Phosphatidylinositol-4-phosphate 5-kinase 1 α Mediates Extracellular Calcium-induced Keratinocyte Differentiation

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Extracellular calcium (Cao) is a major regulator of keratinocyte differentiation, but the mechanism is unclear. Phosphatidylinositol-4-phosphate 5-kinase 1 α (PIP5K1 α) is critical in synthesizing phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂]. In this study, we sought to determine whether PIP5K1 α plays a role in mediating the ability of Cao to induce keratinocyte differentiation. We found that treatment of human keratinocytes in culture with Cao resulted in increased PIP5K1 α level and activity, as well as PI(4,5)P₂ level, binding of phosphatidylinositol 3,4,5-triphosphate [PI(3,4,5)P₃] to and activation of phospholipase C- γ 1 (PLC- γ 1), with the resultant increase in inositol 1,4,5-trisphosphate (IP₃) and intracellular calcium (Cai). Knockdown of PIP5K1 α in human keratinocytes blocked Cao-induced increases in the binding of PI(3,4,5)P₃ to PLC- γ 1; PLC- γ 1 activity; levels of PI(4,5)P₂, IP₃, and Cai; and induction of keratinocyte differentiation markers. Coimmunoprecipitation and confocal studies revealed that Cao stimulated PIP5K1 α recruitment to the E-cadherin–catenin complex in the plasma membrane. Knockdown of E-cadherin or β -catenin blocked Cao-induced activation of PIP5K1 α . These results indicate that after Cao stimulation PIP5K1 α is recruited by the E-cadherin–catenin complex to the plasma membrane where it provides the substrate PI(4,5)P₂ for both PI3K and PLC- γ 1. This signaling pathway is critical for Cao-induced generation of the second messengers IP₃ and Cai and keratinocyte differentiation.

INTRODUCTION

Keratinocytes differentiate in vitro in a manner recapitulating the process of epidermal differentiation in vivo. The normal epidermis displays a characteristic calcium gradient, with low calcium in the basal layer, whereas calcium levels increase progressively toward the granular layer, and decline again in the cornified layer (Menon *et al.*, 1985). This calcium gradient is important for maintaining normal epidermal differentiation because loss of the calcium gradient induced by epidermal barrier disruption causes an increase in proliferation and a decrease in differentiation (Elias *et al.*, 2002). A disturbed calcium gradient has been observed in psoriatic epidermis favoring increased proliferation and decreased differentiation (Menon and Elias, 1991). Normal keratinocytes cultured in low extracellular calcium (Cao; 0.03 mM) conditions do not differentiate and phenotypically resemble basal epidermal cells. Increasing Cao levels above 0.1 mM induces differentiation of keratinocytes, and they

achieve morphological characteristics of the suprabasal epidermal cells (Hennings *et al.*, 1981). Elevated Cao stimulates the expression of several keratinocyte differentiation markers such as keratin 1, keratin 10, involucrin, transglutaminase 1, loricrin, and filaggrin (Hennings *et al.*, 1980, 1983; Yuspa *et al.*, 1989; Pillai *et al.*, 1990; Hohl *et al.*, 1991). Elevated Cao also induces redistribution of cell adhesion molecules from the cytosol to the plasma membrane where they participate in the formation of intercellular contacts (Hennings and Holbrook, 1983; Braga *et al.*, 1995). An early response to high Cao is an increase in intracellular calcium (Cai) concentration (Hennings *et al.*, 1989; Pillai *et al.*, 1990). Blocking this Cai rise with calcium chelators inhibits expression of keratinocyte differentiation markers, suggesting that Cai increase is required for triggering of keratinocyte differentiation (Li *et al.*, 1995). However, the precise mechanism by which Cao induces Cai rise and subsequent keratinocyte differentiation remains unclear. We have previously dissected a signaling pathway responsible for Cao-induced keratinocyte differentiation (Xie and Bikle, 1999, 2007; Xie *et al.*, 2005). The results of these studies suggest that high Cao induces the formation of a p120-catenin–dependent E-cadherin–catenin complex that recruits and activates phosphatidylinositol 3-kinase (PI3K). Phosphatidylinositol 3,4,5-triphosphate [PI(3,4,5)P₃] converted from phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] by PI3K in the plasma membrane and then recruits and activates phospholipase C- γ 1 (PLC- γ 1). PLC- γ 1 hydrolyzes PI(4,5)P₂ to the intracellular messengers inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) to induce keratinocyte differentiation (Xie and Bikle, 1999; Xie *et al.*, 2005; Xie and Bikle, 2007) via a sustained Cai rise (Li *et al.*, 1995), presumably triggered

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Abbreviations used: Cai, intracellular calcium; Cao, extracellular calcium; DAG, diacylglycerol; IP₃, inositol 1,4,5-trisphosphate; PI(3,4,5)P₃, phosphatidylinositol 3,4,5-triphosphate; PI3K, phosphatidylinositol 3-kinase; PIP5K1 α , phosphatidylinositol 4-phosphate 5-kinase 1 α ; PI4P, phosphatidylinositol 4-phosphate; PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C.

by an initial Cai mobilization induced by IP₃ (Bikle *et al.*, 1996). During PI(3,4,5)P₃ synthesis and IP₃ generation, both PI3K and PLC- γ 1 in the plasma membrane use PI(4,5)P₂ as their substrate. This would cause a shortage of PI(4,5)P₂. To maintain the amount of IP₃ produced in cells, compensatory synthesis of PI(4,5)P₂ is necessary. Therefore, stimulation of PI(4,5)P₂ synthesis by Cao could be an essential regulatory step to sustain IP₃ production and keratinocyte differentiation.

Phosphatidylinositol-4-phosphate 5-kinase 1 (PIP5K1) isozymes are critical in synthesizing PI(4,5)P₂ by the phosphorylation of phosphatidylinositol 4-phosphate (PI4P) at the D-5 position of the inositol ring. PI(4,5)P₂ constitutes only a small fraction (0.5–1%) of total cell membrane lipid (Nasuhoglu *et al.*, 2002). Upon cell stimulation, PIP5K1 is activated to increase PI(4,5)P₂ synthesis in the membrane. To date, three isoforms of PIP5K1 have been identified, designated 1 α , 1 β , and 1 γ (Wenk *et al.*, 2001; Di Paolo *et al.*, 2002; Ling *et al.*, 2002). PIP5K1 α and PIP5K1 β are distributed ubiquitously (Ishihara *et al.*, 1996; Loijens and Anderson, 1996), but PIP5K1 γ is expressed only in the brain, lung, and kidneys (Ishihara *et al.*, 1998). Each isoform of PIP5K1 differently localizes in the cell. PIP5K1 α has been reported to localize in the perinucleus or nucleus in unstimulated cells (Chatah and Abrams, 2001; Doughman *et al.*, 2003) and be translocated to the plasma membrane when cells are activated (Doughman *et al.*, 2003), unlike PIP5K1 β , which is present only in the vesicular perinuclear region (Doughman *et al.*, 2003). In the present study, we sought to determine whether PIP5K1 α is required for the Cao-induced synthesis of PI(4,5)P₂ in the plasma membrane, leading to PLC- γ 1 activation as well as providing its substrate for generation of the second messengers ultimately leading to keratinocyte differentiation.

MATERIALS AND METHODS

Cell Culture

Human keratinocytes were isolated from neonatal human foreskins as described previously (Pillai *et al.*, 1988). Primary cultures were established in serum-free medium (medium 154CF with human keratinocyte growth supplement; Cascade Biologics, Portland, OR) containing 0.07 mM Cao. Second passage human keratinocytes were plated with serum-free medium containing 0.03 mM Cao and used in the subsequent experiments.

Small Interfering RNA (siRNA) Transfection

Keratinocytes with 10% confluence were transfected with siRNA for E-cadherin, β -catenin, PIP5K1 α , or negative control (ON-TARGETplus siRNA; Dharmacon RNA Technologies, Chicago, IL) at a concentration of 100 nM in accordance with manufacturer's recommendations using TransIT-siQUEST transfection reagent (Mirus, PanVera, Madison, WI) at a dilution of 1:750 according to the manufacturer's protocol.

Cell Lysate Preparation, Western Analysis, and Coimmunoprecipitation

Total cell lysates were isolated using phosphate-buffered saline (PBS) containing 2% SDS, Complete protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN), and 4-(2-aminoethyl)benzenesulfonfyl fluoride (AEBSEF; EMD Biosciences, San Diego, CA). Plasma membrane lysates were isolated using Mem-PER Eukaryotic Membrane Protein Extraction Reagent kit (Pierce Chemical, Rockford, IL). The protein concentration of the lysate was measured by the bicinchoninic acid (BCA) PROTEIN ASSAY KIT (Pierce Chemical). Equal amounts of protein were electrophoresed through reducing SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto polyvinylidene fluoride (PVDF) microporous membranes (Immobilon-P, 0.45 μ M; Millipore, Billerica, MA). After incubation in blocking buffer (100 mM Tris base, 150 mM NaCl, 5% nonfat milk, and 0.5% Tween 20), the blot was incubated overnight at 4°C with appropriate primary antibodies: polyclonal antibodies against human PIP5K1 α , PLC- γ 1, E-cadherin, p120-catenin, or transglutaminase 1 (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:200; monoclonal antibody (mAb) against human β -catenin (Santa Cruz Biotechnology) at a dilution of 1:200; polyclonal antibody against Akt and its phosphorylated form (serine 473; Cell Signaling Technology, Danvers, MA) at

a dilution of 1:1000; polyclonal antibody against keratin 1 or keratin 5 (Covance Research Products, Denver, PA) at a dilution of 1:10,000; mAb against human involucrin (Sigma-Aldrich, St. Louis, MO) at a dilution of 1:2000; polyclonal antibody against human p85 (Millipore), which is the regulatory subunit of class Ia PI3K at a dilution of 1:1000; and monoclonal antibodies against human integrin α 2 (plasma membrane marker), BIP (endoplasmic reticulum marker), or GM130 (*cis*-Golgi marker) at a dilution of 1:250 (BD Biosciences, San Jose, CA). After incubation with the primary antibody and a series of washes, the membranes were incubated for 1 h with the appropriate anti-immunoglobulin G (IgG) secondary antibody conjugated to horseradish peroxidase (GE Healthcare, Chalfont St. Giles, United Kingdom) in the blocking buffer. After a second series of washes, bound antibody complexes were visualized using the SuperSignal ULTRA chemiluminescent kit (Pierce Chemical) and subsequent exposure to x-ray film. To analyze protein complex formation at the plasma membrane by coimmunoprecipitation, equal amounts of plasma membrane protein (500 μ g) extracted with Mem-PER Eukaryotic Membrane Protein Extraction Reagent kit were incubated with 2 μ g of polyclonal antibody against E-cadherin for 1 h at room temperature or overnight at 4°C and then with 20 μ l of UltraLink immobilized protein A/G (Pierce Chemical) for 1 h at 4°C. The lysate-antibody-agarose beads mixture was washed four times with PBS and then analyzed by Western analysis with antibodies against PIP5K1 α . In a reverse approach, plasma membrane lysates were analyzed by coimmunoprecipitation with PIP5K1 α antibodies followed by Western blotting with E-cadherin antibody.

PIP5K1 α Activity Assay

The activity of PIP5K1 α was determined according to the method previously described by Chong *et al.* (1994), with some modifications. This method detects the formation of PI(4,5)P₂ from PI4P. Briefly, cells in 100-mm dishes were washed three times with ice-cold PBS, and extracted in kinase buffer (25 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 150 mM NaCl, 10% glycerol, 1% NP-40, AEBSEF, Complete protease inhibitors (Roche Molecular Biochemicals), and phosphatase inhibitor cocktail tablets (Roche Molecular Biochemicals). PIP5K1 α was immunoprecipitated from the lysate containing 500 μ g of protein using 2 μ g of polyclonal antibody against PIP5K1 α for 1 h at 4°C and then 20 μ l of UltraLink immobilized protein A/G for 1 h at 4°C. After a series of washes, 10 μ l of conjugated beads were then mixed with 50 μ l of kinase buffer containing sonicated phospholipids (70 μ M PI4P and 35 μ M phosphatidylserine, final concentrations) and preincubated for 15 min at 25°C. Reactions were started by the addition of 20 μ M [γ -³²P]ATP (1 μ Ci/assay). After incubation for 5 min at 25°C, the reactions were stopped by adding 0.3 ml of methanol, 1 N HCl (1:1, vol/vol) and extracted with 0.25 ml of chloroform. The organic layer was dried, resuspended in chloroform, and chromatographed on oxalate-pretreated Silica Gel 60 plates. Plates were developed in chloroform:methanol:2.5 N ammonium hydroxide (9:7:2) (vol/vol/vol). The ³²P-labeled products were visualized by autoradiography.

PLC- γ 1 Activity Assay

PLC- γ 1 activity was determined by measuring accumulation of IP₃ according to the experimental procedure described previously (Sun *et al.*, 1997). Cells in 150-mm dishes were washed with PBS containing 0.1% sodium orthovanadate and 0.1% NaF and then incubated with 1% NP-40 containing phosphatase inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN) and Complete protease inhibitors (Roche Molecular Biochemicals) for 5 min. Cells were scraped into microfuge tubes and incubated at 4°C on a rotator for 1 h. Protein from the supernatant collected after centrifugation was incubated with 2 μ g of polyclonal PLC- γ 1 antibody (BD Biosciences, San Jose, CA) at 4°C overnight and then with 20 μ l of UltraLink immobilized protein G (Pierce Chemical) at 4°C for 1 h. After centrifugation, the pellet was washed with reaction buffer (10 mM HEPES, pH 7.0, 10 mM NaCl, 120 mM KCl, 2 mM EGTA, 0.05% deoxycholate, 5 μ g/ml bovine serum albumin (BSA), and 10 μ M CaCl₂) and resuspended in 200 μ l of reaction buffer. In triplicates, 50 μ l of suspension was incubated with sonicated vesicles containing [³H]PI(4,5)P₂ (PerkinElmer Life and Analytical Sciences, Boston, MA), phosphatidylcholine (Sigma-Aldrich), and phosphatidylserine (Sigma-Aldrich) in a molar ratio of 1:3:3 in 100 μ l of reaction buffer. The reaction was ended by adding 200 μ l of 10% trichloroacetic acid (TCA) at 5 min and then with 200 μ l of 10% BSA. The radioactivity of supernatant after centrifugation was determined by a scintillation counter. We have previously shown that Cao-induced activation of PLC- γ 1 is detectable using PLC- γ 1 immunoprecipitated from cell lysates solubilized by the low stringency detergent NP-40, which leaves the PI(3,4,5)P₃-PLC- γ 1 complex intact and functional in the immunoprecipitate (Xie *et al.*, 2005).

Association of PI(3,4,5)P₃ with PLC- γ 1

The association of PI(3,4,5)P₃ was determined by immunoprecipitation with the PLC- γ 1 antibody followed by PI(3,4,5)P₃ detection with the PI(3,4,5)P₃ antibody as described previously (Xie *et al.*, 2005). Total protein (100 μ g) extracted from human keratinocytes with 1% NP-40 was incubated with PLC- γ 1 antibody at 4°C overnight and then with UltraLink immobilized protein G (Pierce Chemical) for 1 h. PI(3,4,5)P₃ bound to PLC- γ 1 was extracted

PIP5K1 α

Negative control

Figure 1. PIP5K1 α is expressed in all layers of the epidermis. Five sections of paraffin-embedded human adult skin from five different individuals were processed for immunohistochemical staining with an antibody for PIP5K1 α . The figure shows a representative field of these sections. PIP5K1 α expression is highest in the stratum granulosum. PIP5K1 α is stained in brown (left). The omission of primary antibody control resulted in no PIP5K1 α staining (right). Nuclei were counterstained with hematoxylin (blue). Magnification, 100 $\times$.

by chloroform and then spotted onto a dry PVDF membrane. After incubation in blocking buffer (100 mM Tris base, 150 mM NaCl, 5% nonfat milk or 2% bovine serum albumin, and 0.5% Tween 20), the membrane was incubated overnight at 4°C with a mAb against PI(3,4,5)P₃ (Echelon Research Laboratories, Salt Lake City, UT) at a concentration of 1 μ g/ml. After washes in the blocking buffer, the membranes were incubated for 1 h with anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (GE Healthcare) in the blocking buffer. After a second series of washes, bound antibody complexes were visualized using the SuperSignal ULTRA chemiluminescent kit (Pierce Chemical) and subsequent exposure to x-ray film.

PI(4,5)P₂ and IP₃ Assays

PI(4,5)P₂ mass and IP₃ concentrations in keratinocytes were determined using the *D*-myo-IP₃[³H] Biotrak assay system (GE Healthcare). Normal human keratinocytes harvested by trypsinization were incubated with an equal volume of ice-cold 15% (vol/vol) trichloroacetic acid on ice for 15 min. Cells were then sedimented by centrifugation at 4000 $\times$ g for 20 min at 4°C. The supernatant was extracted three times with 10 volumes of water-saturated diethyl ether and neutralized by titration to pH 7.5 with NaHCO₃. IP₃ concentration in the supernatant was determined following the manufacturer's protocol. The assay was based on competition between [³H]IP₃ (the tracer) and unlabeled IP₃ in the standard or samples for binding to a binding protein.

The method for PI(4,5)P₂ measurements involved the conversion of PI(4,5)P₂ in crude lipid extracts into IP₃ by alkaline hydrolysis. Extracts were then neutralized and assayed for IP₃. Briefly, the cell pellet collected by centrifugation was washed with TCA/1 mM EDTA and then water. The pellet was dissolved in 0.94 ml of methanol/chloroform/12 M HCl (80:40:1, vol/vol/vol). The lipid and aqueous phases were resolved by adding 0.31 ml of chloroform and 0.56 ml of 0.1 M HCl and then vortexed. After centrifugation at 1000 $\times$ g for 15 min at room temperature, the lower phase containing the lipid extract was evaporated to dryness in a stream of nitrogen at room temperature. The dried sample was dissolved in 0.25 ml of 1 M KOH and then heated at 100°C for 15 min. After cooling in ice, excess alkali was removed from samples by filtration through columns prepared by adding 0.5 ml of a 50% (wt/vol) slurry of Dowex 50 (200–400 mesh, H⁺ form), and the eluate was collected. The column was washed with 2.25 ml of water, and the total eluate was washed with 2 $\times$ 2 ml of butan-1-ol:light petroleum ether (5:1, vol/vol). One milliliter of lower phase was removed and lyophilized. The lyophilizate was dissolved in 1 ml of distilled water, and aliquots were taken for determination of IP₃ concentration following the manufacturer's protocol. The assay was based on competition between [³H]IP₃ (the tracer) and unlabeled IP₃ in the standard or samples for binding to a binding protein.

Cai Determination

Cai levels of keratinocytes attached to glass coverslips were measured using a dual-wavelength fluorescence imaging system (Intracellular Imaging, Cincinnati, OH). Briefly, the cells were loaded with 12.5 μ M Fura-2-acetoxymethyl ester (Invitrogen, Carlsbad, CA) in 0.1% Pluronic F-127 in buffer A (20 mM HEPES buffer, pH 7.4, 120 mM sodium chloride, 5 mM potassium chloride, 1 mM magnesium chloride, 1 mg/ml sodium pyruvate, and 1 mg/ml glucose) containing 0.07 mM calcium chloride at room temperature for 30 min followed by a 30-min rinse in buffer A. Cells were then washed and measured in buffer A containing 0.03 mM calcium chloride before exposure to 2 mM calcium chloride. The cells were alternately illuminated with 200-ms flashes of 340- and 380-nm light every 10 s, monitoring the emission wavelength of 510 nm. The signals from 20 to 50 single cells for each measurement were recorded. Cai concentration was calculated based on the ratio of emission at the two excitation wavelengths according to the formula developed by Grynkiewicz *et al.* (1985).

Immunohistochemistry

Paraffin-embedded adult human skin was cut into 5- μ m sections. After deparaffinization and rehydration, the sections were boiled in 10 mM citrate buffer for antigen retrieval. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in PBS. Nonspecific binding was blocked with 10 mM Tris buffer, pH 7.6, containing 4% bovine serum albumin, 0.5% fish gelatin, 0.1% Tween 20, and 500 mM NaCl. The sections were then incubated with polyclonal goat anti-human PIP5K1 α antibody at a concentration of 10 μ g/ml. The binding of the PIP5K1 α antibody was detected with biotinylated donkey anti-goat IgG (Vector Laboratories, Burlingame, CA), followed by ATP-binding cassette peroxidase (Vector Laboratories) reagent. Peroxidase activity was revealed with diaminobenzidine substrate (Vector Laboratories) followed by counterstaining with hematoxylin. Negative controls were performed by omitting the first antibody.

Confocal Microscopy

Keratinocytes were cultured on chamber slides, fixed with 4% paraformaldehyde for 20 min at room temperature, and permeabilized with 0.5% Triton X-100/PBS for 10 min. After blocking with 4% BSA and 0.05% Tween 20 in PBS, cells were incubated with 4 μ g/ml antibodies against E-cadherin and PIP5K1 α at 4°C overnight, followed by another incubation with the Alexa 488 donkey anti-goat and Alexa 594 chicken anti-rabbit as secondary antibodies (10 μ g/ml; Invitrogen) at room temperature for 1 h. Slides were washed in PBS, and coverslipped using Gel-Mount (Biomedex, Foster City, CA), and examined with a Leica TCS NT/SP confocal microscope (Leica Microsystems, Heidelberg, Germany).

RESULTS

PIP5K1 α Is Expressed in All Layers of the Epidermis

To investigate whether PIP5K1 α is expressed in the epidermis, we performed immunohistochemistry on adult skin sections. Using a polyclonal anti-human PIP5K1 α antibody, we found that PIP5K1 α is present in all layers of the epidermis, although at highest concentration in the stratum granulosum. Most of the PIP5K1 α was found in the cytoplasm and nucleus, although membrane localization was also apparent. The omission of primary antibody control resulted in no PIP5K1 α staining (Figure 1).

PIP5K1 α siRNA Knockdown Blocks Cao-stimulated Downstream Activation of PLC- γ 1

To determine whether PIP5K1 α is required for Cao-induced downstream activation, cultured human keratinocytes were treated with siRNA for PIP5K1 α for 3 d followed by 1.2 mM Cao for 30 min. The total PI(4,5)P₂ and level, association of PI(3,4,5)P₃ with PLC- γ 1, PLC- γ 1 activity, and IP₃ level were then determined. The results show that Cao significantly increased the activity of PLC- γ 1 (Figure 2A) and association of PI(3,4,5)P₃ with PLC- γ 1 (Figure 2A), PI(4,5)P₂ (Figure 2B), and IP₃ levels (Figure 2C). PIP5K1 α knockdown blocked these inductions (Figure 2, A–C). The siRNA knockdown efficiency was evaluated by Western analysis using an antibody against PIP5K1 α . The endogenous PIP5K1 α was re-

Figure 2. PIP5K1 α siRNA knockdown blocks Cao-stimulated increase in PI(4,5)P₂ mass, Akt phosphorylation, association of PI(3,4,5)P₃ with PLC- γ 1, PLC- γ 1 activation, and IP₃ mass in human keratinocytes. Cultured human keratinocytes were treated with PIP5K1 α siRNA for 72 h and then with Cao for 30 min. Cells were harvested, and cell lysates were isolated. PLC- γ 1 in the total cell lysates was immunoprecipitated with a PLC- γ 1 antibody, and the PLC- γ 1 activity and association of PI(3,4,5)P₃ with PLC- γ 1 were assayed (A). The levels of PLC- γ 1 in the PLC- γ 1 immunoprecipitates, and PIP5K 1 α , Akt and its phosphorylated form (p-Akt, serine-473) in the total cell lysates were determined by Western analysis (A). Association of PI(3,4,5)P₃ with PLC- γ 1 was determined by immunoprecipitation with PLC- γ 1 antibody followed by incubation of the immunoprecipitate-spotted membrane with PI(3,4,5)P₃ antibody (A). The PI(4,5)P₂ (B) and IP₃ (C) levels in the total cell lysates were evaluated as described in *Materials and Methods*. The results of PLC- γ 1 activity and PI(4,5)P₂ and IP₃ levels are expressed as percentages of the control values. Data are mean $\pm$ SD of triplicates within a single representative experiment, * $p < 0.01$ (significantly different from the control in the presence of 0.03 mM Cao and control siRNA). Results shown are representative of 3 experiments with three separate siRNA treatments.

duced to an undetectable level by siRNA for PIP5K1 α (Figure 2A). In contrast, PLC- γ 1 was detected at the same level in all four samples. These data indicate that PIP5K1 α is required for Cao-stimulated PI(4,5)P₂ synthesis, association of PI(3,4,5)P₃ with PLC- γ 1, PLC- γ 1 activation, and IP₃ generation in human keratinocytes.

Having shown that PIP5K1 α is required for association of PI(3,4,5)P₃ and subsequent PLC- γ 1 activation, we then wanted to know whether PIP5K1 α is required for Cao-induced increase in PI(3,4,5)P₃ mass. We have found previously that Cao induces serine phosphorylation of Akt (Xie *et al.*, 2005), a kinase downstream of PI(3,4,5)P₃. Therefore, we examined the effect of knockdown of PIP5K1 α on Cao-induced serine phosphorylation of Akt and found that knockdown of PIP5K1 α blocked Cao-induced serine phosphorylation of Akt (Figure 2A), correlating with the effect of PIP5K1 α knockdown on Cao-induced increase in PI(4,5)P₂ mass, PI(3,4,5)P₃ association with PLC- γ 1, PLC- γ 1 activity, and IP₃ mass.

PIP5K1 α siRNA Knockdown Blocks Cao-induced Cai Rise

To further examine whether PIP5K1 α is required for the downstream IP₃-mediated pathway, cultured human keratinocytes were transfected with PIP5K1 α siRNA, transfected cells were incubated for 3 d, and the response of Cai level to Cao was then determined. The results show that PIP5K1 α siRNA not only reduced the basal level of the Cai but also blocked Cao-induced Cai rise (Figure 3A), indicating that PIP5K1 α is required for the Cao-induced increase in Cai. To determine whether knockdown of PIP5K1 α also affects other

ligand-induced PLC activation and IP₃ production leading to Cai rise, we examined its impact on the Cai response to ATP through the activation of P₂ purinergic receptors. We have shown previously that ATP treatment induces a rapid and transient increase in IP₃ and mobilization of calcium from intracellular sources but inhibits differentiation of keratinocytes (Pillai and Bikle, 1992). In the current study, ATP induced a rapid increase in Cai in keratinocytes pretreated with control siRNA in the presence of 0.03 mM Cao (Figure 3B). Knockdown of PIP5K1 α markedly reduced ATP-evoked Cai release, presumably due to the depletion of membrane PI(4,5)P₂, the substrate for PLC isozymes, although depletion of Cai stores could also lead to this result. To determine whether Cai stores are affected by PIP5K1 α knockdown, the Cai response to ionomycin in the absence of Cao was evaluated. The results showed that ionomycin induced Ca²⁺ release from internal stores, resulting in a rapid and transient rise in Cai in cells treated with control siRNA. Knockdown of PIP5K1 α led to a 75% reduction in the rise in Cai, suggesting that PIP5K1 α is required for the maintenance of Cai stores as well as maintenance of PI(4,5)P₂ for IP₃ production.

PIP5K1 α siRNA Knockdown Blocks Cao-induced Human Keratinocyte Differentiation

To address whether PIP5K1 α plays a role in Cao-induced keratinocyte differentiation, human keratinocytes were pretreated with siRNA for PIP5K1 α for 3 d before exposing them to 1.2 mM Cao for 24 h. Protein levels of PIP5K1 α were evaluated to assess the knockdown efficiency. Protein levels of keratin 5 and signaling molecules involved in keratino-

Figure 3. PIP5K1 α siRNA knockdown suppresses the acute Cao response to elevated Cao and ATP and reduces the Cai pool in keratinocytes. Human keratinocytes cultured on glass coverslips were transfected with PIP5K1 α siRNA in the presence of 0.03 mM Cao. Transfected cells were incubated for 72 h at 37°C, 5% CO₂. Cai was measured in the presence of 0.03 mM Cao, and the response to 2 mM Cao (A) or 100 μ M ATP (B) was determined. To determine the degree of Cai pool emptying, Cai was measured before and after the addition of 20 μ M ionomycin (C). The traces shown in this figure represent the average Cai of 45–60 individual cells during recording. The results shown are from a representative experiment repeated three times with three separate siRNA treatments.

cyte differentiation pathway such as E-cadherin, PI3K-p85, and PLC- γ 1 were examined to monitor the specificity of the PIP5K1 α siRNA. Protein levels of differentiation markers keratin 1, involucrin, and transglutaminase 1 were determined to assess keratinocyte differentiation. The results show that Cao treatment increased the protein level of PIP5K1 α within 24 h (Figure 4A). The siRNA for PIP5K1 α markedly reduced PIP5K1 α protein expression in human keratinocytes and prevented the increase after Cao treatment (Figure 4A). Levels of other signaling molecules, including E-cadherin, PLC- γ 1, and PI3K-p85, were not affected by PIP5K1 α siRNA (Figure 4A), showing that PIP5K1 α siRNA specifically targeted the endogenous PIP5K1 α gene. Keratin 5 expression used as a loading control was not affected by Cao or PIP5K1 α knockdown (Figure 4A). How-

ever, knockdown of PIP5K1 α blocked Cao-induced keratin 1, involucrin, and transglutaminase 1 expression (Figure 4A), indicating that PIP5K1 α is required for Cao-induced human keratinocyte differentiation.

After monolayer growth of control siRNA-treated human keratinocytes for 3 d in 0.03 mM Cao, elevating the Cao concentration to 1.2 mM induced a rapid change in cell-cell contact. Distinct spaces between cells became much less apparent within 4 h. Cells stratified within 24 h (Figure 4B). Pretreatment with siRNA for PIP5K1 α did not block these Cao-induced morphological changes (Figure 4B). These results are consistent with our previous findings showing that E-cadherin or β -catenin knockdown does not affect Cao-induced cell-cell contact, but blocks Cao-induced keratinocyte differentiation (Xie and Bikle, 2007).

Figure 4. PIP5K1 α siRNA knockdown blocks Cao-induced human keratinocyte differentiation. (A) Cultured human keratinocytes were treated with PIP5K1 α siRNA for 72 h and then with Cao for 24 h. Cells were harvested and total cell lysates were isolated. The protein levels of PIP5K1 α , E-cadherin, PI3K-p85, PLC- γ 1, involucrin, transglutaminase 1, keratin 1, and keratin 5 were determined by Western analysis. Keratin 5 was used as a loading control. (B) Cultured human keratinocytes were treated with PIP5K1 α siRNA for 72 h and then with Cao for 24 h. Phase-contrast photographs of cells are shown.

The data shown are representative of three separate experiments.

Cao Induces PIP5K1 α Recruitment to E-cadherin at the Plasma Membrane of Human Keratinocytes

We have shown previously that p120-catenin dependent E-cadherin- β -catenin complex formation is required for PI3K activation and keratinocyte differentiation. In the present study, we sought to test whether high Cao induces PIP5K1 α recruitment to the E-cadherin- β -catenin complex at the plasma membrane. This recruitment would make PIP5K1 α accessible to its substrate PIP at the plasma membrane. To address this issue, the plasma membrane of human keratinocytes treated with 1.2 mM Cao for 0, 5, 15, 30, 120, 240, or 360 min was isolated, and the binding of PIP5K1 α to the E-cadherin complex was analyzed by immunoprecipitation with the E-cadherin antibody followed by Western analysis with the PIP5K1 α antibody. The results show that high Cao induces PIP5K1 α binding to a complex with E-cadherin at the plasma membrane as early as 5 min, and a plateau was reached after 15-min Cao treatment (Figure 5A). To determine whether this complex formation occurs at the plasma membrane, human keratinocytes were treated with Cao for 5 min, and plasma membrane lysates were extracted. The plasma membrane proteins were immunoprecipitated with E-cadherin antibody followed by Western analysis with antibodies against β -catenin, p120-catenin, or PIP5K1 α . The results show that the E-cadherin complex contains not only β -catenin and p120-catenin but also PIP5K1 α (Figure 5A). These data were confirmed by the reverse approach using PIP5K1 α antibody for immunoprecipitation and then antibodies against E-cadherin, β -catenin, or p120-catenin for western analysis of the immunoprecipitate (Figure 5A). IgG and protein G beads alone (no antibody) were included in the coimmunoprecipitation assay, showing that there was no background with these negative controls (Figure 5A). In parallel experiments, human keratinocytes were treated with 1.2 mM Cao for 5 min, and total cell lysates as well as plasma membrane lysates were isolated. The protein levels for E-cadherin, β -catenin, p120-catenin, and PIP5K1 α were determined by Western analysis. The results show that 5 min of Cao exposure of cells raised levels of not only E-cadherin, β -catenin, and p120-catenin but also PIP5K1 α at the plasma membrane, although no changes in the levels of these proteins was found in the total lysate (Figure 5B). Western analysis with antibody against the plasma membrane marker integrin α 2, endoplasmic reticulum marker BIP, or *cis*-Golgi marker GM130 confirmed that only the integrin α 2 antibody immunoreacted with the plasma membrane lysate, confirming the purity of the plasma membrane lysate preparation (Figure 5B). To confirm the results from the immunoprecipitation assay, human keratinocytes were treated with 1.2 mM Cao for 5 min, and cells were examined by confocal microscopy by using antibodies against E-cadherin or PIP5K1 α . The results showed that 1.2 mM Cao treatment within 5 min induced PIP5K1 α colocalization with E-cadherin in the plasma membrane, although a substantial amount of PIP5K1 α is also present in the nucleus in the presence of either 0.03 or 1.2 mM Cao (Figure 5C). These data indicate that high Cao induces PIP5K1 α recruitment to E-cadherin in the plasma membrane. Of interest is that the rise in Cao also increases E-cadherin in the nucleus as well as plasma membrane.

β -Catenin Knockdown Blocks Cao-induced PIP5K1 α Recruitment to E-cadherin at the Plasma Membrane of Human Keratinocytes

We have previously demonstrated previously that β -catenin is required for PI3K recruitment to the E-cadherin complex

in the plasma membrane and keratinocyte differentiation induced by high Cao (Xie and Bikle, 2007). In the present study, we sought to determine whether β -catenin mediates the complex formation between E-cadherin and PIP5K1 α . To address this issue, the expression of β -catenin in human keratinocytes was knocked down by siRNA. Cells were harvested after stimulation with 1.2 mM Cao for 5 min. Plasma membrane lysates were analyzed for complex formation by immunoprecipitation with an E-cadherin antibody followed by Western analysis with a PIP5K1 α antibody or E-cadherin antibody. The total cell lysates were analyzed for β -catenin level by Western analysis. The results show that β -catenin knockdown blocked Cao-induced complex formation between PIP5K1 α and E-cadherin at the plasma membrane (Figure 6A). In parallel experiments, human keratinocytes were treated with siRNA for E-cadherin or β -catenin for 72 h and then with 1.2 mM Cao for 5 min, and plasma membrane lysates were isolated. The protein levels for E-cadherin or β -catenin, PIP5K1 α and integrin α 2 were determined by Western analysis. The results show that knockdown of E-cadherin or β -catenin blocked Cao-induced PIP5K1 α plasma membrane recruitment (Figure 6, B and C). Integrin α 2 used as an internal control was not affected by Cao or E-cadherin or β -catenin knockdown (Figure 6, B and C). These data suggest that E-cadherin and β -catenin are required for Cao-induced recruitment of PIP5K1 α to the plasma membrane.

E-cadherin or β -Catenin Knockdown Blocks Cao-induced PIP5K1 α Activation

To determine whether high Cao induces PIP5K1 α activity, cultured human keratinocytes were treated with 1.2 mM Cao for 5–360 min (Figure 7A). Cells were harvested and total cell lysates were isolated for PIP5K1 α activity assay. Consistent with an increase in PI(4,5)P₂ level, Cao induced PIP5K1 α activity in a time-dependent manner (Figure 7A). Having shown that E-cadherin binds PIP5K1 α through β -catenin, we wanted to know whether E-cadherin and β -catenin were required for the activation of PIP5K1 α . To address this issue, E-cadherin or β -catenin was knocked down by siRNA before Cao treatment. Cells were harvested, and total cell lysates were isolated for PIP5K1 α activity assay. The results show that E-cadherin or β -catenin knockdown blocked Cao-induced PIP5K1 α activation (Figure 7B). These data indicate that both E-cadherin and β -catenin are required for Cao-induced PIP5K1 α activation.

DISCUSSION

Our present studies have investigated the role of PIP5K1 α in mediating the ability of Cao to induce keratinocyte differentiation. The results demonstrate for the first time that PIP5K1 α plays an indispensable role in Cao-induced PI(4,5)P₂ synthesis, PLC- γ 1 activation, IP₃ production, Cai rise, and, ultimately, keratinocyte differentiation. On Cao stimulation, PIP5K1 α is recruited to interact with E-cadherin and β -catenin, which results in its activation. The activated PIP5K1 α then synthesizes more PI(4,5)P₂ in the plasma membrane to provide sufficient substrate for PI3K and PLC- γ 1, which is an essential regulatory step to sustain the activation of PI3K and PLC- γ 1 in keratinocytes. As a result, PI(4,5)P₂ levels are constantly maintained at a high level to enable the generation of IP₃ required for the increase in Cai necessary for initiating keratinocyte differentiation. As expected, the early differentiation marker keratin 1 was more profoundly reduced than the mid-differentiation markers involucrin and transglutaminase when Cao signaling was blocked by PIP5K1 α knockdown because it is more sen-

Figure 5. Cao induces PIP5K1 α recruitment to E-cadherin at the plasma membrane of human keratinocytes. (A) Cultured human keratinocytes were treated with 1.2 mM Cao. The cells were harvested at the time point(s) indicated. The plasma membrane and total cell lysates were isolated, and the lysates were analyzed by immunoprecipitation (IP) with antibodies against E-cadherin followed by Western analysis with antibodies against PIP5K1 α , β -catenin, p120-catenin, or E-cadherin. In a reverse approach, detection of E-cadherin, β -catenin, and p120-catenin in the PIP5K1 α immunoprecipitates was accomplished by Western analysis with the appropriate antibodies. IgG and protein G beads alone were included in the coimmunoprecipitation assay and used as negative controls. The results are from a representative experiment that was repeated four times. (B) Cultured human keratinocytes were treated with 1.2 mM Cao for 5 min. Cells were harvested and the protein levels of E-cadherin, β -catenin, p120-catenin, PIP5K1 α , integrin α 2 (plasma membrane marker), BIP (endoplasmic reticulum marker), and GM 130 (*cis*-Golgi marker) in total cell lysates, and plasma membrane lysates were determined by Western analysis. The results are from a representative experiment that was repeated four times. (C) Cultured human keratinocytes were treated with 1.2 mM Cao for 5 min. Cells were stained with a rabbit polyclonal antibody against E-cadherin, and a goat polyclonal antibody against PIP5K1 α , followed by the appropriate fluorescein-5-isothiocyanate (FITC)- or Texas Red-conjugated secondary antibody. Fluorescent signals were detected with a confocal microscope, and green (FITC) and red (Texas Red) images (single z section) were superimposed, so that sites of staining overlap are visualized as yellow. The results are from a representative experiment that was repeated three times.

sitive to changes in Cai, even at resting levels (100 nM). Together with our previous findings (Xie and Bikle, 1999, 2007; Xie *et al.*, 2005), we thus propose the following model (Figure 8) showing a mechanism by which Cao induces keratinocyte differentiation. Cao induces the formation of a p120-catenin and β -catenin-dependent E-cadherin complex that recruits

both PIP5K1 α and PI3K to the plasma membrane and activates them. Activated PIP5K1 α and PI3K in the plasma membrane synthesize PI(4,5)P₂ and PI(3,4,5)P₃ in succession. PI(3,4,5)P₃ then recruits and activates PLC- γ 1, which hydrolyzes PI(4,5)P₂ into the intracellular messengers IP₃ and DAG to trigger keratinocyte differentiation.

Figure 6. β -Catenin knockdown blocks Cao-induced PIP5K1 α recruitment to E-cadherin at the plasma membrane of human keratinocytes. (A) Cultured human keratinocytes were treated with β -catenin siRNA for 72 h and then with Cao for 5 min. Plasma membrane lysates were analyzed for complex formation by immunoprecipitation with an E-cadherin antibody followed by Western analysis with a PIP5K1 α antibody or E-cadherin antibody. The total cell lysates were analyzed for β -catenin levels by Western analysis. The results are from a representative experiment repeated three times with three separate siRNA treatments. (B and C) Cultured human keratinocytes were treated with siRNA for E-cadherin (B) or β -catenin (C) for 72 h and then with Cao for 5 min. Cells were harvested, and the protein levels of E-cadherin or β -catenin, PIP5K1 α , and integrin α 2 (plasma membrane marker) were determined by Western analysis. The results are from a representative experiment that was repeated three times.

In contrast to the IP_3 increase, $\text{PI}(4,5)\text{P}_2$ showed only a modest increase in response to high Cao. A possible explanation is that Cao treatment activates not only PIP5K1 α but also PI3K and PLC- γ 1. These activations lead to rapid in-

creases in $\text{PI}(4,5)\text{P}_2$ synthesis as well as hydrolysis to IP_3 and conversion to $\text{PI}(3,4,5)\text{P}_3$. As a result, $\text{PI}(4,5)\text{P}_2$ is constantly maintained at an appropriate level to sustain PLC- γ 1 activity. It is also likely that the pool of $\text{PI}(4,5)\text{P}_2$ involved in Cao

Figure 7. E-cadherin or β -catenin knockdown blocks Cao-induced PIP5K1 α activation. (A) Cultured human keratinocytes were treated with Cao for the indicated times. Cells were harvested and total cell lysates were isolated for the PIP5K1 α activity assay. The autoradiograph shown is representative of three experiments with three separate siRNA treatments. The $\text{PI}(4,5)\text{P}_2$ signal intensities were quantitated by Fujifilm General-Purpose Analysis Software Muti Gauge and normalized to the band intensities of PIP5K1 α in the corresponding Western blot. Results are expressed as percentages of the values at time 0. Data are expressed as mean $\pm$ SD of three separate experiments, * p < 0.05 (significantly different from the control at time 0). (B) Cultured human keratinocytes were treated with siRNA for E-cadherin or β -catenin for 72 h and then with Cao for 15 min. Cells were harvested, and total cell lysates were isolated for PIP5K1 α activity assay. The autoradiograph shown is from a representative experiment repeated three times with three separate siRNA treatments. The $\text{PI}(4,5)\text{P}_2$ signal intensities were quantitated by Fujifilm General-Purpose Analysis Software Muti Gauge and normalized to the band intensities of PIP5K1 α in the corresponding Western blot. Results are expressed as percentages of the values in the control lane (the presence of 0.03 mM Cao and control siRNA). Data are expressed as mean $\pm$ SD of three separate experiments, * p < 0.05 (significantly different from the control in the presence of 0.03 mM Cao and control siRNA).

Figure 8. A proposed model for the signaling pathway of Cao-induced keratinocyte differentiation. In high Cao conditions, PIP5K1 α and PI3K are recruited to a p120-catenin dependent E-cadherin complex by β -catenin in the plasma membrane in which they increase the synthesis of PI(4,5) P_2 and PIP(3,4,5) P_3 , respectively. PLC- γ 1 then is recruited to the plasma membrane and activated by PI(3,4,5) P_3 , producing more IP $_3$ that in turn increases Cai concentration to stimulate keratinocyte differentiation.

signaling represents only a fraction of the total PI(4,5) P_2 pool in the cell. This explanation does not exclude the possible contribution of PI(4,5) P_2 synthesized by PIP5K1 β to the basal level of PI(4,5) P_2 in the cell. However, we have found that PIP5K1 β is expressed at low level and is not regulated by Cao in keratinocytes (data not shown).

Previous studies have shown that elevation of Cao results in an acute and sustained increase in Cai in keratinocytes (Hennings *et al.*, 1989; Kruszewski *et al.*, 1991) and the sustained rise in Cai is necessary for keratinocyte differentiation (Li *et al.*, 1995). Our present data indicate that PIP5K1 α is required for Cao-induced acute and sustained Cai rise. Extracellular ATP, acting via P_2 purinergic receptors, induces only an acute increase in Cai by release of calcium from intracellular stores via activation of PLC (Pillai and Bikle, 1992; Lee *et al.*, 2001). The consequence of the transient Cai rise evoked by ATP is increased proliferation and decreased differentiation (Pillai and Bikle, 1992). From our data, it seems that PIP5K1 α also mediates the ATP-induced acute Cai rise, presumably by providing substrate PI(4,5) P_2 for PLC. However, the decrease in Cai in response to ionomycin in the absence of Cao in PIP5K1 α knockdown keratinocytes also suggests that PIP5K1 α is required for maintaining Cai stores, raising the possibility that this process is also mediated by PI(4,5) P_2 , the product of PIP5K1 α . In platelets, PI(4,5) P_2 has been shown to enhance store-operated calcium entry (Jardin *et al.*, 2008), which is essential for refilling the Cai stores (Putney, 1986, 1990). In addition, PLC- γ 1, which has also been shown to be required for the activation of store-operated calcium channels in liver cells (Litjens *et al.*, 2007) and keratinocytes (Tu *et al.*, 2005), may also contribute to the maintenance of the Cai store.

The confocal results show that PIP5K1 α is localized predominantly in the perinucleus and nucleus in the presence of low Cao and is partially recruited to the intercellular contact sites where it is associated with E-cadherin and β -catenin in keratinocytes grown in high Cao medium. The localization of PIP5K1 α and its product PI(4,5) P_2 in the perinucleus and nuclear speckles has been reported in other cell types (Boronenkov *et al.*, 1998; Doughman *et al.*, 2003). Although the PI(4,5) P_2 downstream pathway at these non-membrane structures has not been elucidated yet, it seems clear that the nuclear pool of PI(4,5) P_2 plays essential roles in several nuclear processes ranging from chromatin remodeling and pre-mRNA splicing to growth and proliferation (Bunce *et al.*, 2006). These processes are conceivably required for basal cellular activities of keratinocytes given that PIP5K1 α is expressed in all layers of the epidermis. When keratinocytes are stimulated by high Cao, PIP5K1 α is rapidly translocated to the plasma membrane where the substrate PI4P is situated to synthesize more membrane PI(4,5) P_2 , which activates downstream signaling leading to differentiation. Surprisingly, our data show that E-cadherin

is also translocated to the nucleus and colocalized with nuclear PIP5K1 α , in response to high Cao. These results suggest a heretofore unappreciated function for the E-cadherin–catenin complex in the nucleus. Further investigations are required to determine the mechanism and the functional significance of this observation.

Similar to the PI3K recruitment to the E-cadherin–catenin complex (Calautti *et al.*, 2005; Xie and Bikle, 2007), PIP5K1 α is also recruited to E-cadherin via β -catenin in response to high Cao concentrations. However, how β -catenin mediates these recruitments remains unclear. It seems unlikely that both kinases bind competitively to the same site of β -catenin in that knockdown of PIP5K1 α does not cause up-regulation of PI3K signaling. In fact, PI(3,4,5) P_3 association with PLC- γ 1 is down-regulated by PIP5K1 α knockdown as a result of reduction in PI(4,5) P_2 synthesis. By this recruitment mechanism, PI3K is able to cooperate nicely with PIP5K1 α to synthesize more PI(3,4,5) P_3 required for PLC- γ 1 activation. However, the region of PIP5K1 α required for its recruitment is not known. PIP5K1 α does not contain known protein–protein interaction domains and lacks any obvious means of regulation or interaction with other proteins. Additional studies will be necessary to determine the precise mechanism that mediates this interaction.

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