The novel protein kinase C (nPKC) isoforms are important regulators of human involucrin (hINV) gene expression during keratinocyte differentiation (Efimova, T., and Eckert, R. L. (2000) J. Biol. Chem. 275, 1601–1607). Although the regulatory mechanism involves mitogen-activated protein kinase (MAPK) activation, the role of individual nPKC isoforms has not been elucidated. We therefore examined the effects of individual nPKCs on MAPK activation. We observe unique changes whereby nPKC expression simultaneously increases p38 activity and decreases ERK1 and ERK2 activity. Although p38α, p38β, and p38δ are expressed in keratinocytes, only a single isoform, p38δ, accounts for the increased p38 activity. Parallel studies indicate that this isoform is also activated by treatment with the keratinocyte regulatory agents, 12-O-tetradecanoylphorbol-13-acetate, calcium, and okadaic acid. These changes in MAPK activity are associated with increased C/EBPα transcription factor expression and DNA binding to the hINV promoter and increased hINV gene expression. Expression of PKCδ, PKCε, or PKCγ causes a 10-fold increase in hINV promoter activity, whereas C/EBPα expression produces a 25-fold increase. However, simultaneous expression of both proteins causes a synergistic 100-fold increase in promoter activity. These responses are eliminated by the dominant-negative C/EBP isoform, GADD153, and are also inhibited by dominant-negative forms of Ras, MEKK1, MEK3, and p38. These results suggest that the nPKC isoforms produce a unique shift in MAPK activity via a Ras, MEKK1, MEK3 pathway, to increase p38δ and inhibit ERK1/2 and ultimately increase C/EBPα binding to the hINV promoter and hINV gene expression.

Protein kinase C (PKC) family members are classified into three major groups. The conventional/classical PKCs (cPKCs), cPKCβI, cPKCβII, and cPKCγ are calcium-, diacylglycerol-, and phospholipid-dependent kinases; the atypical PKC kinases (aPKCs) are calcium- and diacylglycerol-independent (1–3); and the novel PKCs (nPKCs, nPKCζ, nPKCη, nPKCδ, and nPKCø), are calcium-independent enzymes (1–3). Each PKC isoform has unique co-factor requirements, tissue distribution, subcellular localization, and substrate specificity (1, 3, 4). Epidermal keratinocytes express α, δ, ε, η, and ζ isoforms (5–9). However, the role of each isoform in regulating differentiation is not well understood.

In previous studies, we demonstrated that the novel PKC isoforms PKCδ, PKCε, and PKCη, but not the conventional and atypical PKC forms, activate keratinocyte differentiation as measured by effects on human involucrin (hINV) gene expression (10, 11). Involucrin is a precursor of the keratinocyte-cornified envelope and a marker of early keratinocyte differentiation (12). Dominant-negative PKCδ inhibits this response (10). This pathway appears to operate by triggering a cascade that includes Ras, MEKK1, and MEK3. Although this pathway is known to require MAPK activity, whether individual nPKCs activate different MAPKs has not been explored. In the present study we activate the signaling cascades at the PKC level by expression of individual nPKC isoforms in keratinocytes and monitor the effects on MAPK function and downstream responses. Our results show that nPKC activation results in increased p38 MAPK activity and reduced ERK1/2 activity. No change in activity of other p38 isoforms (p38α and p38β) is observed. This combination of changes leads to increased C/EBPα transcription factor expression and activity and increased hINV gene expression. These results suggest that nPKC inhibits ERK1/2 expression and activates p38δ via a Ras, MEKK1, MEK3 pathway that targets C/EBPα to increase hINV gene expression.

MATERIALS AND METHODS

Reagents—Keratinocyte serum-free medium, gentamicin, trypsin, and Hanks’ balanced salt solution were obtained from Invitrogen. Dif- fuse was obtained from Roche Molecular Biochemicals. Phorbol ester (12-O-tetradecanoylphorbol-13-acetate (TPA)) and Me 2SO were pur- chased from Sigma. Biotinylmaleimide and okadaic acid were from Calbiochem. The pGL2-Basic plasmid and the chemiluminescent lucif- erase assay system were obtained from Promega. Chemiluminescence

human involucrin; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; caMEK, constitutively active MEK; MEK, MEK kinase; TPA, 12-O-tetradeca- noylphorbol-13-acetate; MOI, multiplicity of infection; JNK, c-Jun N- terminal kinase; dn, dominant-negative; C/EBP, CCAAT/enhancer-binding protein.
was measured using a Berthold luminometer. Oligonucleotides for construction of mutant promoter sequences were synthesized using an Applied Biosystems DNA synthesizer. PKC isoform-selective (PKCδ, sc-837; PKCθ, sc-215) and C/EBPα-selective (C/EBPα, sc-61x) antibodies were obtained from Santa Cruz Biotechnology. The rabbit anti-ino- 

Plasmids—We have previously published the structure of the hINV promoter construct pINV-241, which include nucleotides −241/−7 of the hINV promoter, linked to the luciferase reporter gene in pGL2-Basic (14). All of the positions are define relative to the hINV gene transcription start site. The structure of the mutated C/EBP site in pINV-241 (C/EBPΔα) has been described (15). PKC expression vectors were a generous gift from Dr. S. Ohno (7, 16). The gel mobility shift assay was performed exactly as previously outlined (14, 15). Transfection efficiency was monitored using a green fluorescent protein expression plasmid. Recombinant adenovirus Vectors—nPKC-expressing adenoviruses were previously constructed (26). Adenoviruses encoding constitutively active MEK6 and wild type FLAG-tagged p38 MAPK isoforms α, β, γ, and δ were obtained from Dr. Y. Wang (27, 28). A control (empty) adenovirus, Ad5-EV, was generated by recombining pCA3 plasmid with the pJM17 adenovirus backbone. Recombinant adenoviruses were propagated in 293 cells and purified by cesium chloride centrifugation. The optimal multiplicity of adenoviral infection was determined using the green fluorescent protein-encoding adenovirus. The adenoviruses were administered at the indicated MOI in the presence of 2.5 μg/ml polybrene.

Immunoblot Analysis—Total cell or nuclear extracts were prepared from normal human epidermal keratinocytes as described previously (15). Equal quantities of protein were electrophoresed on a 10% denaturing SDS-polyacrylamide gels and transferred to nitrocellulose. The membranes were blocked, incubated with an indicated primary antibody, washed, and exposed to an appropriate horseradish peroxidase-conjugated secondary antibody. Secondary antibody binding was visualized using chemiluminescent detection methods (29). MAPK activities of MAPK-expressing keratinocytes were measured using nonisotopic p44/42 (ERK1/2), p38 MAPK, and JNK/stress-activated protein kinase assay methods (New England Biolabs) (25). Briefly, keratinocytes total cell lysates were prepared under nondenaturing conditions. Equal amounts of total protein (200 μg) were used per each kinase assay. Immobilized, dual phosphorylase ERK1/2 and phosphorylase p38 MAPK monoclonal antibodies were used to selectively immunoprecipitate active (phosphorylated) ERK1/2 and p38 kinases, respectively. JNK/stress-activated protein kinase was selectively precipitated from cell lysates using c-Jun fusion protein glutathione-Sepharose beads. Precipitated kinases were then allowed to phosphorylate substrate proteins (Elk-1 for ERK1/2, ATF-2 for p38, and c-Jun for JNK/stress-activated protein kinase) in a kinase reaction performed in the presence of ATP. Phosphorylation of the substrate proteins was analyzed by immunoblotting using phosphorylated substrate-specific antibodies. The activity of adenovirus-delivered FLAG-tagged p38 MAPK isoforms was measured by precipitating the tagged kinases using anti-FLAG M2 mouse monoclonal antibody (F3165, Sigma). Expression of individual FLAG-p38 isoforms was confirmed by immunoblot using anti-FLAG antibody. To measure the activity of endogenous p38 isoform, p38-specific antibody (SC-7585) was used to selectively immunoprecipitate this enzyme followed by a kinase assay performed as outlined above.

Gel Mobility Supershift Analysis of C/EBPα Binding—The gel mobility shift assay was performed exactly as previously outlined (14, 15) using a double-stranded, 32P end-labeled oligonucleotide, 5'-GGTTTTT-GCTGCTTAAGATGCGCCTG-3' (C/EBP site is in bold), that encodes the hINV C/EBP-binding site region (15).

RESULTS

Regulation of MAPK Activity by PKCδ and PKCγ—To identify the mechanism whereby the nPKC isoforms regulate gene expression during keratinocyte differentiation, we transfected keratinocytes with empty adenovirus or adenovirus encoding PKCδ or PKCγ. At 48 h, the cells were harvested, and an in vitro kinase assay was performed by immunoprecipitating the activated forms of all p38 isoforms using anti-phospho-p38. Kinase activity was then measured based on the ability of the precipitated kinase to phosphorylate ATF-2. Parallel assays were performed to measure ERK1/2 and JNK1/2 activity. As shown in Fig. 1A, PKCδ and PKCγ markedly increase p38 activity. In contrast, ERK1/2 activity is suppressed (PELK1). JNK activity was not detected (P-c-Jun). To assure that the observed changes in kinase activity were not due to regulation of kinase level, we measured endogenous kinase levels by immunoblot. Fig. 1B shows that nPKC expression does not alter the level of p38, JNK1/2, or ERK1/2. To evaluate whether the decline in overall ERK activity is selectively associated with reduced ERK1 or ERK2 activity, we compared total ERK1/2 and P-ERK1/2 levels by immunoblot. Fig. 1C shows that although total ERK1/2 levels are not altered by PKCδ or γ treatment, both isoforms are proportionately reduced in activity as measured by diminished levels of phosphorylated ERK1/2 (P-ERK1/2). Fig. 1A shows that JNK1/2 is not active. Thus, as a positive control to assure that the JNK assay is functional, we treated keratinocytes for 24 h with okadaic acid, prepared extracts, and assayed JNK activity. As shown in Fig. 1D, okadaic acid-dependent JNK activity is readily detected, confirming the validity of the assay. Finally, it is important to confirm that the vector-delivered proteins are expressed. Thus, extracts were prepared from adenovirus expression vector-infected cells, and the level of PKC isoform expression was monitored by immunoblot. The blot shown in Fig. 1E confirms that PKCδ and η are expressed. It should be noted that, as expected (10), endogenous PKCγ expression is detected when the film exposure time is extended, and overexpression of selected PKC isoforms does not affect the expression of the endogenously expressed PKCs (not shown). Taken together, these results provide evidence that nPKC isoforms δ and η activate p38, inhibit ERK1/2, and have no effect on JNK1/2 activity.

Activation of p38 Isoforms by PKCδ and PKCγ—p38 MAPK exists as a family of four distinct isoforms (α, β, δ, and γ) that have different biological functions (30). To identify which p38 isoform is activated by PKC, keratinocytes were infected with FLAG-tagged p38α, β, γ, or δ in the presence of empty virus or virus encoding PKCγ or PKCδ. After 48 h, the cells were harvested, and individual FLAG-p38 isoforms were precipitated using anti-FLAG antibody and assayed for the ability to phosphorylate ATP-2 (29, 31). Fig. 2A shows that adenovirus-delivered FLAG-p38α, γ, and δ are active in control (empty virus infected) cultures and that p38δ and γ activities are increased in the presence of empty virus or virus encoding PKCγ or PKCδ. After 48 h, the cells were harvested, and individual FLAG-p38 isoforms were precipitated using anti-FLAG antibody and assayed for the ability to phosphorylate ATP-2 (29, 31). Fig. 2A shows that adenovirus-delivered FLAG-p38α, γ, and δ are active in control (empty virus infected) cultures and that p38δ and γ activities are increased in the presence of empty virus or virus encoding PKCγ or PKCδ. Thus, the only p38 isoform that is increased in activity in response to nPKC is p38δ. To confirm that the vector-delivered p38 isoforms are expressed, we prepared cell extracts and performed immunoblotting using a FLAG-specific antibody. Fig. 2B shows that, with the exception of p38γ, which is expressed at higher levels, each FLAG-p38 isoform is expressed at a relatively similar level. The above results suggest that p38δ is the major p38 isoform activated by PKCδ and η. To determine whether endogenous p38δ behaves in a similar manner, we infected keratinocytes

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PKC\(\eta\) and \(\delta\) regulate MAPK activity. A, normal human keratinocytes were infected with empty adenovirus (EV) or infected with adenovirus encoding PKC\(\eta\) or \(\delta\) at a MOI of 15, and 48 h later the cells were lysed for extract preparation. Activated p38, JNK1/2, and ERK1/2 were precipitated, respectively, using mouse monoclonal anti-phospho-p38, \(\alpha\), \(\beta\), \(\gamma\), or \(\delta\) (New England Biolabs 9181), and 30 \(\mu\)l of protein A/G-agarose (Santa Cruz Biotechnology, sc-2003), and p38 activity was monitored based on the ability of the precipitated kinase to phosphorylate ATF-2. B, immunoblot showing that adenovirus-delivered p38 \(\alpha\), \(\beta\), \(\gamma\), and \(\delta\) are expressed at comparable levels in keratinocytes. C, endogenous p38 activity is regulated by nPKCs. The keratinocytes were infected with empty adenovirus (EV) or adenovirus encoding PKC\(\eta\), PKC5, dnPKC\(\eta\), or dnPKC\(\delta\). After 48 h, the cells were harvested and endogenous p38 was precipitated using goat anti-p38 (Santa Cruz sc-7383, 5 \(\mu\)g/precipitation), p38 kinase activity was monitored based on ability to phosphorylate ATF-2 (P-ATF-2) as described for Fig. 1. In parallel, the p38 levels were monitored by immunoblot using goat anti-p38 at a dilution of 1:1000. \(\beta\)-Actin levels were monitored to control for gel loading.

FIG. 2. Regulation of p38 MAPK isoforms by PKC\(\eta\) and \(\delta\). A, to measure the enzymatic activity of individual p38 isoforms in response to nPKC expression, keratinocytes were co-infected with empty adenovirus vector (EV) or adenovirus encoding PKC\(\delta\) or PKC\(\eta\) and with FLAG-p38\(\alpha\), \(\beta\), \(\gamma\), or \(\delta\). After 48 h, individual FLAG-tagged p38 isoforms were immunoprecipitated (200 \(\mu\)g of protein/sample) using mouse monoclonal anti-FLAG antibody M2 (Sigma F3165, 5 \(\mu\)g/precipitation) and 30 \(\mu\)l of protein AG-agarose (Santa Cruz Biotechnology, sc-2003), and p38 activity was monitored based on the ability of the precipitated kinase to phosphorylate ATF-2. B, immunoblot showing that adenovirus-delivered p38 \(\alpha\), \(\beta\), \(\gamma\), and \(\delta\) are expressed at comparable levels in keratinocytes. C, endogenous p38 activity is regulated by nPKCs. The keratinocytes were infected with empty adenovirus (EV) or adenovirus encoding PKC\(\eta\), PKC5, dnPKC\(\eta\), or dnPKC\(\delta\). After 48 h, the cells were harvested and endogenous p38 was precipitated using goat anti-p38 (Santa Cruz sc-7385, 5 \(\mu\)g/precipitation). p38 kinase activity was monitored based on ability to phosphorylate ATF-2 (P-ATF-2) as described for Fig. 1. In parallel, the p38 levels were monitored by immunoblot using goat anti-p38 at a dilution of 1:1000. \(\beta\)-Actin levels were monitored to control for gel loading.

with empty vector or vectors encoding PKC\(\delta\) or PKC\(\eta\). After 48 h, endogenous p38\(\delta\) was selectively immunoprecipitated, and p38\(\delta\) activity was assayed based on the ability to phosphorylate ATF-2. As shown in Fig. 2C, PKC\(\delta\) and \(\eta\) stimulate the activation of endogenous p38\(\delta\). In contrast, the dominant-negative forms of PKC\(\delta\) and \(\eta\) (dnPKC\(\delta\) and dnPKC\(\eta\)) do not regulate p38\(\delta\) activity, suggesting that this regulation requires nPKC activity. To assure that these changes in p38\(\delta\) activity are not the results of altered p38 levels, we prepared extracts 1:5000, rabbit anti-JNK1/2 (Sigma J4500 diluted 1:2000), and rabbit anti-ERK1/2 (Sigma M5670 diluted 1:5000) antibodies, followed by detection with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences NA934 diluted 1:10,000). These experiments were repeated a minimum of three times with similar results. \(\beta\)-Actin levels were monitored as a control for loading. C, cells were treated as in A, and total ERK1/2 level and phosphorylated ERK1/2 level were monitored by immunoblot using rabbit anti-ERK1/2 (Sigma M5670) and mouse monoclonal anti-phospho-ERK1/2 (Santa Cruz Biotechnology, sc-7383) antibodies, respectively. D, keratinocytes were treated for 24 h in the absence (−) or presence (+) of 100 \(\mu\)M okadaic acid, and JNK1/2 activity was monitored as outlined above. E, normal keratinocytes were infected with empty adenovirus (EV) or PKC\(\delta\) or PKC\(\eta\)-encoding adenovirus. After 48 h, the cell extracts were prepared, and PKC\(\delta\) and PKC\(\eta\) levels were measured by immunoblot using rabbit anti-PKC\(\delta\) (Santa Cruz Biotechnology, sc-937), and rabbit anti-PKC\(\eta\) (Santa Cruz Biotechnology, sc-215).
from cells at 48 h after nPKC-encoding virus infection and measured p38δ levels by immunoblot. The middle panel of Fig. 2C shows that PKC expression does not alter p38δ levels. The bottom panel is a β-actin immunoblot that is included to assure even loading.

**Regulation of ERK1/2 and p38δ Activity by Differentiating Agents**—If nPKC activation is important for response to agents that regulate keratinocyte differentiation, we would anticipate that treatment with these agents would produce a change in MAPK activity similar to that of expression of nPKC isoforms. To address this issue, cells were infected with a FLAG-p38δ encoding adenovirus. After 24 h, the cells were treated with 0.3 mM calcium, 100 nM okadaic acid, or 50 ng/ml TPA, agents that regulate nPKC activity or the activity of enzymes immediately downstream of the nPKCs. At the indicated times, the extracts were prepared, and FLAG-38δ was precipitated and assayed for activity. As measured by the ability to phosphorylate ATF-2, each of these agents induced strong and sustained activation of p38δ; however, the time course of the calcium-dependent activation was slower (Fig. 3A). It should be noted that okadaic acid- and TPA-dependent p38δ activity was maximally increased by 6 h and remained maximal until 24 h (not shown). ERK1/2 activity was measured directly by monitoring the level of phosphorylated ERK1/2 following treatment for the time indicated. Fig. 3B shows that phosphorylated ERK1 and ERK2 levels are reduced by TPA and okadaic acid treatment. However, calcium did not reduce the level of phosphorylated ERK1 or ERK2. These changes in p38δ and ERK1/2 activity were not associated with changes in p38δ or ERK1/2 levels as measured by immunoblot (not shown).

**PKCs and PKCη Regulate C/EBPα Expression**—The above results suggest that both differentiating agents and nPKC isoforms regulate MAPKs in similar manners. An important aspect of how the nPKC-dependent MAPK cascades regulate keratinocyte differentiation is identification of the downstream targets that mediates activation of differentiation-dependent gene expression. C/EBPα has been proposed as a key transcriptional regulator of differentiation (15, 32, 33). Our previous study suggests that TPA-dependent regulation of differentiation-associated gene expression requires C/EBPα activity (15). We next determined whether nPKC isoforms regulate C/EBP function. We infected keratinocytes with native or dominant-negative forms of PKCδ or η, and after 48 h monitored C/EBPα level. Fig. 4A shows that C/EBPα, consistent with previous reports (15), is expressed in control (empty vector-infected) cells. The present experiment shows that this expression is markedly increased by PKCδ and PKCη and markedly reduced by expression of dnPKCδ or dnPKCη. Thus, C/EBPα levels increase in a PKCδ and PKCη-dependent manner. Based on the above results, we suggest that PKCδ and η increase C/EBPα expression via a p38δ-dependent mechanism. To test this hypothesis, we infected keratinocytes with empty adenovirus or adenoviruses encoding p38δ, constitutively active MEK6 (caMEK6), or both. After 48 h, the cells were harvested, and the C/EBPα levels were monitored by immunoblot. As shown in Fig. 4B, adenovirus-dependent expression of p38δ causes a marked increase in C/EBPα level. This experiment also demonstrates that caMEK6 enhances C/EBPα expression. MEK6 is a MAPK kinase that is an immediate upstream activator of p38δ (30, 31). Thus, the fact that p38δ increases C/EBPα expression that is further increased when both MEK6 and p38δ are present further suggests that the C/EBPα increase is p38δ-dependent.

**Synergistic Activation of Target Gene Expression by nPKC Isoforms and C/EBPα**—The above studies suggest that nPKCs, via activation of p38δ and C/EBPα, regulate differentiation-associated gene expression in keratinocytes. To evaluate the impact of this regulation on differentiation, we studied involucrin gene expression. Involucrin is a marker of keratinocyte differentiation that is regulated via a C/EBPα-dependent mechanism (15). We transfected keratinocytes with pINV-241, a luciferase-linked hINV promoter reporter plasmid (14). The cells were then treated with expression plasmids encoding PKCδ, PKCε, PKCη, or C/EBPα. As shown in Fig. 5A, C/EBPα expression causes a 25-fold increase in promoter activity, whereas each nPKC isoform (nPKCδ, ε, and η) produces a 10-fold activity increase. Remarkably, simultaneous expression of nPKC with C/EBPα results in a >100-fold activation. The
PKCs and PKC- and p38 MAPK Regulate Expression of the Endogenous hINV Gene—The above studies demonstrate that the novel PKC isoforms activate hINV promoter expression. To confirm the physiological relevance of this observation, we examined the effects of PKCγ and p38 on expression of endogenous hINV. Keratinocytes were infected with empty expression vector or vectors encoding PKCγ, p38, or both. After 48 h, the cells were harvested, and the total cell extracts were prepared for immunoblot. Fig. 5E shows that involucrin levels are minimal in cells infected with empty vector. However, hINV levels are markedly increased in cells expressing PKCγ or p38 and further increased in cells expressing both PKCγ and p38.

p38 MAPK and ERK1/2 Regulation of nPKC-dependent hINV Promoter Activity—The results presented in Figs. 1 and 2 are consistent with the idea that p38 functions as an activator and ERK1/2 as an inhibitor of the nPKC-dependent regulation. To test this, we monitored the ability of dominant-negative forms of p38, ERK1, and ERK2, to alter the PKC-dependent increase in hINV promoter. The keratinocytes were transfected with PKCδ in the absence and presence of dnp38, dnERK1, or dnERK2. As shown in Fig. 6A, PKCδ produces an 8-fold increase in hINV promoter activity. Co-expression of dnp38 with PKCδ completely inhibits this response. In contrast, dnERK1 markedly enhances the PKCδ-dependent activation. dnERK2 does not alter the regulation. These results suggest that p38 is a mediator of the PKCδ-dependent regulation, and ERK1 is an inhibitor. Identical results were observed for PKCe- and PKCγ-dependent gene activation (not shown).

Ras, MEKK1, and MEK3 Activity Are Required for nPKC-dependent Regulation—To identify kinases that mediate transfer of the nPKC-dependent signal to the MAPKs, we transfected keratinocytes with PKCδ in the absence and presence of dnRas, dnRaf1, dnMEKK1, dnMEK3, and dnJNK. Fig. 6B shows that dnRas, dnMEKK1, and dnMEK3 inhibit the PKCδ-dependent increase in hINV promoter activity. In contrast, dnRaf1 and dnJNK do not influence this increase. Identical results were observed for PKCe and γ (not shown).

**DISCUSSION**

Novel PKC Isoforms Drive Keratinocyte Differentiation via Activation of p38 MAPK—Epidermal keratinocytes and other stratified squamous surface epithelia undergo a regulated process of differentiation (12). They begin as proliferative cells and are ultimately converted to nonproliferating cells called corneocytes that assemble the epidermal surface (12). This change involves a remarkable number of gene activation events that result in the expression of a group of proteins, including involucrin, that are designed to construct this barrier (12, 37, 38). Specific PKC isoforms are implicated in this process. For example, recent studies show that the nPKC isoforms activate keratinocyte differentiation (10, 26) and that this response is associated with growth inhibition (26). This response is also associated with increased expression of differentiation-associated marker genes, including type I transglutaminase (26) and hINV (10). A major goal is identification of the cascades that link nPKC activation to increased expression of target genes.

In this regard, we have shown that an nPKC, Ras, MEKK1, MEK3, and p38 cascade regulates hINV gene expression (10, 25). Although these studies showed that p38 is an important intermediary in this cascade, they did not identify which of the four known p38 isoforms are required (25). Identifying the active isoform is important, because individual p38 isoforms are known to produce varying biological responses in a cell type-specific manner (39–42), and three p38 isoforms, α, β, and δ, are expressed in keratinocytes (29). Our present study indicates that, in keratinocytes, the nPKCs selectively activate p38δ and not p38α or p38β. These results are in agreement...
Fig. 5. A, novel PKC and C/EBPα synergistically increase hINV promoter activity. Cultured human epidermal keratinocytes were transfected with 2 μg of pINV-241 in the presence of 2 μg of control plasmid or plasmid encoding C/EBPα, PKCδ, PKCe, or PKCη. The final concentration of plasmid was adjusted to 4 μg/group for all treatments using empty plasmid. After 48 h, the cells were harvested, and the extracts were prepared and assayed for luciferase activity. The inset is an immunoblot demonstrating that each expression vector produces the appropriate encoded protein. B, keratinocytes were transfected with 2 μg of pINV-241 in the presence of 2 μg of control plasmid or plasmid encoding GADD153, PKCδ, PKCe, or PKCη. The final concentration of plasmid was adjusted to 4 μg/group for all treatments using empty plasmid. After 48 h, the cells were harvested, and luciferase activity was monitored as outlined in A. C, keratinocytes were transfected with pINV241(C/EBPm) in the presence of C/EBPα, PKCδ, PKCe, or PKCη. After 48 h, luciferase activity was monitored as in A. D, to measure the effects of PKCδ and PKCη expression on C/EBPα binding to DNA, the cells were infected with empty adenovirus or adenovirus encoding PKCδ or PKCη. After 48 h, the cells were harvested, and the nuclear extracts were prepared. To detect C/EBPα binding to DNA, nuclear extract (2 μg) was incubated with double-stranded, 32P end-labeled oligonucleotide encoding the hINV promoter C/EBP site (5'-GGTTTGCTGCTTAAGATGCCTG, C/EBP-binding site in bold type) (15). The lane labeled – shows the migration of free probe in the absence of nuclear extract. The other lanes contain nuclear extract prepared from cells treated with the indicated adenovirus. A 100-fold molar excess of radioinert homologous oligonucleotide was included in the reaction mixture, as indicated, to demonstrate specificity of the binding. Additional controls demonstrating that the binding is due to C/EBPα are included in our
with the findings of a previous study showing that PKC-activating agents increase p38δ activity in HeLa cells (43). We further confirm that stimuli that enhance keratinocyte differentiation via PKC-associated mechanisms, including calcium and TPA, increase p38δ activity. Thus, these results confirm that p38δ is an important pro-differentiation isoform in several contexts.

In addition to the selective activation of the p38δ isoform, nPKC isoforms also suppress ERK1/2 activity. This suggests that a reduction in ERK1/2 activity may also be essential for keratinocyte differentiation and for inhibition of proliferation. In contrast, JNK1/2 activity is not altered. Agents that regulate keratinocyte differentiation, including calcium, TPA, and okadaic acid, shared the common ability to increase p38δ activity. However, these agents differ with respect to suppression of ERK1/2 activity, because TPA and okadaic acid inhibited ERK1/2, but calcium treatment did not. Thus, it appears that nPKCs activate a subset of the differentiation-related signaling changes. However, it is a response (i.e. p38δ activation) that is shared by all of the differentiating agents and appears to be necessary for activation of hINV gene expression. It is possible that separate cascades regulate different aspects of the differentiation process, and additional studies will be necessary to sort this out.

**Novel PKCs Increase C/EBPα Level and Binding to the hINV Promoter**—Regulation of hINV gene expression in keratinocytes requires input from several transcriptional regulators, including the C/EBP transcription factors (14, 44–46). The observation that C/EBPα binding to the hINV promoter C/EBP-binding site is increased in TPA-treated keratinocytes suggests a role for protein kinase C in this regulation (15). Our present studies directly test this possibility and show that the nPKCs have an important role. nPKC isoform expression results in increased C/EBPα level and DNA binding, suggesting that

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**FIG. 6.** Ras, MEKK1, MEK3, and p38 kinases are required for nPKC-dependent regulation of differentiation. A, keratinocytes were transfected with 2 μg of pINV-241 in the presence or absence of 1 μg of plasmid encoding PKCδ (+PKCδ) or and 1 μg of control plasmid (−PKCδ). In addition, each group was transfected with control plasmid (C) or plasmid encoding dnP38, dnERK1, or dnERK2. After 48 h, the cells were harvested and assayed for luciferase activity. The total quantity of plasmid in each group was maintained constant by the addition of control plasmid. B, keratinocytes were transfected with 2 μg of pINV-241 in the presence or absence of 1 μg of plasmid encoding PKCδ (+PKCδ) or 1 μg of control plasmid (−PKCδ). In addition, each group was transfected with additional control plasmid (C) or plasmid encoding dnRas, dnRaf1, dnMEK1, dnMEK3, or dnJNK. After 48 h, the cells were harvested and assayed for luciferase activity. The total quantity of plasmid in each group was maintained constant by addition of control plasmid. C, pathway of C/EBPα factor and hINV gene regulation by nPKC, Ras, MEKK1, MEK3, and p38δ and ERK1/2. The model indicates that stimuli that activate ERK1/2 have a negative effect on hINV gene transcription, whereas a pathway that includes nPKC, Ras, MEKK1, MEK3, and p38δ activates C/EBPα level and DNA binding and increases hINV gene expression.
nPKCs control C/EBPα level/activity. Consistent with this idea, dominant-negative forms of PKCδ and γ do not increase C/EBPα level, and the nPKC-dependent activation of hINV gene expression is inhibited by GADD153, a dominant-negative C/EBP isoform. In addition, dominant-negative kinase studies indicate that the nPKC- and C/EBPα-dependent activation of gene expression requires Ras, MEKK1, and MEK3 activity. Based on these results, we propose the pathway of regulation shown in Fig. 6C in which a nPKC, Ras, MEKK1-dependent signal is transferred to C/EBPα via MEK3 and p38. Further evidence indicates that ERK1/2 activity is simultaneously suppressed and that this suppression is due to inhibition of activity of both ERK1 and ERK2. Inhibition of ERK1/2 activity using dominant-negative kinases results in increased hINV promoter activity, consistent with an inhibitory role for ERK. ERK1 appears to be the stronger suppressor. Thus, these findings tie nPKC activation to increased p38 activity and tie reduced ERK1 activity to increased C/EBP binding at the hINV promoter C/EBP-binding site and to increased hINV gene activation.

p38 also regulates differentiation via activation of C/EBP factors in other cell types. In adipocytes p38 MAPK phosphorylates C/EBP-homologous protein via activation of p38α and β (47), suggesting that CHOP serves as a link between p38 MAPK and regulation of adipose cell differentiation (48, 49). In contrast, our studies clearly identify p38β as carrying the signal downstream to the C/EBPβ protein. This may reflect a cell type-specific difference in the wiring of the signaling cascades and in the selection of the target C/EBP factor.

Role of C/EBP during in Vivo Keratinocyte Differentiation—C/EBP factors are expressed in a regulated manner during keratinocyte differentiation. C/EBPα is expressed in the suprabasal, differentiated compartments, whereas C/EBPδ is present in the undifferentiated basal layers (50). C/EBPβ and CHOP are in the nuclei of mid-layer cells (51). In keratinocyte cultures differentiated by suspension in semi-solid media, both C/EBPα and C/EBPβ are present, whereas only C/EBPβ is present in adherent, undifferentiated, cells (52). Thus, in general, C/EBPα levels are increased with increased differentiation. The present results are consistent with a model wherein C/EBPα enhances hINV gene expression. In contrast, C/EBPβ and δ inhibit hINV expression in cultured cells (15) or does not influence hINV expression in C/EBPβ knockout mice (38). This may, at least in part, explain why hINV is expressed in the suprabasal epidermal layers, where C/EBPα is expressed, and not in the basal layers, where C/EBPβ predominates. Although our results with hINV are consistent with this model, the regulation is not simple, because other markers of keratinocyte differentiation, K1 and K10, are increased by C/EBPβ (33, 38, 51), and C/EBPβ is a negative regulator of human papillomavirus type 11 promoter transcription (53). It is possible that this difference in regulatory potential of the various C/EBP isoforms may be related to other transcription factors associated with C/EBP in the regulatory complex. Thus, whether C/EBP acts with API factors, as appears to be the case for hINV, or other factors, may determine the regulatory outcome. Additional studies will be necessary to understand the role of these interactions.

In summary, our results indicate that nPKC isoforms simultaneously inhibit ERK1/2 activity and selectively activate the δ isoform of p38 MAPK via a cascade that includes Ras, MEKK1, and MEK3. The results are consistent with a mechanism whereby p38α increases C/EBPα transcription factor level and C/EBPα DNA binding to hINV promoter regulator elements to increase hINV gene expression.