Protein Arginine Methyltransferase 5 (PRMT5) Signaling Suppresses Protein Kinase Cδ- and p38δ-dependent Signaling and Keratinocyte Differentiation*

Received for publication, December 7, 2011 Published, JBC Papers in Press, December 23, 2011, DOI 10.1074/jbc.M111.331660

Santosh R. Kanade† and Richard L. Eckert‡§

From the Departments of †Biochemistry and Molecular Biology, ‡Dermatology, and §Obstetrics and Gynecology and Reproductive Sciences, University of Maryland School of Medicine, Baltimore, Maryland 21201

Background: MAPK signaling is an important mechanism controlling keratinocyte differentiation.

Results: PRMT5 and p38δ interact as part of a multiprotein signaling complex, and PRMT5 and p38δ produce opposing actions in regulating differentiation.

Conclusion: PRMT5 modulates p38δ MAPK kinase phosphorylation and signaling.

Significance: This is a novel mechanism that links p38δ MAPK signaling and PRMT5 signaling.

PRMT5 is an arginine methyltransferase that symmetrically dimethylates arginine residues on target proteins to alter target protein function. We show that PRMT5 knockdown is associated with increased p38δ expression, and PRMT5 dimethylates proteins in the p38δ signaling complex. At a functional level we show that PRMT5 inhibits the PKCδ- or 12-O-tetradecanoylphorbol-13-acetate-dependent increase in human involucrin expression, and PRMT5 dimethylates proteins in the p38δ complex. Moreover, PKCδ expression reduces the PRMT5 level, suggesting that PKCδ activates differentiation in part by reducing PRMT5 level. These studies indicate antagonism between the PKCδ and PRMT5 signaling in control of keratinocyte differentiation.

Mitogen-activated protein kinases (MAPK) are dual-specificity serine/threonine kinases that drive intracellular signal transduction (1). MAPK family kinases share sequence similarity and conserved structural domains and include the extracellular signal-regulated kinases (ERK), Jun N-terminal kinases (JNK), and p38 MAPK. ERK is activated by mitogens and growth factors (2), whereas JNK and p38 kinases are typically activated in response to cellular stress (3). MAPKs play a central role in control of keratinocyte cell fate, and the balance between ERK and p38δ activity is a key determinant of keratinocyte survival status. Enhanced ERK activity is associated with survival, whereas enhanced p38δ activity is associated with differentiation and apoptosis (4–10). Three p38 isoforms, p38α, p38β, and p38δ, are expressed in keratinocytes (5, 8). Among these, p38δ has a key role as a positive regulator of keratinocyte differentiation (7). p38δ mediates the response to differentiating agents including phorbol ester, calcium, okadaic acid, and green tea polyphenol (7,10–15).

It is clear that MAPK signaling is regulated by cross-talk from other signaling cascades; however, this regulation is not well understood. This is because the key cascades that cross-talk with MAPK signaling are not well defined, are likely cell type- and context-specific, and the impact of this cross-talk on biological outcome is not well understood. In this study we demonstrate a role for protein arginine methyltransferase five (PRMT5)² in modulating MAPK signaling in keratinocytes. PRMT5 is an enzyme that dimethylates protein-bound arginine residues (16). Protein methylation is receiving increasing attention as an important post-translational modification. Protein arginine methyl transferases (PRMTs) are evolutionarily conserved enzymes that catalyze transfer of methyl groups from S-adenosyl methionine to the guanidino nitrogen of protein-bound arginine. Eight functional PRMT proteins are encoded in the mammalian genome (17). These enzymes mono- and dimethylate arginine residues in proteins and are classified as type I (PRMT1, 2, 3, 4, 6, and 8) and type II (PRMT5 and 7) enzymes. Type I enzymes catalyze formation of asymmetrically dimethylated arginine (16, 18).

PRMT5 is the sole type II member of the PRMT family that catalyzes formation of symmetrically dimethylated arginine (SDMA) (16). PRMT5 was discovered by yeast two-hybrid screening as Janus kinase-interacting protein 1 (16). PRMT5 catalyzes formation of symmetrically dimethylated arginine (SDMA) (16). PRMT5 dimethylates a variety of histone and non-histone proteins. Histone targets include histones H3 and H4 (19, 20), whereas non-

---

* This work was supported, in whole or in part, by National Institutes of Health Grant R01 AR046494 (to R. L. E.).

† A John F. B. Weaver Distinguished Professor. To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Maryland School of Medicine, 108 N. Greene St., Baltimore, MD 21201. Tel.: 401-706-3220; Fax: 401-706-8297; E-mail: recket@umaryland.edu.

‡ The abbreviations used are: PRMT, protein arginine methyltransferase; TPA, 12-O-tetradecanoylphorbol-13-acetate; KEδ, normal human keratinocyte; SDMA, symmetric dimethylarginine; hINV, involucrin; KSFM, keratinocyte serum-free medium.
PRMT5 and Keratinocyte Differentiation

histone targets include small heterodimer partner (21), myelin basic protein (22), and a host of others. PRMT5 interacts in a number of protein complexes that regulate RNA processing, signal transduction, and transcription (19, 23–29). PRMT5 is a critical determinant of circadian period in Arabidopsis (30), and as a component of the androgen receptor cofactor complex, PRMT5 positively modulates androgen receptor-driven transcription independent of its methyltransferase activity (31, 32). PRMT5 modulates enhanced GFR-mediated ERK activation (33) and is required for p53 expression and induction of p53 targets (34). PRMT5 also binds to death receptor 4 (35). An important study shows that PRMT1 modulates p38 MAPK regulation of differentiation in megakaryocytes (36). In addition to these functions in signal transduction, PRMT5 also participates in the assembly of the transcriptional repressor complex on various eukaryotic promoters (37). Thus, PRMT5 and protein arginine dimethylation are emerging as important regulators of cell function.

Involutrin is a keratinocyte structural protein that functions as a precursor of the cornified envelope and is expressed in the suprabasal layers of epidermis (38, 39). Regulation of involucrin gene expression has been extensively studied as a model for understanding regulation of differentiation-dependent gene expression in epidermis (5). A PKC, Ras, MEKK1, MEK3/MEK6 signaling cascade has been implicated as a key control pathway in regulating involucrin expression (5). In this study we use this system to study cross-talk between PRMT5 and MAPK signaling in regulating keratinocyte differentiation. We show that PRMT5 reduces involucrin expression in normal human keratinocytes (KErn). These studies further show that PRMT5 is part of a p38-ERK signaling complex and that PRMT5 modification of proteins in this complex is associated with reduced p38 phosphorylation. The net impact is that PRMT5 antagonizes p38-dependent keratinocyte differentiation.

EXPERIMENTAL PROCEDURES

Reagents—Keratinocyte serum-free medium (KSFM), trypsin and Hanks’ balanced salt solution were purchased from Invitrogen. Rabbit polyclonal antibodies specific for ERK1/2 (sc-94), PKCδ, PKCa (sc-213), PKCε (sc-208), p38δ (sc-7587), p38α (sc-7572), p38β (sc-6176), PRMT5 (sc-22132), PRMT1 (sc-59648), and peroxidase-conjugated anti-goat IgG (sc-213) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin (A5441), anti-FLAG M2 mouse monoclonal antibody (F-3165), anti-FLAG M2 affinity gel (A2202), and 12-O-tetradecanoylphorbol-13-acetate (TPA) were obtained from Sigma. Peroxidase-conjugated anti-mouse IgG (NA931) and peroxidase-conjugated anti-rabbit IgG (NA934V) were purchased from GE Healthcare. The antibody for detection of symmetric dimethyl arginine (07-412) was from Millipore (Bedford, MA). Knockdown of PKCδ and PRMT5 was achieved using siRNA. Pooled PKCδ-siRNA was from Santa Cruz (sc-36253) and from Dharmacon (I-003524-08). We also used individual siRNAs that target PKCδ mRNA sequences 5’-AGAAGGCGGACCAUGUAUU (PKCδ-1) and 5’-GUUGAUGCUGUUCAGUU (PKCδ-2) (40). All were equally effective at reducing PKCδ level. Pooled PRMT5-siRNA (NM_001039619) was from Santa Cruz (sc-41073) and Origene (SR307063). In addition, we used individual siRNA that target the PRMT5 mRNA sequences 5’-CAGGACUGAGGACACUCUGGAU (PRMT5-1) and 5’-CGCGCUACUUGAGACUGUCCUU (PRMT5-2) (35). All were equally effective at reducing PRMT5 levels. Rabbit anti-human involucrin was previously described (42, 43). Rabbit anti-phospho-p38 (9211S) was from Cell Signaling (Danvers, MA), and mouse monoclonal anti-phospho-ERK1/2 (M8159) was from Sigma. The involucrin promoter luciferase reporter construct, pINV-2473, was previously described (44).

Primary Keratinocyte Culture and Adenovirus Production—Newborn foreskin epidermis was separated from dermis with dispase, and the keratinocytes were dispersed with trypsin and cultured in KSFM supplemented with epidermal growth factor and pituitary extract (44, 45). Adenoviruses encoding wild-type FLAG-p38δ, PKCδ, and empty control adenovirus (Ad5-FLAG-p38δ, Ad5-PKCδ, Ad5-EV) were prepared as previously described by propagation in 293 cells and purification by cesium chloride centrifugation (9, 46). For infection, keratinocytes were treated with multiplicity of infection 10 of adenovirus in the presence of 2.5 μg/ml Polybrene for 4 h before the addition of fresh KSFM.

Affinity Purification of FLAG-p38δ and Mass Spectrometry—Keratinocytes were infected with Ad5-FLAG-p38δ and after 24 h extracts were prepared in lysate buffer (20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-mercaptoethanol, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and proteinase inhibitor mixture). Anti-FLAG M2 affinity gel was washed with a large volume of lysis buffer followed by two bed volumes of 20 mM glycine-HCl, pH 2.8, and then re-equilibrated with lysis buffer. Cell lysate (120 mg of total protein) was incubated with M2 gel for 12 h at 4 °C with shaking and then packed into PD10 column and washed with lysis buffer until A280 < 0.02 to remove unbound protein. FLAG-p38δ-associated proteins were eluted with 20 mM glycine-HCl, pH 2.8, and the collected fractions (200 μl) were neutralized by the addition of 1 M Tris before storage at −20 °C.

Affinity-purified FLAG-p38δ-associated proteins, prepared as outlined above, were concentrated by trichloroacetic acid precipitation, and samples were separated by SDS-PAGE followed by Coomassie Blue staining. Gel bands were excised and digested with trypsin, and the tryptic peptides were analyzed by liquid chromatography-tandem mass spectroscopy using a Bruker Omnistar benchtop MALDI-TOF MS/MS. For protein identification, the monoisotopic mass maps of tryptic peptides were searched in databases using the MASCOT search engine.

Immunoprecipitation and Immunoblot—Total extracts in lysis buffer were incubated with appropriate primary antibody and protein A/G-agarose overnight at 4 °C and washed 4 times with Tris-buffered saline, pH 7.4. After the final wash the beads were collected, boiled with sample buffer, and centrifuged, and supernatant was electrophoresed and transferred to nitrocellulose membrane. The blots were blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with appropriate primary and horse-
radish peroxidase-conjugated secondary antibody, and antibody binding was visualized by chemiluminescent detection (Amersham Biosciences).

**Keratinocyte Electroporation**—Keratinocytes were electroporated with plasmids using an Amaxa electroporator and the VPD-1002 nucleofection kit (Amaxa, Cologne, Germany). Keratinocytes were harvested with trypsin and replated 1 day before use. On the day of electroporation, 1.2 million replated cells were harvested with trypsin and suspended in KSFM. The cells were collected at 2000 × g, washed with 1 ml of sterile phosphate-buffered saline, and suspended in 100 μl of keratinocyte nucleofection solution. The cell suspension, which included 3 μg of plasmid or siRNA, was mixed by gentle up and down pipetting and electroporated using the T-018 program. KSFM (500 μl) was added, and the suspension was transferred to a 60-mm cell culture dish.

**Construction of pcDNA3-myc-hPRMT5**—The pOTB7-hPRMT5 (GenBank™ BC025979) plasmid (MHS1011-7508890) was obtained from Open Biosystems (Huntsville, AL). hPRMT5 was amplified to create a BamHI/NotI fragment using forward (5′-GATCGAATTCGGATCCATGGAA-CAAAAACTTATTTCTGAAGAAGATCTGATGGCGGCGATGGCGGTCCGGG3) and reverse (5′-GATCTCTAGAGCGGCGGCTAGAGCCATGTATATGAGGCGGCGCTGT3) primers. The ATG start codon (double underline) and myc epitope (underlined) are indicated. The insert was then cloned into pcDNA3 to create pcDNA3-myc-hPRMT5.

**Quantitative PCR**—RNA was isolated using RNAspin Mini kit (25-0500-71) (GE Healthcare). RNA (1 μg) was used for cDNA synthesis with Moloney murine leukemia virus transcriptase (Invitrogen). Gene expression was measured by real time PCR using Light Cycler 480 SYBR Green I Master mix (04-707 516 001) from Roche Diagnostics and specific primers included 3′-ACCCTTT. CTGGCGTCTTCACC; reverse, 5′/H11032ATGAGCATCTCGTACA), and GAPDH (forward, 5′/H11032ATGAGCATCTCGTACA), and GAPDH (forward, 5′-GATCGAATTCGGATCCATGGAA-CAAAAACTTATTTCTGAAGAAGATCTGATGGCGGCGATGGCGGTCCGGG3) and reverse (5′-GATCTCTAGAGCGGCGGCTAGAGCCATGTATATGAGGCGGCGCTGT3) primers. The ATG start codon (double underline) and myc epitope (underlined) are indicated. The insert was then cloned into pcDNA3 to create pcDNA3-myc-hPRMT5.

**RESULTS**

**Identification of FLAG-p38δ-associated Proteins**—Our previous studies describe a p38δ-ERK signaling complex in keratinocytes that regulates keratinocyte differentiation (6). Although these studies show that p38δ interacts with ERK1/2, it is likely that p38δ also interacts with other proteins and that these proteins influence function. To identify additional interaction partners, extracts were prepared from FLAG-p38δ-expressing keratinocytes, and p38δ-associated proteins were purified by FLAG affinity column chromatography. The column was loaded with 120 mg of total cell lysate and washed with loading buffer, and affinity-bound proteins were eluted with glycine buffer. Fig. 1A shows a Coomassie-stained gel illustrating the affinity purification of FLAG-p38δ. The fractions show the lysate (L) that was loaded onto the column, the column wash (W), and column elution fractions (F1, etc.). The Coo-
These findings suggest that PRMT5 is a binding partner for p38δ. To confirm this, we prepared total cell extracts from human keratinocytes and immunoprecipitated endogenous p38δ with anti-p38δ. The precipitate was electrophoresed, and the gel was stained with anti-PRMT5. Fig. 1 shows that PRMT5 co-precipitates with p38δ whether the precipitation is performed with anti-p38δ or anti-PRMT5, thereby providing additional evidence for p38δ/PRMT5 intracellular interaction.

Role of PRMT5 in Regulating Keratinocyte Differentiation—Our previous studies demonstrate that a PKCδ signaling cascade increases p38δ activity, which leads to increased cell differentiation (5, 6, 10, 47, 48). Because PRMT5 interacts with p38δ, we hypothesized that it may regulate differentiation-associated responses that depend upon p38δ activity. To assess this we monitored the impact of PRMT5 knockdown and overexpression on p38δ expression. hINV is a marker protein that is increased during keratinocyte differentiation and in response to differentiation stimuli (5, 6, 10, 47, 48). We began by assessing the impact of PRMT5 knockdown and overexpression on hINV mRNA and protein levels. Fig. 2A shows that treatment with PRMT5-pooled (Santa Cruz, sc-41073) or individual (PRMT5-1, PRMT5-2) siRNA (35) reduces PRMT5 levels, and this is associated with a marked increase in hINV protein levels. Fig. 2B shows this is due to changes in mRNA levels. Treatment
with PRMT5-pooled (P), PRMT5-1 (J) or PRMT5-2 (2) siRNA reduces PRMT5 mRNA, and this is associated with increased hINV mRNA. Consistent with these findings, the converse is also observed, as overexpression of PRMT5 reduces hINV mRNA and protein level (Fig. 2, D and E).

PRMT5 is a protein arginine methyltransferase that catalyzes modification of arginine residues in target proteins to form SDMA, a modification that modulates target protein function (16, 18). We, therefore, examined the impact of PRMT5 knockdown and overexpression on SDMA formation. Extracts from PRMT5-siRNA-treated and PRMT5-overexpressing cells were prepared for anti-SDMA immunoblot. Proteins ranging in molecular mass from 50 to 300 kDa are detected (Fig. 2, C and E). Immunostaining of a limited number of proteins covering a broad range of molecular weights is typical of anti-SDMA reagents (49). Using this as a crude assay of PRMT5 activity, we observe a 10–15% reduction in average SDMA intensity in PRMT5-siRNA-treated cells and a similar 10–15% increase in extracts derived from PRMT5-overexpressing cells. We do not presently know which proteins are identified in this assay, but these findings suggest that increased PRMT5-dependent SDMA formation is associated with suppression of involucrin gene expression.

**Regulation of hINV Expression by PKCδ and PRMT5**—The above studies indicate that PRMT5 suppresses hINV mRNA and protein levels. We next compared the action of PRMT5 against two treatments that increase differentiation, PKCδ overexpression and TPA treatment. PKCδ promotes keratinocyte differentiation and increases involucrin gene expression via activation of p38 (4, 5, 47). pINV-2473 encodes the full-length hINV promoter linked to a luciferase reporter gene such that increased promoter activity is reflected in increased luciferase activity (44). Keratinocytes were transfected with pINV-2473 in the presence of control-siRNA or PRMT5-siRNA and empty or PKCδ-encoding expression vector. In the presence of control-siRNA, PKCδ increases hINV promoter activity 3-fold (Fig. 3A). In contrast, when PRMT5-siRNA is present and PRMT5 is reduced (Fig. 3C), the induction is 6.5-fold (Fig. 3A). Moreover, the PKCδ induction of hINV promoter activity is suppressed (Fig. 3B) in PRMT5 overexpressing (Fig. 3C) cells.

TPA is a pharmacological agent that increases PKC activity and stimulates differentiation (44). We used TPA treatment as a second method of PKCδ activation. Keratinocytes were transfected with pINV-2473 and treated with TPA in the presence of control- or PRMT5-siRNA. TPA at 5 and 10 ng/ml increases hINV promoter activity, and a further 2-fold increase in promoter activity is observed in PRMT5 knockdown cells (Fig. 4A). In the converse experiment in which PRMT5 is overexpressed, the TPA-dependent increase in hINV promoter activity is suppressed by 2-fold (Fig. 4B). These studies suggest that PRMT5 opposes the pro-differentiation action of TPA and PKCδ.

It is well established that PKCδ enhances keratinocyte differentiation (4–6, 47). We wondered whether part of this mechanism may involve PKCδ suppression of PRMT5 expression. To test this we manipulated PKCδ level and monitored the impact on PRMT5 expression. Fig. 5A shows that PRMT5 RNA level is increased 4-fold when PKCδ mRNA levels is reduced by treating KERn with PKCδ-pooled (Santa Cruz) or individual (PKCδ-1 or PKCδ-2) siRNA. Moreover, the reduction in PKCδ level is associated with increased PRMT5 protein expression and SDMA formation (Fig. 5B). In the inverse experiment, PKCδ overexpression reduces PRMT5 RNA and protein level (Fig. 5, C and D), and this is associated with reduced SDMA formation (Fig. 5D). As noted in Fig. 2, the anti-SDMA blots shown in Fig. 5, B and D, provide a generally index of PRMT5 activity (49).

**PRMT5 Knockdown Enhances Total p38 Phosphorylation**—PKCδ or TPA stimulation of differentiation is associated with

---

**FIGURE 3. Impact of PRMT5 on PKCδ-dependent hINV promoter activity.** A, KERn were electroporated with 3 μg of control- or PRMT5-pooled (Santa Cruz) siRNA, and after 48 h the cells were trypsinized and electroporated with 1.5 μg of pINV-2473 (involucrin promoter luciferase reporter) in the presence of 1.5 μg of empty vector (pEGFN1) or PKCδ-encoding plasmid (pPKCδ-EGFN1). After an additional 18 h, cell extract was prepared for assay of luciferase activity. Values are the mean ± S.E., n = 3. The control-siRNA EV and PKCδ values are significantly different, p < 0.001; the PRMT5-siRNA EV and PRMT5-siRNA PKCδ values are significantly different, p < 0.001. B, KERn were electroporated with 1.5 μg of pcDNA3 or pcDNA3-PRMT5 expression vector, and after 48 h cells were re-electroporated with 1.5 μg of pINV-2473 and 0.5 μg of pEGFN1 or pPKCδ-EGFN1. After an additional 18 h, extracts were prepared for luciferase assay. The values are the mean ± S.E., n = 3. The pcDNA3 EV and PKCδ values are significantly different, p < 0.001. C, KERn were incubated with the indicated expression vectors or siRNA, and after 24 h extracts were prepared for detection of the indicated proteins by immunoblot. Similar results were observed at 48 and 72 h (not shown).
increased p38 MAPK activity as evidenced by increased p38 phosphorylation (4, 6, 10, 15). We hypothesized that PRMT5 may regulate differentiation by modulating the extent of this activation. Because no antibody is available that specifically detects phosphorylation of the individual p38α, -β, and -δ isoforms, we began by monitoring total p38 phosphorylation (i.e. phosphorylation of p38α, -β, and -δ) (9, 46). Keratinocytes were electroporated with control-siRNA or PRMT5-siRNA, and the level of total phosphorylated p38 was measured. Fig. 6 shows that PRMT5 knockdown increases total p38 phosphorylation (p38T-P). We also monitored the impact of PRMT5 knockdown on the phosphorylation status of other key regulators. p38α, -β, and -δ are expressed in keratinocytes (9, 46), but p38δ is the major form phosphorylated by stimuli that drive differentiation (4, 10, 47). ERK1/2 is also an important regulator, and ERK1/2 phosphorylation enhances keratinocyte survival (4). Fig. 6 shows that PKCδ, p38δ, and ERK1/2 levels and ERK1/2 phosphorylation are not altered by PRMT5 knockdown. p38α and β levels were also not altered by PRMT5 knockdown (not shown). Thus, a major impact of PRMT5 knockdown is to enhance total p38 phosphorylation.

PRMT5-dependent Covalent Modification of Proteins in p38δ Complex Is Associated with Increased p38δ Phosphorylation—Given that PRMT5 knockdown enhances total p38 phosphorylation, a key question is whether PRMT5 specifically modulates phosphorylation of the p38δ isoform. This could be expected, as previous studies indicate that p38δ is the key p38 isoform involved in regulation of differentiation (6, 7, 9). A second key issue is whether any such ability of PRMT5 to regulate p38δ phosphorylation is associated with altered SDMA modification of proteins in the p38δ signaling complex. To assess this, we performed two types of experiments. We first treated cells with control or PRMT5-pooled PRMT5-1 or PRMT5-2 siRNA for 48 h and then immunoprecipitated p38δ and assessed phosphorylation status. In Fig. 7A shows that p38δ is phosphorylated in PRMT5 knockdown but not control cells. As a second method to confirm this result, we monitored the phosphorylation state of vector-expressed FLAG-p38δ. Cells were treated with control- or PRMT5-siRNA and then infected with Ad5-EV or Ad5-FLAG-p38δ. At 24 h post-infection, FLAG-p38δ was immunoprecipitated with anti-FLAG, and the p38δ phosphorylation state was measured. Fig. 7B confirms that PRMT5 knockdown is associated with increased p38δ phosphorylation.

We next monitored whether the p38δ complex contains SDMA modified proteins. We infected cells with Ad5-EV or Ad5-FLAG-p38δ and after 24 h immunoprecipitated with anti-FLAG and monitored the level of SDMA in the precipitate. Fig. 7C shows that SDMA modified proteins are present in the p38δ complex. We next assessed whether this SDMA modification requires PRMT5. We treated cells with control- or PRMT5-siRNA and after 48 h prepared extracts for immunoprecipitation with anti-p38δ. Fig. 7D shows that a SDMA-modified 60-kDa protein is associated with the p38δ complex. Moreover, PRMT5 knockdown reduced the level of this protein or the extent of its SDMA modification. This protein has a molecular mass of 60 kDa, suggesting that it is a protein other than p38δ. As a control for assay integrity, we confirm appropriate immunoprecipitation of p38δ and PRMT5. Taken together, these findings suggest that PRMT5 suppresses p38δ phosphorylation and that this is associated with increased SDMA modification of a 60-kDa protein present in the p38δ complex.

DISCUSSION

Epidermal Differentiation—The epidermal keratinocyte, the major cell type present in the epidermis, undergoes a complex and choreographed program of differentiation. This process requires a balance among keratinocyte proliferation, differentiation, and apoptosis. Ultimately, this process leads to the formation of a multilayered epidermis that contains a proliferative basal zone beneath several layers of cells that are at various stages in the differentiation process (50). Involucrin is a marker of this process that is increased in differentiated cells (5) and is an important component of the scaffold of the cornified envelope (43, 51–53). Understanding how this process is regulated to produce a multilayered tissue is a major goal in epidermal biology, as disorders in this process are observed in a host of epidermal disease states and also in cancer.

p38δ-ERK Signaling Complex—Previous studies show that a p38δ-ERK complex regulates expression of key apoptosis, differentiation, and proliferation-associated genes in keratinocytes (4–6, 10, 11). p38δ activity is increased, and ERK1/2 activity is reduced in differentiated keratinocytes (6). The importance of interaction within this complex is illustrated by the role of p38δ and ERK1/2. p38δ and ERK1/2 are associated either directly or indirectly in this complex, and increased p38δ phosphorylation and reduced ERK1/2 activity is observed in
response to treatment with differentiation stimuli (4–7). We propose that this complex serves to integrate incoming signals and that the net output then determines cell fate. For example, activation of PKC\(\delta\) increases p38\(\delta\) activity and cell differentiation and death, whereas activation of ERK1/2 by delivery of constitutively active Raf antagonizes this response and promotes cell survival (5). The fact that a p38\(\delta\)-ERK complex is involved in this regulation suggests that other proteins may also be partners in this regulatory complex.

An important goal is identification of additional partners that are part of this complex and influence the signaling activity. To achieve this we expressed FLAG-p38\(\delta\) in keratinocytes and identified proteins associated with p38\(\delta\) by FLAG-affinity chromatography and mass spectrometry. One of the proteins that copurified with p38\(\delta\) was PRMT5. This interaction was confirmed by co-immunoprecipitation of PRMT5 and p38\(\delta\) from keratinocyte extracts using a p38\(\delta\)-specific antibody. This confirmation is important, as it has been reported in one study that PRMT5 can interact with anti-FLAG (16). Protein arginine methyltransferases are a family of proteins that dimethylate arginine residues in target proteins (16). Arginine modification in this context influences target protein structure, function, and activity (16). PRMTs have a role in numerous cellular processes including regulation of cell signaling and gene expression (17, 17).

**FIGURE 5. PKC\(\delta\) regulates PRMT5 level.** A, KERn were electroporated with 3 \(\mu\)g of control- and PKC\(\delta\)-pooled (P, Santa Cruz) or individual (PKC\(\delta\)-1, PKC\(\delta\)-2) (40) siRNA, and after 24 h PKC\(\delta\) and PRMT5 mRNA levels were monitored as outlined in Fig. 2. B KERn were electroporated with 3 \(\mu\)g of control- or PKC\(\delta\)-pooled (Santa Cruz) siRNA, and after 24 h extracts were prepared for detection of PKC\(\delta\), PRMT5, and SDMA by immunoblot. The values are the mean \pm S.D. of three replicates, and similar results were observed in each of three experiments. C and D, KERn were infected with the indicated adenovirus, and after 24 h mRNA and protein extracts were prepared for detection of PKC\(\delta\) and PRMT5 RNA and protein. SDMA was also monitored by immunoblot. The arrows in the SDMA blot indicate the major SDMA-modified proteins. The bar graph values are the mean \pm S.D. of three replicated samples. Similar results were observed in each of three experiments.

**FIGURE 6. PRMT5 suppresses total p38 phosphorylation.** Keratinocytes were electroporated with 3 \(\mu\)g of control- or PRMT5-siRNA, and after 48 h cell extracts were prepared and electrophoresed for immunoblot detection of the indicated proteins. Similar results were observed in each of two experiments.
32, 33, 54–57). However, additional studies are needed to explore the physiological mechanisms whereby PRMTs regulate cells’ survival, differentiation, and apoptosis. Despite the recognized importance of PRMT-dependent modification in a host of cell types, the role of PRMT in regulating keratinocyte function has not been studied.

**PRMT5 Regulates Basal Involucrin Expression**—Our previous studies show that keratinocyte differentiation requires activity in a PKCδ, Ras, MEK1, MEK3 cascade that triggers changes in activity in a p38δ-ERK complex to increase p38δ activity relative to ERK activity (4–7, 9–11, 15, 47, 48, 58). Activation of this cascade is associated with cessation of cell proliferation and increased morphological differentiation (8, 10, 15), and prolonged stimulation can cause apoptosis (4, 8). Stimulation of MAPK activity by this cascade is not associated with changes in expression of p38δ or ERK1/2 level but is associated with increased p38δ activity and reduced ERK1/2 activity (6). Involucrin is a marker of differentiation that is increased in differentiated cells and is expressed at a fixed basal level in resting keratinocytes (5). An interesting and unexpected finding is that PRMT5 knockdown increases basal involucrin expression including increasing hINV mRNA and protein levels and promoter activity. Moreover, the converse is also observed in that PRMT5 overexpression reduces basal involucrin mRNA and protein levels.

**PRMT5 Influences Stimulus-dependent Involucrin Expression**—We also monitored the impact of PRMT5 on response to agents that activate p38δ signaling to increase differentiation. Overexpression of PKCδ and treatment with TPA enhance p38δ phosphorylation and increase involucrin expression (4, 6). TPA is a diacylglycerol analog that enhances PKCδ activity and induces differentiation (44, 48). We show that PRMT5 knockdown enhances the differentiation-promoting ability of these agents and the PRMT5 overexpression suppresses the differentiation-promoting response. These findings suggest that PRMT5 is a negative regulator of keratinocyte differentiation. Thus, these studies identify an important new PRMT5-mediated regulatory circuit in keratinocytes that functions to sup-

---

**FIGURE 7.** PRMT5 regulates p38δ phosphorylation and SDMA modification of p38δ-associated proteins. A, KERn were electroporated with 3 μg of control, PRMT5-pooled, PRMT5-1, or PRMT5-2 siRNA, and after 48 h extracts were prepared for immunoprecipitation (IP) with anti-p38δ. The precipitated material was then examined by immunoblot to detect p38δ and phosphorylated p38δ. B, KERn were electroporated with 3 μg of control- or PRMT5-pooled (Santa Cruz) siRNA and after 24 h infected with multiplicity of infection 10 of Ad5-EV or Ad5-p38δ, and after an additional 24 h extracts were prepared for immunoprecipitation with anti-FLAG. The precipitated proteins were electrophoresed, and the level of FLAG-p38δ (anti-FLAG) and phosphorylated p38δ was monitored by immunoblot. C, KERn (70% confluent) was infected with multiplicity infection 10 Ad5-EV or Ad5-FLAG-p38δ, and after 24 h lysate was prepared, and 200 μg of protein was immunoprecipitated with anti-FLAG antibody. The resulting pellets were electrophoresed in a parallel lane. The blot was then incubated with anti-SDMA. D, KERn were electroporated with 3 μg of control- or PRMT5-pooled (Santa Cruz) siRNA, and after 48 h extracts were prepared for immunoprecipitation with anti-IgG or anti-p38δ. The precipitates were then electrophoresed for immunodetection of p38δ, PRMT5, and SDMA. The arrows indicate the major SDMA modified bands. E, PKCδ and PRMT5 regulation of p38δ activity; a working model. The model is explained in the “Discussion.” We realize that multiple complexes may exist and that some may lack one or more of the components, and that it is likely that other proteins are also present in this complex. We also note that we do not know whether there is direct interaction between the indicated components. The arrows indicate relative change in level. The dashed arrow indicates PRMT5 SDMA modification of target proteins.
press differentiation. In addition to an important role in normal cells, these findings suggest a role for PRMT5 in skin cancer. PRMT5 levels are increased (29), and PKCδ levels are decreased in cancer (59, 60). Thus, the balance between these signaling inputs is likely to be important in disease development, and we anticipate that PRMT5 may serve to enhance skin cancer cell survival. The possibility that PRMT5-related regulation is increased is an important area for further investigation in skin cancer. PRMT5 may also link the p38 pathway and also co-immunoprecipitates with p38 MAPK, indicating that no previous reports described an impact of PRMT5 on MAPK signaling. The only available report relates an impact of PRMT1, a type I PRMT that asymmetrically dimethylates arginine (16, 18), on p38α function in megakaryocyte differentiation (36).

**Working Model**—Our previous studies show that PKCδ activates involucrin gene expression (keratinocyte differentiation) by stimulating a cascade that regulates a p38δ and ERK1/2-containing complex to increase p38δ and decrease ERK1/2 phosphorylation (4–6, 10, 48). This change in the balance of p38δ and ERK1/2 activity ultimately leads to increased nuclear AP1 factor levels, increased AP1 factor interaction with specific sites on the hINV promoter, and increased transcription (41, 44, 61, 62).

Based on the studies presented in this proposal, we propose that PRMT5 is a new component of this signaling complex. The role of PRMT5 in this complex is to inhibit p38δ activity and suppress differentiation by catalyzing SDMA modification at the arginine residue(s) of a 60-kDa protein (and perhaps other proteins) that are present in this complex. We propose that SDMA modification of these targets alters the complex in a way that leads to reduced p38δ phosphorylation. Thus, when PRMT5 levels are high and PRMT5 is active, differentiation-dependent gene (hINV) expression is suppressed. Under conditions where cells are exposed to a differentiation stimulus, PKCδ activity triggers signaling events that drive differentiation. We propose that PKCδ acts to increase p38δ phosphorylation by two potential mechanisms (Fig. 7E). One mechanism is stimulating signaling events that lead to increased p38δ phosphorylation (6). Our present studies suggest that a second mechanism is suppression of PRMT5 mRNA and protein levels, thereby reducing PRMT5 modification of proteins in the p38δ complex. Our studies show that reduced PRMT5 levels are associated with increased p38δ phosphorylation. Thus, the present studies identify a new signaling protein present in the p38δ complex that suppresses differentiation-associated p38δ activity.

**Acknowledgment**—We thank Dr. Austin Yang, Director of University of Maryland School of Medicine Proteomics Facility, for assistance with mass spectrometry and informatics analysis.

**REFERENCES**

1. Schaeffer, H. J., and Weber, M. J. (1999) Mitogen-activated protein kinases. Specific messages from ubiquitous messengers. *Mol. Cell. Biol.* 19, 2435–2444
2. Roux, P. P., and Blenis, J. (2004) ERK and p38 MAPK-activated protein kinases. A family of protein kinases with diverse biological functions. *Microbiol. Mol. Biol. Rev.* 68, 320–344
3. Enslen, H., Branco, D. M., and Davis, R. J. (2000) Molecular determinants that mediate selective activation of p38 MAP kinase isoforms. *EMBO J.* 19, 1301–1311
4. Efimova, T., Broome, A. M., and Eckert, R. L. (2004) Protein kinase Cδ regulates keratinocyte death and survival by regulating activity and subcellular localization of a p38 extracellular signal-regulated kinase 1/2 complex. *Mol. Cell. Biol.* 24, 8167–8183
5. Eckert, R. L., Crish, J. F., Efimova, T., Dashiti, S. R., Deucher, A., Bone, F., Adhikary, G., Huang, G., Gopalakrishnan, R., and Balasubramanian, S. (2004) Regulation of involucrin gene expression. *J. Invest. Dermatol.* 123, 13–22
6. Efimova, T., Broome, A. M., and Eckert, R. L. (2003) A regulatory role for...
PRMT5 and Keratinocyte Differentiation

p38 MAPK in keratinocyte differentiation. Evidence for p38-ERK1/2 complex formation. J. Biol. Chem. 278, 34277–34285

7. Eckert, R. L., Efimova, T., Balasubramanian, S., Crish, J. F., Bone, F., and Dashi, S. (2003) p38 mitogen-activated protein kinases on the body surface. A function for p38. J. Invest. Dermatol. 120, 823–828

8. Eckert, R. L., Efimova, T., Dashi, S. R., Balasubramanian, S., Deucher, A., Crish, J. F., Sturniolo, M., and Bone, F. (2002) Keratinocyte survival, differentiation, and death. Many roads lead to mitogen-activated protein kinase. J. Investig. Dermatol. Symp. Proc. 7, 36–40

9. Dashi, S. R., Efimova, T., and Eckert, R. L. (2001) MEK7-dependent activation of p38 MAP kinase in keratinocytes. J. Biol. Chem. 276, 8059–8063

10. Efimova, T., LaCelle, P., Welter, J. F., and Eckert, R. L. (1998) Regulation of human involucrin promoter activity by a protein kinase C, Ras, MEKK1, MEK3, p38/RK, AP1 signal transduction pathway. J. Biol. Chem. 273, 24387–24395

11. Kraft, C. A., Efimova, T., and Eckert, R. L. (2007) Activation of PKCα and p38 MAPK during okadaic acid-dependent keratinocyte apoptosis. Arch. Dermatol. Res. 299, 71–83

12. Eckert, R. L., Crish, J. F., Efimova, T., and Balasubramanian, S. (2006) Opposing action of curcumin and green tea polyphenol in human keratino
cytes. Mol. Nutr. Food Res. 50, 123–129

13. Balasubramanian, S., and Eckert, R. L. (2007) Keratinocyte proliferation, differentiation, and apoptosis. Differential mechanisms of regulation by curcumin, EGCG, and apigenin. Toxicol. Appl. Pharmacol. 224, 214–219

14. Hong, S., Song, H. R., Lutz, K., Kerstetter, R. A., Michael, T. P., and Mc-

Clung, C. R. (2010) Type II protein arginine methyltransferase 5 (PRMT5) is required for circadian period determination in Arabidopsis thaliana. Proc. Natl. Acad. Sci. U.S.A. 107, 21211–21216

15. Tanaka, H., Hoshikawa, Y., Oh-hara, T., Koike, S., Naito, M., Noda, T., Arai, H., Tsuru, T., and Fujita, N. (2009) PRMT5, a novel TRAIL recep-
tor-binding protein, inhibits TRAIL-induced apoptosis via nuclear fac-
tor-κB activation. Mol. Cancer Res. 7, 557–569

16. Chang, Y. I., Hua, W. K., Yao, C. L., Huang, S. M., Hung, Y. C., Kuan, C. J., Leou, J. S., and Lin, W. J. (2010) Protein-arginine methyltransferase 1 suppresses megakaryocytic differentiation via modulation of the p38 MAPK pathway in K562 cells. J. Biol. Chem. 285, 20595–20606

17. Cesario, D., De Cegli, R., Medugno, L., Florio, F., Grosso, M., Lupo, A., Izzo, P., and Costanzo, P. (2009) The Kruppel-like zinc finger protein ZNF224 recruits the arginine methyltransferase PRMT5 on the transcriptional repressor complex of the aldolase A gene. J. Biol. Chem. 284, 32321–32330

18. Rice, R. H., and Green, H. (1979) Presence in human epithelial cells of a soluble protein precursor of the cross-linked envelope. Activation of the cross-linking by calcium ions. Cell 18, 681–694

19. Rice, R. H., and Green, H. (1977) The cornified envelope of terminally differentiated human epidermal keratinocytes consists of cross-linked protein. Cell 11, 417–422

20. Cai, Q., Li, J., Gao, T., Xie, J., and Evers, B. M. (2009) Protein kinase C6 negatively regulates hedgehog signaling by inhibition of Gli1 activity. J. Biol. Chem. 284, 2150–2158

21. Crish, J. F., and Eckert, R. L. (2008) Synergistic activation of human involu-
crin gene expression by Fra-1 and p300. Evidence for the presence of a multiprotein complex. J. Invest. Dermatol. 128, 530–541

22. LaCelle, P. T., Lambert, A., Ekambaram, M. C., Robinson, N. A., and Eck-
ert, R. L. (1998) In vitro cross-linking of recombinant human involucrin. Skin Pharmacol. Appl. Skin Physiol. 11, 214–226

23. Robinson, N. A., LaCelle, P. T., and Eckert, R. L. (1996) Involution is a covalently cross-linked constituent of highly purified epidermal corneocyte. Evidence for a common pattern of involution cross-linking in vivo and in vitro. J. Invest. Dermatol. 107, 101–107

24. Welter, J. F., Crish, J. F., Agarwal, C., and Eckert, R. L. (1995) Fos-related antigen (Fra-1), JunB, and junD activate human involucrin promoter transcription by binding to proximal and distal AP1 sites to mediate phorbol ester effects on promoter activity. J. Biol. Chem. 270, 12614–12622

25. Banks, E. B., Crish, J. F., Welter, J. F., and Eckert, R. L. (1998) Characterization of human involucrin promoter distal regulatory region transcription.
tional activator elements. A role for Sp1 and AP1 binding sites. Biochem. J. 331, 61–68.

46. Dashti, S. R., Efimova, T., and Eckert, R. L. (2001) MEK6 regulates human involucrin gene expression via a p38α- and p38δ-dependent mechanism. J. Biol. Chem. 276, 27214–27220.

47. Efimova, T., Deucher, A., Kuroki, T., Ohba, M., and Eckert, R. L. (2002) Novel protein kinase C isoforms regulate human keratinocyte differentiation by activating a p38α mitogen-activated protein kinase cascade that targets CCAAT/enhancer-binding protein α. J. Biol. Chem. 277, 31753–31760.

48. Efimova, T., and Eckert, R. L. (2000) Regulation of human involucrin promoter activity by novel protein kinase C isoforms. J. Biol. Chem. 275, 1601–1607.

49. Boisvert, F. M., Côté, J., Boulanger, M. C., and Richard, S. (2003) A proteomic analysis of arginine-methylated protein complexes. Mol. Cell. Proteomics 2, 1319–1330.

50. Eckert, R. L., Crish, J. F., and Robinson, N. A. (1997) The epidermal keratinocyte as a model for the study of gene regulation and cell differentiation. Physiol. Rev. 77, 397–424.

51. Robinson, N. A., Lapic, S., Welte, J. F., and Eckert, R. L. (1997) S100A11, S100A10, annexin I, desmosomal proteins, small proline-rich proteins, plasminogen activator inhibitor-2, and involucrin are components of the cornified envelope of cultured human epidermal keratinocytes. J. Biol. Chem. 272, 12035–12046.

52. Nemes, Z., Marekov, L. N., and Steinert, P. M. (1999) Involucrin cross-linking by transglutaminase 1. Binding to membranes directs residue specificity. J. Biol. Chem. 274, 11013–11021.

53. Steinert, P. M., and Marekov, L. N. (1999) Initiation of assembly of the cell envelope barrier structure of stratified squamous epithelia. Mol. Biol. Cell 10, 4247–4261.

54. Liu, F., Zhao, X., Perna, F., Wang, L., Koppiar, P., Abdel-Wahab, O., Harr, M. W., Levine, R. L., Xu, H., Tefferi, A., Deblasio, A., Hatlen, M., Menendez, S., and Nimer, S. D. (2011) JAK2V617F-mediated phosphorylation of PRMT5 down-regulates its methyltransferase activity and promotes myeloproliferation. Cancer Cell 19, 283–294.

55. Rank, G., Cerruti, L., Simpson, R. J., Moritz, R. L., Jane, S. M., and Zhao, Q. (2010) Identification of a PRMT5-dependent repressor complex linked to silencing of human fetal globin gene expression. Blood 116, 1585–1592.

56. Igarashi, H., Kuwahara, K., Yoshida, M., Xing, Y., Maeda, K., Nakajima, K., and Sakaguchi, N. (2009) GAPN suppresses the arginine methyltransferase PRMT5 regulating IL-4-mediated STAT6-signaling to IgE production in B cells. Mol. Immunol. 46, 1031–1041.

57. Guderian, G., Peter, C., Wiesner, J., Sickmann, A., Schulze-Osthoff, K., Fischer, U., and Grimmel, M. (2011) BioK1, a new interactor of protein arginine methyltransferase 5 (PRMT5), competes with pICln for binding and modulates PRMT5 complex composition and substrate specificity. J. Biol. Chem. 286, 1976–1986.

58. Deucher, A., Efimova, T., and Eckert, R. L. (2002) Calcium-dependent involucrin expression is inversely regulated by protein kinase Cα and PKCζ. J. Biol. Chem. 277, 17032–17040.

59. Reno, E. M., Haughian, J. M., Dimitrova, I. K., Jackson, T. A., Shroyer, K. R., and Bradford, A. P. (2008) Analysis of protein kinase Cζ expression in endometrial tumors. Hum. Pathol. 39, 21–29.

60. Yadav, V., Yanez, N. C., Fenton, S. E., and Denning, M. F. (2010) Loss of protein kinase Cζ gene expression in human squamous cell carcinomas. A laser capture microdissection study. Am. J. Pathol. 176, 1091–1096.

61. Crish, J. F., Zaim, T. M., and Eckert, R. L. (1998) The distal regulatory region of the human involucrin promoter is required for expression in epidermis. J. Biol. Chem. 273, 30460–30465.

62. Crish, J. F., Gopalakrishnan, R., Bone, F., Gilliam, A. C., and Eckert, R. L. (2006) The distal and proximal regulatory regions of the involucrin gene promoter have distinct functions and are required for in vivo involucrin expression. J. Invest. Dermatol. 126, 305–314.