or keratinocytes transfected with PKN\(\gamma\)-ACCcDNA (or vector alone) were incubated with HA (50 \mu g/ml) at 37°C for 10 min (or pretreated with anti-CD44 antibody followed by adding HA (50 \mu g/ml) or incubated with no HA). These cells were then immunoprecipitated with anti-PLC\(_1\) (or anti-cortactin) antibody followed by immunoblotting with anti-phosphoserine or anti-phosphothreonine, respectively. Subsequent blots were then developed using the ECL reagent according to the manufacturer’s instructions. During these immunological analyses, an equal amount of cellular protein (50 \mu g/ml) immunoprecipitated with the antibody was applied to SDS-PAGE followed by immunoblot analyses.

In some experiments, keratinocytes (e.g. untransfected or transfected with His-tagged PKN\(\gamma\)-ACCcDNA or vector only) were incubated with HA (50 \mu g/ml) at 37°C for 10 min (or pretreated with anti-CD44 antibody followed by adding HA (50 \mu g/ml) or incubated with no HA). These cells were then immunoprecipitated with rabbit anti-PKN\(\gamma\) IgG (or mouse anti-His IgG) followed by immunoblotting with mouse anti-

**TABLE I**

**Detection of Rac1 activation in keratinocytes.**

| Treatments                          | Amount of \[^{35}S\]GTP\(_S\) bound to Rac1 | % control (

**FIG. 1.** Characterization of PKN\(\gamma\) and CD44-PKN\(\gamma\) complex in human keratinocytes. A, detection of PKN\(\gamma\) in keratinocytes. Keratinocytes were solubilized by 1% Nonidet P-40 buffer followed by anti-PKN\(\gamma\)-mediated immunoblotting analyses. Lane 1, immunoblot of keratinocytes with preimmune serum; lane 2, immunoblot of keratinocytes with anti-PKN\(\gamma\)-mediated immunoblotting using anti-PKN\(\gamma\) antibody (a) or anti-CD44 antibody (b), respectively. Lane 3, detection of PKN\(\gamma\) in the complex isolated from untreated keratinocytes by anti-CD44-mediated immunoprecipitation followed by immunoblotting with anti-PKN\(\gamma\) antibody (a) or anti-CD44 antibody (b), respectively. Lane 4, detection of PKN\(\gamma\) in the complex isolated from untreated keratinocytes by anti-CD44-mediated immunoprecipitation followed by immunoblotting with anti-PKN\(\gamma\) antibody (a) or anti-CD44 antibody (b), respectively. B, demonstration of RhoGT-Pase binding properties of PKN\(\gamma\). PKN\(\gamma\) (isolated from keratinocytes) was incubated with GDP- or GTP-loaded forms of either Rac1 or RhoA-GST-conjugated beads. Proteins associated with Rac1/RhoA-GST beads were then analyzed by immunoblotting with anti-PKN\(\gamma\) as described under “Materials and Methods.” C, analysis of the CD44-PKN\(\gamma\) complex in keratinocytes. Keratinocytes (untreated or treated with HA) were solubilized by 1% Nonidet P-40 buffer and immunoprecipitated (IP) with anti-CD44 antibody followed by immunoblotting using anti-PKN\(\gamma\) antibody (a) or anti-CD44 antibody (b), respectively. Lane 1, detection of PKN\(\gamma\) in the complex isolated from untreated keratinocytes by anti-CD44-mediated immunoprecipitation followed by immunoblotting with anti-PKN\(\gamma\) antibody (a) or reblotting with anti-CD44 antibody (b) as a loading control. Lane 2, detection of PKN\(\gamma\) in the complex isolated from untreated keratinocytes by anti-CD44-mediated immunoprecipitation followed by immunoblotting with anti-PKN\(\gamma\) antibody (a) or reblotting with anti-CD44 antibody (b) as a loading control.

Rac1 IgG/mouse anti-RhoA IgG or reblotted with anti-PKN\(\gamma\) IgG (or anti-His IgG) followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (or goat anti-rabbit IgG) (1:10,000 dilution). Cell lysates of keratinocytes (e.g. transfected with His-tagged PKN\(\gamma\)-ACCcDNA or vector only) were also immunoblotted with anti-PKN\(\gamma\) IgG and anti-His IgG, respectively. These blots were then developed using ECL reagent (Amersham Biosciences).

**PKN\(\gamma\)-mediated Protein Phosphorylation Assay in Vitro**—The PKN\(\gamma\) kinase reaction was carried out in 50 \mu l of the reaction mixture containing 40 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM dithiorethiol, 7 mM MgCl\(_2\), 0.1% CHAPS, 0.4 \mu M calyculin A, 100 \mu M ATP, purified enzymes (e.g. 100 ng of PKN\(\gamma\) keratinocytes isolated from untransfected keratinocytes or keratinocytes transfected with His-tagged PKN\(\gamma\)-ACCcDNA or vector only) and 1 \mu g of PLC\(_1\) keratinocytes (or 1 \mu g of keratinocytes cortactin) in the presence of RhoA or Rac1 (incubated with 200 \mu M GDP}
and PKN anti-cortactin beads) were revealed by anti-phosphoserine/anti-phosphoserine/anti-phospho-protein kinase (PK) blot (B, c immunoblot (Lane 2, c). RhoA/Rac1-mediated immunoblot (Lane 1, c). Lane 3, PLC-1 phosphorylation by GDP-Rac1-treated PKNγ using anti-phosphothreonine (a)/anti-phosphoserine (b)-mediated immunoblot or anti-PLC-1-mediated immunoblot (c). Lane 4, PLC-1 phosphorylation by GTP-Rac1-treated PKNγ using anti-phosphothreonine (a)/anti-phosphoserine (b)-mediated immunoblot or anti-PLC-1-mediated immunoblot (c). B, keratinocytes treated with HA (or pretreated with anti-CD44 plus HA or no HA) were solubilized by Nonidet P-40 and immunoprecipitated with anti-PLC-γ antibody followed by immunoblotting with anti-phosphothreonine/anti-phosphoserine, respectively. Lane 1, detection of PLC-1 phosphorylation by anti-PLC-1-mediated immunoprecipitation followed by immunoblotting with anti-phosphothreonine (a)/anti-phosphoserine (b) or (replotting with anti-PLC-1 as a loading control) in keratinocytes treated with no HA. Lane 2, detection of PLC-1 phosphorylation by anti-PLC-1-mediated immunoprecipitation followed by immunoblotting with anti-phosphothreonine (a)/anti-phosphoserine (b) or (replotting with anti-PLC-1 as a loading control) in keratinocytes treated with HA. Lane 3, detection of PLC-1 phosphorylation by anti-PLC-1-mediated immunoprecipitation followed by immunoblotting with anti-phosphothreonine (a)/anti-phosphoserine (b) or (replotting with anti-PLC-1 as a loading control) in keratinocytes pretreated with anti-CD44 plus HA or no HA treated with HA (50 μg/ml) at 37 °C for 10 min (or pretreated with anti-CD44 antibody followed by adding HA (50 μg/ml), or incubated with no HA), was also incubated in this enzyme assay mixture. Reactions were carried out for 15 min and terminated by adding trichloroacetic acid and 2 mg/ml BSA. Precipitated proteins were removed by centrifugation. Radioactive [3H]IP3 (liberated from [3H]PIP2) in the trichloroacetic acid-soluble supernatant fraction was analyzed by scintillation counting. PIP2 hydrolysis was linear for 15 min under the conditions used. The values expressed represent an average of triplicate determinations of 3–5 experiments with an S.D. of less than ±5%.

**PLC-γ Activity Assay**—The procedures for analyzing the activity of unphosphorylated or phosphorylated PLC-1 were similar to those described previously (47). Briefly, keratinocytes were solubilized in Nonidet P-40 buffer in the presence of protease inhibitors plus 1 μM Na3VO4, 1 μM sodium orthovanadate, and 1 μM okadaic acid. Lysates were spun at 5000 × g, and the supernatants were collected. PLC-1 was immunoprecipitated from the supernatants using rabbit anti-PLC-1-conjugated beads. These PLC-1-conjugated beads were then phosphorylated with PKNγ in the presence of GDP/GTP-bound RhoA or GDP/GTP-bound Rac1 or no PKNγ (as described above). Subsequently, unphosphorylated or RhoA/Rac1-PKNγ phosphorylated PLC-1 immunobeads were incubated with vesicles containing 100 μM phosphatidylethanolamine, 10 μM phosphatidyserine, 10 μM PI, and 0.028 μM [3H]PIP2 in PLC activity buffer (25 mM HEPES, 80 mM KCl, 3 mM EGTA, 0.5 mM dithiothreitol, pH 7.0) at 37 °C. In some cases, PLC-γ1 isolated from keratinocytes (transfected or transfected with PKNγ-ACC1DNA or vector alone), which were treated with HA (50 μg/ml) at 37 °C for 10 min (or pretreated with anti-CD44 antibody followed by adding HA (50 μg/ml) or incubated with no HA), was also incubated in this enzyme assay mixture. Reactions were carried out for 15 min and terminated by adding 10% trichloroacetic acid and 2 mg/ml bovine serum albumin. Precipitated proteins were removed by centrifugation. Radioactive [3H]IP3 (liberated from [3H]PIP2) in the trichloroacetic acid-soluble supernatant fraction was analyzed by scintillation counting. PIP2 hydrolysis was linear for 15 min under the conditions used.

**Double Immunofluorescence Staining**—Keratinocytes (transfected with PKNγ-ACC1DNA or vector alone) were incubated with HA (50 μg/ml) at 37 °C for 36 h (or pretreated with anti-CD44 antibody followed by adding HA (50 μg/ml) or incubated with no HA) followed by fixing with 2% paraformaldehyde. Subsequently, these cells were stained with Texas Red-labeled rat anti-CD44 IgG, Texas Red-labeled cells were then rendered permeable by ethanol treatment followed by incubating with fluorescein (FITC)-conjugated immunoreagents (e.g. anti-PKNγ IgG, anti-PLC-γ1 IgG, anti-cortactin IgG). To detect nonspecific antibody binding, Texas Red-conjugated anti-CD44 or FITC-conjugated immunoreagents (e.g. anti-PKNγ IgG, anti-PLC-γ1 IgG, and anti-cortactin IgG) were added after the cells were stained with Texas Red-labeled rat anti-CD44 IgG. Texas Red-labeled cells were then examined with a confocal laser scanning microscope.
HA/CD44 and Rac1-PKNγ in Keratinocyte PLCγCA2+ Signaling

Measurement of Intracellular Ca2+ Mobilization—Keratinocytes (untreated or pretreated with the various inhibitors U73122 (1 μM), Xestospongin C (1 μM), BAPTA/AM (1 μM), cytochalasin D (20 μg/ml), and colchicine (1 × 10−5 M) at 37°C for 1 h or transfected with PKNγ-ACCcDNA or vector alone) were first incubated with 10 μM Fura-2/AM (Calbiochem) for 1 h at room temperature in a buffer solution containing 145 mM NaCl, 5 mM KCl, 0.1 mM MgCl2, 5 mM glucose, and 15 mM HEPES (pH 7.3) in the presence or absence of 1 mM CaCl2. Cells were subsequently washed three times with the same buffer. Cells (105 cells/ml) (with or without rat anti-CD44 antibody (1 μg/ml)) resuspended in 0.1 M phosphate-buffered saline (pH 7.0) were incubated simultaneously with an equal volume of 0.1 M phosphate-buffered saline (pH 7.0) containing HA (50 μg/ml) (Sigma) into a 20-μl chamber placed along with unphosphorylated cortactin isolated from vector-transfected cells (unphosphorylated cortactin (control) is designated as 100%). In some cases, unphosphorylated cortactin isolated from vector-transfected cells was centrifuged at 25,000 × g for 10 min, and reprecipitated by combining 250 μl of buffer to the supernatants by using rabbit anti-PLCγ1 antibody followed by adding HA (50 μg/ml), or incubated with no HA) was also incubated with this enzyme assay mixture. Reactions were performed for 15 min and terminated by adding trichloroacetic acid and 2 mM EDTA. Precipitated proteins were removed by centrifugation. Radioactive [3H]PPIγ (liberated from [3H]PPIγ) in the trichloroacetic acid-soluble supernatant fraction was analyzed by using scintillation counting. PPIγ hydrolysis was linear for 15 min under the conditions used. The values expressed represent an average of triplicate determinations of 3–5 experiments with an S.D. of less than ±5%.

RESULTS

HA-stimulated Rac1 Signaling and CD44-PKNγ Kinase Association in Keratinocytes—The binding of HA to CD44 is known to induce important changes in certain RhoGTPases such as Rac1 (22, 23, 26). By using an in vitro [35S]GTPγS binding assay, we have determined that Rac1, isolated from human keratinocytes, displays specific guanine nucleotide binding activity (Table I). In particular, we have demonstrated that the addition of HA to CD44-containing keratinocytes causes almost a 3-fold increase in the binding of [35S]GTPγS to Rac1 as compared with the amount of binding present in untreated keratinocytes (Table I) or in keratinocytes pretreated with anti-CD44 antibody followed by HA treatment (Table I). These findings suggest that HA and CD44 are directly involved in the activation of Rac1 in human keratinocytes.

Eukaryotic cells contain a large number of protein serine/threonine kinases, which appear to play important roles in signal transduction required for various cell functions. One of the known downstream targets for the GTP-bound (activated) form of Rac1 is PKNγ kinase (also called PRK2 kinase) (29–31). By using a specific anti-PKNγ-mediated immunoblot technique, we have found significant levels of the PKNγ (molecular mass = 120 kDa) are expressed in keratinocytes (Fig. 1A, lane 1). We believe that the PKNγ detected in keratinocytes revealed by anti-PKNγ-mediated immunoblot is specific because no protein is detected in these cells using preimmune rabbit IgG (Fig. 1A, lane 1).

Several lines of evidence indicate that PKNγ binds to RhogT-Pases (e.g. Rac1 and RhoA) and that the activity of PKNγ is up-regulated in the presence of the GTP-bound form of Rac1 or RhoA (29–31). In this study we have incubated purified PKNγ (isolated from keratinocytes) with GDP- or GTP-loaded forms of either Rac1- or RhoA-GST conjugated beads. Proteins associated with Rac1 or RhoA-GST beads were then analyzed by immunoblotting with anti-PKNγ (Fig. 1B). Our results indicate that a large amount of PKNγ is associated with GTP-bound Rac1-GST beads (Fig. 1B, lane 4), whereas very little PKNγ is bound to GDP-bound Rac1 (Fig. 1B, lane 3). We have also noted that PKNγ binds GDP-bound RhoA (Fig. 1B, lane 1) and GTP-bound RhoA (Fig. 1B, lane 2) equally well. These results are consistent with previous findings that determined that PKNγ-Rac1 interaction is GDP-dependent but PKNγ-RhoA binding is GDP-independent (30).

In addition, we have addressed the question of whether there is an interaction between CD44 and PKNγ in human keratinocytes. To this end we first carried out anti-CD44-mediated immunoprecipitation followed by anti-PKNγ immunoblot (Fig. 1C, a) or anti-CD44 immunoblot (Fig. 1C, b), respectively, using untreated keratinocytes. Our results indicate that a low level of PKNγ (Fig. 1C, a, lane 1) is present in the anti-CD44-immunoprecipitated materials (Fig. 1C, a, lane 1). Subsequently, we have determined that HA treatment causes the recruitment of a significant amount of PKNγ (Fig. 1C, a, lane 2) into the CD44-PKNγ complex (Fig. 1C, b, lane 2). These findings clearly establish that CD44 and PKNγ are closely associated with each other in vivo, particularly following HA treatment of the keratinocytes.
PLC1 Serves as a New Cellular Substrate for Rac1-dependent PKNγ Kinase and Regulates Ca2+ Signaling in Keratinocytes—HA-CD44 interaction has been shown to be tightly coupled with intracellular Ca2+ mobilization pathways in many different cell types (34–36). In these signaling events, PLCγ1 initially hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) into IP3 which leads to Ca2+ release from intracellular stores (37–39). In searching for a possible linkage between HA/CD44-mediated Rac1 signaling, PKNγ kinase, and intracellular Ca2+ regulation, we have demonstrated that PKNγ isolated from keratinocytes is capable of phosphorylating PLCγ1 at threonine residues (as detected by anti-PLCγ1-mediated immunoprecipitation followed by anti-phosphothreonine-mediated immunoblot) (Fig. 2A, a) but not serine residues (as detected by anti-PLCγ1-mediated immunoprecipitation followed by anti-phosphoserine-mediated immunoblot) (Fig. 2A, b). These PKNγ-mediated PLCγ1 phosphorylation experiments were carried out in the presence of RhoA (either unactivated RhoA (GDP-bound RhoA) (Fig. 2A, a, lane 1, and A, b, lane 1) or activated RhoA (GTP-bound RhoA) (Fig. 2A, a, lane 2, and A, b, lane 2)) or Rac1 (either unactivated Rac1 (GDP-bound Rac1) (Fig. 2A, a, lane 3, and A, b, lane 3) or activated Rac1 (GTP-bound Rac1) (Fig. 2A, a, lane 4 and A, b, lane 4)). Specifically, the level of PKNγ-mediated threonine phosphorylation (but not serine) of PLCγ1 is significantly up-regulated if activated Rac1 (GTP-bound Rac1) is present (Fig. 2A, a, lane 4, and A, b, lane 4). A much lesser up-regulation of PKNγ-mediated PLCγ1 phosphorylation is detected in the presence of GDP-bound Rac1 or GDP/GTP-bound RhoA (Fig. 2A, a, lanes 1–3, and A, b, lanes 1–3). These findings indicate that PLCγ1 serves as a cellular substrate for Rac1 (and to a lesser extent RhoA)-dependent kinases such as PKNγ in vitro.

FIG. 3. Measurement of Ca2+ mobilization in keratinocytes. A, intracellular Ca2+ mobilization was measured by a fluorescence spectrophotometer using cells loaded with 10 μM Fura-2/AM as described under “Materials and Methods.” Subsequently, Fura-2-labeled cells (preincubated with 0.1 mM EGTA) were treated with HA (indicated by arrowhead) (A), pretreated with anti-CD44 antibody followed by HA treatment (indicated by arrowhead) (B), pretreated with U-73122 followed by HA treatment (indicated by arrowhead) (C), pretreated with Xestospongin C (Xesto. C) followed by HA treatment (indicated by arrowhead) (D), or pretreated with BAPTA followed by HA treatment (indicated by arrowhead) (E).
It is noted that the level of threonine phosphorylation of PLCγ1 in vivo (as detected by anti-PLCγ1-mediated immunoprecipitation followed by anti-phosphothreonine-mediated immunoblot) is also greatly enhanced in keratinocytes treated with HA (Fig. 2B, a, lane 2). In contrast, PLCγ1 threonine phosphorylation is very low in untreated keratinocytes (Fig. 2B, a, lane 1) or in those keratinocytes pretreated with anti-CD44 antibody followed by HA treatment (Fig. 2B, a, lane 3). Very little serine phosphorylation of PLCγ1 is present in keratinocytes treated with HA (Fig. 2B, b, lane 1) (or pretreated with anti-CD44 antibody followed by HA treatment (Fig. 2B, b, lane 3) or no HA treatment (Fig. 2B, b, lane 1)). Therefore, we believe that threonine (but not serine) phosphorylation of PLCγ1 also occurs in vivo during HA-CD44 interaction in keratinocytes.

Next, we examined the potential impact of PKNγ-mediated phosphorylation on the regulation of PLCγ1 activity (measured by PLCγ1-mediated IP3 production). Our results indicate that the level of IP3 production is slightly elevated using PLCγ1 phosphorylated with GDP-RhoA or GTP-RhoA-activated PKNγ (Table II). PLCγ1 activity is greatly stimulated using PLCγ1 treated with GTP-bound Rac1-activated PKNγ (Table II) (but not PKNγ in the presence of GDP-bound Rac1 (Table II)). The amount of IP3 production induced by PLCγ1 activated by GTP-Rac1-PKNγ is also significantly higher than PLCγ1-activated by GDP-RhoA-PKNγ or GTP-RhoA-PKNγ (Table II). In control experiments, we found that unphosphorylated PLCγ1 (in the absence of PKNγ) (Table II) can induce only a basal level or very minimal amounts of IP3 production. Therefore, we conclude that Rac1-activated (and to a lesser extent RhoA-activated) PKNγ phosphorylation of PLCγ1 is directly involved in the activation of PLCγ1 activity. Moreover, we have determined that the stimulation of PLCγ1 activity occurs immediately after HA addition to CD44-expressing keratinocytes (Table III). Preincubation with anti-CD44 antibody blocks PLCγ1 activation in keratinocytes treated with HA (Table III) indicating that HA induces PLCγ1 activity in a CD44-dependent manner in keratinocytes.

It has been well documented that PLCγ1-mediated IP3 production triggers IP3 receptor-regulated intracellular Ca2+ mobilization (37–39). In this study, we have used the fluorescence indicator, Fura-2, to measure the intracellular free Ca2+ concentration after HA binding to CD44-containing keratinocytes. The ratio of the fluorescence signal from Fura-2 at 340 and 380 nm excitation was monitored and used to determine the intracellular Ca2+ concentration. Our results clearly show that the intracellular Ca2+ concentration is elevated after the addition of HA to keratinocytes followed by a period of continuous Ca2+ influx (Fig. 3A). These data indicate that intracellular Ca2+ mobilization is one of the early signaling events to occur following HA binding to keratinocytes. Pretreatment of keratinocytes with anti-CD44 antibody (Fig. 3B) or various inhibitors (e.g. U73122 (a PLC inhibitor) (Fig. 3C), Xestospongin C (a membrane-permeable blocker of IP3 receptor-mediated Ca2+ release) (Fig. 3D), or BAPTA (a membrane-permeable Ca2+ chelator) (Fig. 3E)) effectively blocks HA-mediated intracellular Ca2+ elevation. These findings strongly suggest that Ca2+ signaling in keratinocytes involves HA/CD44-dependent and PLC-regulated processes.

Cortactin Serves As Another New Cellular Substrate for Rac1-dependent PKNγ Kinase and Interacts with F-actin—Although GTPase-activated PKNγ has been shown to regulate actin cytoskeleton organization (29–31), the specific cytoskeletal components regulated by PKNγ in HA-mediated CD44 signaling in keratinocytes have yet to be clearly defined. In this study, we have observed that PKNγ kinase isolated from keratinocytes is capable of phosphorylating cortactin at both threonine (Fig. 4A, a) and serine residues (Fig. 4A, b) (as detected by anti-phosphothreonine and anti-phosphoserine-mediated immunoblot), respectively, followed by reblotting with anti-cortactin anti-

![Fig. 4. Detection of cortactin phosphorylation by PKNγ in vitro (A) and in vivo (B).](http://www.jbc.org/)

Lane 1, cortactin phosphorylation by GDP-RhoA-treated PKNγ using anti-phosphothreonine (a)/anti-phosphoserine (b)-mediated immunoblot or anti-cortactin-mediated immunoblot (c). Lane 2, cortactin phosphorylation by GTP-RhoA-treated PKNγ using anti-phosphothreonine (a)/anti-phosphoserine (b)-mediated immunoblot or anti-cortactin-mediated immunoblot (c). Lane 3, cortactin phosphorylation by GDP-Rac1-treated PKNγ using anti-phosphothreonine (a)/anti-phosphoserine (b)-mediated immunoblot or anti-cortactin-mediated immunoblot (c). Lane 4, cortactin phosphorylation by GTP-Rac1-treated PKNγ using anti-phosphothreonine (a)/anti-phosphoserine (b)-mediated immunoblot or anti-cortactin-mediated immunoblot (c). B, keratinocytes treated with HA (or pretreated with anti-CD44 plus HA or no HA) were solubilized by Nonidet P-40 and immunoprecipitated with anti-cortactin antibody followed by immunoblotting with anti-phosphothreonine/anti-phosphoserine, respectively. Lane 1, detection of cortactin phosphorylation by anti-cortactin-mediated immunoprecipitation followed by immunoblotting with anti-phosphothreonine (a)/anti-phosphoserine (b) or (reblotting with anti-cortactin (as a loading control) (c) in keratinocytes treated with no HA. Lane 2, detection of cortactin phosphorylation by anti-cortactin-mediated immunoprecipitation followed by immunoblotting with anti-phosphothreonine (a)/anti-phosphoserine (b) or (reblotting with anti-cortactin as a loading control) (c) in keratinocytes treated with HA. Lane 3, detection of cortactin phosphorylation by anti-cortactin-mediated immunoprecipitation followed by immunoblotting with anti-phosphothreonine (a)/anti-phosphoserine (b) or (reblotting with anti-cortactin as a loading control) (c) in keratinocytes pretreated with anti-CD44 followed by HA treatment.
body (Fig. 4A, c)) in the presence of activated Rac1 (GTP-bound Rac1) (Fig. 4A, a, lane 4, and A, b, lane 4) (and to a lesser extent GDP-Rac1 (Fig. 4A, a, lane 3, and A, b, lane 3)). The level of threonine/serine phosphorylation of cortactin appears to be relatively low when PKNγ was incubated with GDP-bound RhoA (Fig. 4A, a, lane 1, and A, b, lane 1) or GTP-bound RhoA (Fig. 4A, a, lane 2, and A, b, lane 2). These results suggest that cortactin phosphorylation by PKNγ requires Rac1 in a GTP-dependent manner and involves RhoA in a GTP-independent fashion in vitro.

Further analyses indicate that the level of threonine and serine phosphorylation of cortactin in vivo (as detected by anti-cortactin-mediated immunoprecipitation followed by immunoblotting with anti-threonine (Fig. 4B, a), anti-serine (Fig. 4B, a), or anti-cortactin (Fig. 4B, c), respectively) is significantly enhanced in keratinocytes treated with HA (Fig. 4B, a, lane 2, and B, b, lane 2). In contrast, cortactin threonine and serine phosphorylation is relatively low in keratinocytes without any HA treatment (Fig. 4B, a, lane 1, and B, b, lane 1) or those keratinocytes pretreated with anti-CD44 antibody followed by HA treatment (Fig. 4B, a, lane 3, and B, b, lane 3). These observations strongly support the conclusion that HA-mediated cortactin phosphorylation is CD44-dependent.

Cortactin is known to be an actin-binding protein (48, 49). Our data indicate that unphosphorylated cortactin (in the absence of PKNγ) (Fig. 5a) or cortactin with low level phosphorylation by PKNγ, in the presence of unactivated RhoA (GDP-bound RhoA) (Fig. 5b), activated RhoA (GTP-bound RhoA) (Fig. 5c), or unactivated Rac1 (GDP-bound Rac1) (Fig. 5d), is capable of cross-linking the actin filaments into bundles in vitro. However, serine/threonine phosphorylation of cortactin by Rac1 (GTP-bound form)-activated PKNγ downregulates its ability to cross-link filamentous actin (Fig. 5e). These results are consistent with previous findings suggesting cortactin (phosphorylated versus unphosphorylated forms) plays an important role as an F-actin modulator required for cytosome reorganization.

Effects of PKNγ-ACC Domains on PLCγ1/Cortactin Phosphorylation, Ca2+ Signaling, Cytoskeleton Interaction, Keratinocyte Cell-Cell Adhesion, and Differentiation—Previous studies (29–31) have indicated that the N-terminal ACC domains of PKNγ (Fig. 6A, a) bind to RhoGTPases (e.g. Rac1) and up-regulate the activity of the PKNγ in the presence of the GTP-bound form of Rac1. These findings suggest that PKNγ-ACC domains play an important role in regulating the activation of Rac1-dependent PKNγ kinase. In order to demonstrate the role of PKNγ-ACC domains in regulating keratinocyte signaling, PKNγ-ACC domain cDNA was cloned into a His-tagged expression vector pcDNA 3.1 (Fig. 6A, b) followed by a transient transfection of PKNγ-ACCcDNA into keratinocytes. First, we incubated purified His-tagged PKNγ-ACC domains with GDP- and GTP-loaded forms of either Rac1- or RhoA-GST-conjugated beads. Bound proteins were detected by immunoblotting with anti-His antibody (Fig. 6B). Our results indicate that PKNγ-ACC domains interact with GDP-bound RhoA (Fig. 6B, lane 1) and GTP-bound RhoA (Fig. 6B, lane 2) equally well. However, PKNγ-ACC domains appear to preferentially bind to GTP-bound Rac1 (Fig. 6B, lane 4) but not GDP-bound Rac1 (Fig. 6B, lane 3). These results have confirmed the hypothesis that PKNγ-ACC domains are interacting with Rac1 in a GTP-dependent manner but are associating with RhoA in a GTP-independent manner. We have also observed that both endogenous Rac1 (Fig. 6C, a, lane 2) and RhoA (Fig. 6C, b, lane 2) are bound to His-tagged PKNγ-ACC fragments in keratinocytes transfected with PKNγ-ACCcDNA, but no association between Rac1 (Fig. 6C, a, lane 1/RhoA (Fig. 6C, b, lane 1) and His-PKNγ-ACC fragment is detected in vector-transfected cells using anti-His-mediated immunoprecipitation followed by anti-Rac1 or anti-RhoA-mediated immunoblot, respectively. These results establish the existence of a close association between RhoGTPases (e.g. Rac1 and RhoA) and PKNγ-ACC fragments in vivo.

In order to examine the effects of PKNγ-ACC fragment overexpression on Rac1-RhoA interaction with endogenous PKNγ, we have transfected keratinocytes with His-tagged PKNγ-ACCcDNA (or vector alone). Our data indicate that endogenous PKNγ is expressed at comparable levels in vector-transfected (Fig. 6D (I), lanes 1–3) and PKNγ-ACCcDNA-transfected keratinocytes (Fig. 6D (I), lanes 4–6) treated with HA, pretreated with anti-CD44 antibody plus HA, or no HA treatment. Furthermore, we have observed that a low level of endogenous Rac1 and PKNγ is co-precipi-

![Fig. 5. Measurement of the F-actin cross-linking activity of cortactin.](http://www.jbc.org)
FIG. 6. Interaction between the PKNγ-ACC fragment and Rac1/RhoA in vitro and detection of PKNγ-Rac1-RhoA complex in PKNγ-ACC cDNA-transfected/vector-transfected cells. A, illustration of PKNγ full-length (a) and His-tagged PKNγ-ACC cDNA construct (b). B, characterization of the binding interaction between His-tagged PKNγ-ACC and GST Rac1/GST-RhoA beads in vitro. Lane 1, anti-His-mediated immunoblot of PKNγ-ACC fragment associated with GDP-bound RhoA beads. Lane 2, anti-His-mediated immunoblot of PKNγ-ACC fragment associated with GTP-bound RhoA beads. Lane 3, anti-His-mediated immunoblot of PKNγ-ACC fragment associated with GDP-bound Rac1 beads. Lane 4, anti-His-mediated immunoblot of PKNγ-ACC fragment associated with GTP-bound Rac1 beads. C, analyses of PKNγ-ACC-RhoA-Rac1 complex formation in keratinocyte transfectants. Keratinocytes (transfected with His-tagged PKNγ-ACC cDNA or vector alone) were solubilized by 1% Nonidet P-40 buffer. Cell lysates were then used for anti-His-mediated immunoprecipitation followed by immunoblotting with anti-Rac1/RhoA antibody or anti-His antibody, respectively, as described under “Materials and Methods.” a, Anti-Rac1-mediated immunoblot of anti-His-mediated immunoprecipitated materials isolated from keratinocytes transfected with vector alone (lane 1) or with His-tagged PKNγ-ACC cDNA (lane 2). b, anti-RhoA-mediated immunoblot of anti-His-mediated immunoprecipitated materials isolated from keratinocytes transfected with vector alone (lane 1) or with His-tagged PKNγ-ACC cDNA (lane 2). c, anti-His-mediated immunoblot of anti-RhoA-mediated immunoprecipitated materials isolated from keratinocytes transfected with vector alone (lane 1) or with His-tagged PKNγ-ACC cDNA (lane 2). D, analyses of PKNγ expression (I) and PKNγ-ACC-RhoA-Rac1 complex formation (II) in keratinocyte transfectants. Keratinocytes transfected with His-tagged PKNγ-ACC cDNA or vector alone (untreated, treated with HA, or pretreated with anti-CD44 followed by HA addition) were solubilized by 1% Nonidet P-40 buffer. Cell lysates were then used for anti-PKNγ-mediated immunoblot or anti-PKNγ-mediated immunoprecipitation followed by immunoblotting with anti-Rac1/RhoA antibody or anti-PKNγ antibody, respectively, as described under “Materials and Methods.” (I), anti-PKNγ-mediated immunoblot of the cell lysate isolated from keratinocytes transfected with vector alone (untreated lane 1), treated with HA (lane 2) or pretreated with anti-CD44 followed by HA addition (lane 3) or His-tagged PKNγ-ACC cDNA (untreated lane 4), treated with HA (lane 5), or treated with...
tated in untreated vector-transfected keratinocytes (Fig. 6D (II), a, lane 1, and (II), c, lane 1) or pretreated with anti-CD44 antibody followed by HA treatment ((Fig. 6D (II), a, lane 3, and (II), c, lane 3)). Moreover, we have demonstrated that HA is capable of promoting an additional recruitment of endogenous Rac1 (Fig. 6D (II), a, lane 3) into a complex with PKNY (Fig. 6D (II), c, lane 2) in vector-transfected keratinocytes. Although endogenous RhoA is found to be complexed with PKNY in vector-transfected keratinocytes treated with no HA (Fig. 6D (II), b, lane 1) or pretreated with anti-CD44 antibody followed by adding HA (Fig. 6D (II), b, lane 3), very little HA-induced recruitment of endogenous RhoA (Fig. 6D (II), b, lane 2) into PKNY (Fig. 6D (II), c, lane 2) is detected in these cells. These results suggest that the recruitment of endogenous Rac1 (and to a lesser extent RhoA) into PKNY in keratinocytes is HA-dependent and CD44-specific. Moreover, our observations indicate that transfection of keratinocytes with PKNY-ACCcDNA not only causes a significant inhibition of HA/CD44-mediated recruitment of endogenous Rac1 (Fig. 6D (II), a, lane 5) to PKNY (Fig. 6D (II), c, lane 5) but also blocks the basal level of endogenous Rac1/RhoA association with PKNY (Fig. 6D (II), a and b, lane 4, and (II), a and b, lane 6). These findings established the fact that the PKNY fragment containing the ACC domains acts as a potent competitive inhibitor for endogenous Rac1 binding to PKNY in vivo during HA-mediated CD44 signaling.

Furthermore, we have demonstrated that threonine (but not serine) phosphorylation of PLCγ1 (Fig. 7, a–c, lane 2) is greatly

![Diagram](image.png)
enhanced in vector-transfected keratinocytes treated with HA as compared with those observed in untreated vector-transfected cells (Fig. 7, a–c, lane 1) or pretreated with anti-CD44 antibody followed by HA treatment (Fig. 7, a–c, lane 3). Both PLCγ1 activity (measured by the production of IP$_3$) and intracellular Ca$^{2+}$ mobilization are also up-regulated in vector-transfected keratinocytes treated with HA (Table IV). In contrast, the level of PLCγ1 phosphorylation, PLCγ1-mediated IP$_3$ production, and Ca$^{2+}$ mobilization is relatively low in untreated vector-transfected keratinocytes (Table IV) or pretreated with anti-CD44 antibody plus HA (data not shown). However, overexpression of the PKNγ-ACC fragment by transfecting keratinocytes with PKNγ-ACCcDNA not only significantly reduces the ability of PLCγ1 to respond to HA/CD44-stimulated phosphorylation (Fig. 7, a–c, lanes 4–6) but also down-regulates enzymatic activity (measured by PLCγ1-mediated IP$_3$ production) and Ca$^{2+}$ mobilization (Table IV). Therefore, we believe that the ACC fragment of PKNγ by interfering endogenous Rac1 association with PKNγ (Fig. 6D, II, a, lanes 4–6) functions as a potent dominant-negative mutant that effectively inhibits HA/CD44-induced Rac1-PKNγ activation and PLCγ1 phosphorylation required for Ca$^{2+}$ signaling.

In addition, we have determined that both threonine and serine phosphorylation of cortactin (Fig. 7, d–f, lane 2) are stimulated in vector-transfected keratinocytes treated with HA. In contrast, very little cortacin phosphorylation is observed in untreated vector-transfected cells (Fig. 7, d–f, lane 1) or pretreated with anti-CD44 antibody followed by HA treatment (Fig. 7, d–f, lane 3). Transfection of keratinocytes with the His-tagged PKNγ-ACC fragment (Fig. 7g, lanes 4–6) effectively inhibits HA-mediated and CD44-dependent cortacin phosphorylation (Fig. 7, d–f, lanes 4–6). Further analyses indicate that unphosphorylated cortacin (treated by PKNγ kinase isolated from vector-transfected cells in the absence of HA) promotes F-actin cross-linking activity in vitro (Table IV). In contrast, PKNγ kinase-phosphorylated cortacin (treated by PKNγ kinase isolated from vector-transfected cells in the presence of HA) significantly reduces its ability to cross-link F-actin (Table IV). Moreover, we have shown that unphosphorylated cortacin (isolated from keratinocytes transfected with PKNγ-ACC fragment cDNA in the presence or absence of HA) retains its F-actin cross-linking properties (Table IV). These results suggest that the ACC fragment of PKNγ by blocking Rac1 binding to PKNγ (Fig. 6D, II, a, lanes 4–6) acts as a dominant-negative mutant that down-regulates HA/CD44-induced Rac1-PKNγ activation and cortacin phosphorylation required for cytoskeletal function.

By using double immunofluorescence staining and confocal microscopic analyses, we have determined that CD44 is expressed on the cell surface of vector-transfected keratinocytes (Fig. 8A, a) and that PKNγ kinase (Fig. 8A, c) is diffusely distributed in both the cell membrane and the cytoplasm of untreated vector-transfected keratinocytes. A low level of CD44-PKNγ co-localization is detected in these keratinocytes in the absence of HA (Fig. 8A, e). We have also observed that both PLCγ1 (Fig. 8B, c) and cortatin (Fig. 8C, c) are distributed in the cytosol and not complexed with CD44 (Fig. 8B, a, and C, a) in the plasma membrane in untreated vector-transfected keratinocytes. Very little CD44 co-localization with PLCγ1 (Fig. 8B, c and c) and cortatin (Fig. 8C, c) was observed in the untreated keratinocytes. Upon HA addition, keratinocyte cell-cell adhesion occurs (Fig. 8A, b, d, and f) and there is a dramatic recruitment of PKNγ (Fig. 8A, d) from the cytosol to CD44-containing plasma membranes (Fig. 8A, b and f) in vector-transfected keratinocytes. These results demonstrate that HA induces a close association between CD44 and PKNγ in the plasma membrane of vector-transfected keratinocytes. Both PLCγ1 (Fig. 8B, d) and cortatin (Fig. 8C, d) are also co-localized with CD44 (Fig. 8, B, b, d, and f) in the plasma membranes and cell-cell adhesion junction in vector-transfected keratinocytes treated with HA. In contrast, we have noted that overexpression of PKNγ-ACC by transfecting keratinocytes with PKNγ-ACCcDNA significantly reduces HA-mediated cell-cell adhesion (Fig. 8, D–F; Tables V–VII) and PKNγ (Fig. 8D, c and d) co-localization with CD44 (Fig. 8, D, a and b, and D, e and f). Similarly, the interaction between CD44 (Fig. 8, E, a and b, and F, a and b) and PLCγ1 (Fig. 8E, c and d) was observed at the cell-cell adhesion site (Fig. 8, E, e and f, and F, e and f) is greatly inhibited in these transfected cells in the absence or the presence of HA. Treatment of keratinocytes with rat anti-CD44 antibody (but not normal rat IgG) or various agents such as U73122 (a PLC inhibitor), Xestospongin C (an IP$_3$ receptor blocker), BAPTA (a membrane-permeable Ca$^{2+}$ chelator), or cytochalasin D (a microfilament disrupting agent known to prevent actin polymerization) effectively inhibits HA/CD44-induced cell-cell adhesion (Tables V–VII). Transfection of keratinocytes with PKNγ-ACCcDNA also readily impairs HA-dependent cell-cell adhesion of keratinocytes (Tables V–VII). These observations strongly suggest that CD44-mediated PKNγ activation together with PLCγ1-regulated Ca$^{2+}$ signaling and cytoskeleton function are involved in HA-dependent cell-cell adhesion in keratinocytes.

Expression of both involucrin and transglutaminase has been closely associated with the progression of keratinocyte differentiation (1, 2, 51–53). Immunoblot analyses using anti-involucrin and anti-transglutaminase antibody indicate that the binding of HA to CD44 promotes the expression of early differentiation markers such as involucrin (Fig. 9A, a, lanes 1 and 2) and a terminal differentiation marker such as transglutaminase (Fig. 9A, b, lanes 1 and 2). The fact that treatment of keratinocytes with rat anti-CD44 antibody (but not normal rat IgG) (Fig. 9A, a and b, lanes 3 and 4) or various drugs (e.g. the PLC inhibitor U73122 (Fig. 9A, a and b, lanes 5 and 6), the IP$_3$ receptor blocker Xestospongin C (Fig. 9, a and b, lanes 7 and 8), Ca$^{2+}$ chelator BAPTA (Fig. 9, a and b, lanes 9 and 10), and cytoskeleton drug cytochalasin D (Fig. 9, a and b, lanes 11 and 12)) also effectively blocks HA and CD44-mediated keratinocyte differentiation (Fig. 9A). These results indicate that PLC, IP$_3$ receptor-mediated Ca$^{2+}$ activity, and cytoskeletal
HA/CD44 and Rac1-PKNγ in Keratinocyte PLCγ/CA2⁺ Signaling

DISCUSSION

HA is a well known constituent of connective tissue extracellular matrices and, in particular, is abundant in stratified squamous epithelia including the epidermis of skin (1-3). HA is often anchored to CD44, a structurally/functionally important surface receptor that contains HA-binding site(s) (1-12). Transgenic mice expressing an antisense CD44 construct in their skin showed a significant reduction in endogenous HA on the keratinocyte cell surface and loss of keratinocyte functions (54). These findings suggest that both HA and CD44 play an important role in normal epidermal physiology and keratinocyte differentiation. However, current knowledge concerning the mechanism of HA/CD44 interaction in epidermal keratinocytes and the role that this interaction may have in keratinocyte functions and epidermal physiology remains not well understood.

Previous studies by Bikle and co-workers (40) have shown that a multiphasic increase in intracellular CA2⁺ concentration following the addition of exogenous 1-2 mM Ca2⁺ to keratinocytes is required for Ca2⁺-induced keratinocyte differentiation. Inhibition of intracellular Ca2⁺ mobilization by treatment with the Ca2⁺ chelator BAPTA prevents Ca2⁺-induced differentiation (41). These findings suggest that intracellular Ca2⁺ mobilization plays a critical role in regulating keratinocyte differentiation (40, 41). The binding of HA to CD44 increases intracellular Ca2⁺ levels in many cell types including keratinocytes (Fig. 3). The mechanism by which keratinocytes respond to HA/CD44-induced Ca2⁺ signaling is not fully established. PLCγ1 is known to generate 2-s messengers from the hydrolysis of phosphatidylinositol 4,5-bisphosphate, diacylglycerol, and IP3. Diacylglycerol is an activator of protein kinase C, and IP3 interacts with intracellular membrane receptors (e.g. IP3 receptors) leading to an increased release of stored Ca2⁺ ions (37-39). Most important, targeted deletion of PLCγ1 causes embryonic lethality in mice (55). These findings suggest that PLCγ1 plays a critical role in Ca2⁺ signaling and developmental processes. In fact, keratinocytes are known to possess functionally active inositol lipid signaling systems, one of which involves PLCγ1 signaling and developmental processes.

Involucrin (Fig. 9A, lanes 4) and transglutaminase (Fig. 9B, lanes 3 and 4) and transglutaminase (Fig. 9B, lanes 3 and 4) and transglutaminase (Fig. 9B, lanes 3 and 4). Taken together, we conclude that the ACC fragment of PKNγ (by interfering with the endogenous Rac1 interaction with PKNγ) (Fig. 6D (II), a, lanes 4-6) functions as a potent dominant-negative mutant that effectively inhibits HA/CD44-induced Rac1-PKNγ activation and PLCγ1/cortactin phosphorylation required for Ca2⁺- and cytoskeleton-regulated keratinocyte cell-cell adhesion and differentiation.

FIG. 8. Immunofluorescence staining of CD44, PKNγ, PLCγ1, and cortactin in keratinocyte transfectants. Keratinocytes transfected with vector alone or PKNγ-ACCCDNA were fixed by 2% paraformaldehyde. Subsequently, cells were rendered permeable by ethanol treatment and stained with various immunoreagents as described under “Materials and Methods.” A, Texas Red-labeled anti-CD44 (red color) (a), FITC-labeled anti-PKNγ (green color) (c), and co-localization of Texas Red anti-CD44 and FITC-PKNγ (e) (an overlay image of a and c) in vector-transfected keratinocytes treated with no HA. B, Texas Red-labeled anti-CD44 (red color) (a), FITC-labeled anti-PLCγ1 (green color) (c), and co-localization of Texas Red anti-CD44 and FITC-PLCγ1 (e) (an overlay image of a and c) in vector-transfected keratinocytes treated with no HA. Texas Red-labeled anti-CD44 (red color) (b), FITC-labeled anti-PKNγ (green color) (d), and co-localization of Texas Red anti-CD44 and FITC-PKNγ (f) (an overlay image of b and d) in vector-transfected keratinocytes treated with HA. C, Texas Red-labeled anti-CD44 (red color) (a), FITC-labeled anti-cortactin (green color) (c), and co-localization of Texas Red anti-CD44 and FITC-cortactin (e) (an overlay image of a and c) in vector-transfected keratinocytes treated with no HA. D, Texas Red-labeled anti-CD44 (red color) (a), FITC-labeled anti-PKNγ (green color) (c), and co-localization of Texas Red anti-CD44 and FITC-PKNγ (e) (an overlay image of a and c) in PKNγ-ACCCDNA-transfected keratinocytes treated with no HA. E, Texas Red-labeled anti-CD44 (red color) (a), FITC-labeled anti-PLCγ1 (green color) (c), and co-localization of Texas Red anti-CD44 and FITC-PLCγ1 (e) (an overlay image of b and d) in PKNγ-ACCCDNA-transfected keratinocytes treated with HA. F, Texas Red-labeled anti-CD44 (red color) (a), FITC-labeled anti-cortactin (green color) (c), and co-localization of Texas Red anti-CD44 and FITC-cortactin (e) (an overlay image of a and c) in PKNγ-ACCCDNA-transfected keratinocytes treated with no HA. Texas Red-labeled anti-CD44 (red color) (b), FITC-labeled anti-cortactin (green color) (d), and co-localization of Texas Red anti-CD44 and FITC-cortactin (f) (an overlay image of b and d) in PKNγ-ACCCDNA-transfected keratinocytes treated with HA.
Thus, it appears that Rac1-activated PKN mediates phosphorylation of PLC during HA stimulation (Table I and Fig. 2). These results clearly indicate that PKN acts as one of its cellular targets. Both Rac1 signaling (Fig. 4) and PKN (Fig. 7) blocks HA/CD44-mediated Ca\textsuperscript{2+} signaling (Table IV), and down-regulates keratinocyte cell-cell adhesion and differentiation (Figs. 8 and 9; Tables V–VII). These results strongly suggest that the ACC domain of PKN is a potent inhibitor of PKN-mediated PLC\textgamma-phosphorylation and Ca\textsuperscript{2+} signaling required for HA/CD44-mediated keratinocyte function. Our observations are consistent with previous findings suggesting that small GTPases (e.g. Rac1) are important for Ca\textsuperscript{2+} sensitization in hamster muscle resistance arteries (58). By using dominant-negative and dominant-active forms of Rac1, Hong-Geller and Cerione (44) have also suggested that these small GTpases regulate signal transduction at the level of PLC\textgamma and IP\textsubscript{3} production.

The cytoskeletal protein cortactin contains a repeat domain and a C-terminal SH3 domain resembling neufectin, a F-actin-associated protein (59). In fact, cortactin is considered to be an actin-binding protein (48, 49). Cortactin not only serves as a cellular substrate for c-Src and Fyn but also localizes with actin at cell adhesion sites during Ca\textsuperscript{2+}-induced keratinocyte differentiation (60). Tyrosine phosphorylation of cortactin is greatly reduced in keratinocytes derived from Fyn knock-out mice, indicating Fyn kinase may be directly involved in cortactin phosphorylation (60). Our previous study demonstrated that HA activates Src kinase-mediated cortactin phosphorylation and modulates cortactin-F-actin binding in tumor cells (61). In this study, we have determined that cortactin is a cellular substrate for Rac1-activated PKN\textgamma kinase in keratinocytes (Fig. 4). Serine/threonine phosphorylation of cortactin by Rac1

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**Table V**

**Measurement of HA-dependent and CD44-specific keratinocyte cell-cell adhesion**

| Treatment | Cell-cell adhesion index \(\langle N_c - N_{NC}\rangle / N_{NC}\) | \(\%\) |
|-----------|---------------------------------|-----|
| No treatment (control) | 20 | |
| HA treatment | 88 | |
| Anti-CD44 IgG + HA treatment | 21 | |

**Table VI**

**Effects of various drugs on HA-dependent keratinocyte cell-cell adhesion**

| Cells | Cell-cell adhesion index \(\langle N_c - N_{NC}\rangle / N_{NC}\) | \(\%\) |
|-------|---------------------------------|-----|
| No HA addition | 22 | 86 |
| U-73122 treatment | 18 | 19 |
| Xestospongin C treatment | 17 | 18 |
| BAPTA treatment | 18 | 16 |
| Cytochalasin B treatment | 16 | 15 |

**Table VII**

**Effect of PKN\textgamma-ACC overexpression on HA-dependent keratinocyte cell-cell adhesion**

| Cells | Cell-cell adhesion index \(\langle N_c - N_{NC}\rangle / N_{NC}\) | \(\%\) |
|-------|---------------------------------|-----|
| Vector-transfected cells | 17 | 84 |
| PKN\textgamma-ACC overexpressed cells | 18 | 19 |

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(measured by PLC\textgamma-mediated IP\textsubscript{3} production) do not correlate well in certain case(s) (38), we were prompted to explore additional mechanisms including post-translational modifications of PLC\textgamma by certain serine/threonine kinase(s), such as the PKN family of kinases, during HA/CD44-mediated activation in keratinocytes.

PKN belongs to a family of serine/threonine kinase that displays high sequence homology to that of the protein kinase C family members (29). There are at least three different isoforms of PKN (PKN\textalpha PAK-1/PRK-1, PKN\textbeta, and PKN\textgamma/PRK2) detected in mammals (29). PKN isoforms appear to be involved in a variety of cellular functions (29–31). PKN\textgamma is a serine/threonine kinase known to interact with Rac1 in a GDP-dependent manner and shares a great deal of homology to protein kinase C in the C-terminal region (29). PKN\textgamma also has a unique regulatory region containing ACC domains that bind to the small GTpases (29–31). In a previous study PKN\textgamma has been shown to be involved in Fyn kinase activity and the cell-cell adhesion in mouse keratinocytes treated with exogenously added Ca\textsuperscript{2+} (33). By using a PKN\textgamma-specific antibody, we have confirmed the presence of PKN\textgamma in human keratinocytes (Fig. 1). Furthermore, we have demonstrated that PKN\textgamma (by binding to activated Rac1) is capable of inducing marked threonine phosphorylation of PLC\textgamma (isolated from keratinocytes) in vitro (Fig. 2). The ability of PKN to phosphorylate PLC\textgamma in the presence of unactivated Rac1 appears to be greatly reduced (Fig. 2). These results clearly indicate that PKN acts as one of the downstream effectors of Rac1 signaling and utilizes PLC\textgamma as one of its cellular targets. Both Rac1 signaling (Table I) and PLC\textgamma phosphorylation in vivo (Fig. 2) can be stimulated by HA and blocked by cells pretreated with anti-CD44 antibody during HA stimulation (Table I and Fig. 2).

Thus, it appears that Rac1-activated PKN\textgamma-mediated phosphorylation of PLC\textgamma is closely coupled with HA-mediated CD44 activation in keratinocytes. Most important, PKN\textgamma-mediated phosphorylation of PLC\textgamma up-regulates PLC\textgamma activity (measured by PLC\textgamma-mediated IP\textsubscript{3} production) (Table II) required for Ca\textsuperscript{2+} signaling.

The ACC domain of PKN\textgamma has been shown to be involved in the regulation of GTP-dependent PKN function (29–31). In Fig. 6, we have detected that the ACC domain is closely associated with Rac1 as a complex. Transfection of keratinocytes with the ACC domain cDNA of PKN\textgamma (Fig. 6) effectively competes for endogenous activated Rac1 binding to PKN\textgamma (Fig. 6), inhibits the ability of PKN\textgamma to phosphorylate PLC\textgamma (Fig. 7), blocks HA/CD44-mediated Ca\textsuperscript{2+} signaling (Table IV), and down-regulates keratinocyte cell-cell adhesion and differentiation (Figs. 8 and 9; Tables V–VII). These results strongly suggest that the ACC domain of PKN\textgamma is a potent inhibitor of PKN\textgamma-mediated PLC\textgamma phosphorylation and Ca\textsuperscript{2+} signaling required for HA/CD44-mediated keratinocyte function. Our observations are consistent with previous findings suggesting that small GTpases (e.g. Rac1) are important for Ca\textsuperscript{2+} sensitization in hamster muscle resistance arteries (58).
Contactin in keratinocytes transfected with PKN/H9253 fragment by transfecting keratinocytes with PKN/contactin and PLC/e.g. related proteins (e.g. protein kinase C isoforms (e.g. protein kinase Ca, -, -, -z, and - isoforms) (62), which regulate various signal cascades and serve as regulators of keratinocyte differentiation-dependent gene expression (e.g. involucrin and/or transglutaminase gene expression) (63–67). HA/CD44-induced intracellular Ca\(^{2+}\) may alter its ability to cross-link filamentous actin (Table IV). In contrast, the reduction of cortactin phosphorylation (isolated from keratinocytes transfected with PKN\(\gamma\)-ACCcDNA with or without HA treatment) is sufficient to promote cross-linking of actin filaments into bundles \(\text{in vitro}\) (Table IV). These results are consistent with previous findings suggesting cortactin plays an important role as a regulator for F-actin-based cytoskeleton function.

To elucidate further the CD44 and PKN\(\gamma\) interaction with cortactin and PLC\(\gamma\)1 \(\text{in vivo}\), we have used immunocytochemical staining and confocal microscopy to monitor morphological changes and the intracellular distribution of various signaling proteins (e.g. CD44, endogenous PKN\(\gamma\), PLC\(\gamma\)1, and cortactin) in keratinocytes transfected with PKN\(\gamma\)-ACCcDNA or vector alone. Our results indicate that that HA promotes co-localization of cortactin and PLC\(\gamma\)1 with CD44 and PKN\(\gamma\) at the plasma membrane region and cell-cell adhesion sites in vector-transfected keratinocytes (Fig. 8). In contrast, overexpression of the dominant-negative form of PKN\(\gamma\) (PKN\(\gamma\)-ACC fragment) by transfecting keratinocytes with PKN\(\gamma\)-ACCcDNA effectively inhibits HA-mediated recruitment of cortactin, PLC\(\gamma\)1, or PKN\(\gamma\) kinase to CD44 (Fig. 8) at the cellular membranes and impairs HA-induced cell-cell adhesion (Fig. 8; Tables V–VII). These findings demonstrate that the ability of cortactin/PLC\(\gamma\)1 to be recruited into CD44-associated membrane region and cell-cell adhesion sites is tightly coupled with an active form of PKN\(\gamma\) kinase in keratinocytes during HA signaling. Treatment of keratinocytes with various inhibitors (e.g. the PLC inhibitor (U73122), the IP\(_3\) receptor blocker (Xestospongin C), the Ca\(^{2+}\) chelator (BAPTA), or the microfilament inhibitor (cytochalasin D)) also displays marked inhibitory effects on HA/CD44-induced cell-cell adhesion (Tables V–VII). These data also strongly suggest that CD44-mediated PKN\(\gamma\) activation is required for PLC\(\gamma\)1-regulated Ca\(^{2+}\) signaling and cytoskeleton function during HA-dependent cell-cell adhesion in keratinocytes.

The early events of keratinocyte differentiation (shortly after HA/CD44-induced Ca\(^{2+}\) signaling) are accompanied by the expression of keratinocyte differentiation markers including involucrin (precursor for the cornified envelope). Within 24–48 h after HA binding to CD44, transglutaminase is also expressed and activated (Fig. 9). One of the mechanisms by which intracellular Ca\(^{2+}\) triggers differentiation during HA-CD44 interaction may involve the Ca\(^{2+}\)-dependent activation of protein kinase C isoforms (e.g. protein kinase Ca, -, -, -z, and - isoforms) (62), which regulate various signal cascades and serve as regulators of keratinocyte differentiation-dependent gene expression (e.g. involucrin and/or transglutaminase gene expression) (63–67).
HA/CD44-mediated Rac1-PKN\(\gamma\) activation and PLC\(\gamma\)-regulated Ca\(^{2+}\) signaling/ cortactin-cytoskeleton binding during keratinocyte cell-cell adhesion and differentiation. CA2\(^{+}\) signaling/cortactin-cytoskeleton binding during keratinocyte cell-cell adhesion and differentiation.

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