MEK6 Regulates Human Involucrin Gene Expression via a p38α- and p38δ-dependent Mechanism

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A signaling cascade that includes protein kinase C (PKC), Ras, and MEK1 regulates involucrin (hINV) gene expression in epidermal keratinocytes (Efimova, T., LaCelle, P., Welter, J. F., and Eckert, R. L. (1998) J. Biol. Chem. 273, 24387–24395 and Efimova, T., and Eckert, R. L. (2000) J. Biol. Chem. 275, 1601–1607). Because signal transfer downstream of MEK1 may involve several MAPK kinases (MEKs), it is important to evaluate the regulatory role of each MEK isoform. In the present study we evaluate the role of MEK6 in transmitting this signal. Constitutively active MEK6 (caMEK6) increases hINV promoter activity and increases endogenous hINV levels. The caMEK6-dependent increase in gene expression is inhibited by the p38 MAPK inhibitor, SB203580, and is associated with a marked increase in p38α MAPK activity; JNK and ERK kinases are not activated. In addition, hINV gene expression is inhibited by dominant-negative p38α and increased when caMEK6 and p38α are co-expressed. caMEK6 also activates p38δ, but p38δ inhibits the caMEK6-dependent activation. These results suggest that MEK6 increases hINV gene expression by regulating the balance between activation of p38α, which increases gene expression, and p38δ, which decreases gene expression.

Involucrin is a component of the keratinocyte cornified envelope that is expressed in the suprabasal layers of stratifying squamous epithelia and serves as a marker of keratinocyte differentiation (1). Differentiation-dependent hINV1 gene activation involves a signaling cascade that includes the novel protein kinase C, Ras, and MEKK1 (2, 3). The MEKK1-associated signal is transmitted via several MAPK kinases (MEKs) (2, 3). These in turn activate p38 kinases, which regulate hINV gene expression (2, 3). Several lines of evidence support this mechanism. For example, dominant-negative forms of protein kinase C, Ras, MEKK1, and p38 inhibit basal and differentiation-dependent hINV gene expression in cultured keratinocytes (2, 3).

Although MEKs are structurally related (4), they can differentially regulate downstream responses. For example, MEK6 is reported to activate all four p38 isoforms (5), whereas MEK3 activates p38α, δ, and γ, but not p38δ (5). Although MEK3 and MEK7 have been implicated as regulators of hINV gene expression (2, 3), the role of the other MEKs has not been examined. Because of the central role of MEK and p38 kinases in regulation of hINV gene expression, it is important to determine how each p38 isoform is regulated by each MEK and how these events influence hINV gene expression. In the present study, we examined the role of MEK6 in conjunction with the p38 isoforms as regulators of hINV gene expression. We demonstrate that MEK6 regulates p38 MAPK activity and that hINV gene expression is influenced by the balance between MEK6-dependent p38α and p38δ activation. These results suggest that the selective activation of specific p38 isoforms is one mechanism whereby the MAPK cascade influences differentiation-dependent gene expression in keratinocytes.

MATERIALS AND METHODS

Tissue Culture, Cell Transfection, and hINV Promoter Assay—Normal human foreskin keratinocytes were cultured as described previously (6). Third passage keratinocytes, grown in 35-mm dishes, were utilized when 50% confluent. Cells were transfected with 2 μg of pINV-2475, a plasmid that encodes the full-length hINV promoter fused to the firefly luciferase gene (7), in the presence of 4 μl of FuGENE6 transfection reagent (8). At 24 h posttransfection, adenoviruses encoding protein-encoding expression plasmid to normalize transfection efficiency (8, 9). Each experiment was repeated a minimum of three times.

EMK1/2, p38, and JNK Kinase Activity Assays—p38, ERK1/2, and JNK activity were assayed using a non-radioactive method. Cell lysates were prepared in 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Kinase activity was monitored in buffer containing 25 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, and 10 mM MgCl2. For total p38 activity assessment, an agarose-conjugated monoclonal antibody (New England Biolabs number 9219) that binds to the phosphorylated form (Thr180/ Tyr182) of all p38 isoforms was used to immunoprecipitate active p38 kinase. The precipitate was assayed for p38 activity as measured by ability to phosphorylate ATF-2. ATF-2 phosphorylation was measured by immunoblot using a rabbit anti-phospho-ATF-2 (New England Biolabs number 9212S). ERK1/2 activity was monitored by immunoprecipitation using an agarose-conjugated monoclonal antibody (New England Biolabs number 9109) that binds phospho-ERK1/2. Activity of the precipitated enzyme was assayed based on ability to phosphorylate ELK-1. Phospho-ELK-1 was detected using a rabbit anti-phospho-ELK1 (New England Biolabs number 9181S). To measure JNK/SAPK

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activity, activated JNK was precipitated using c-jun fusion protein beads (New England Biolabs number 9811) (10), and the activity of the precipitated enzyme was monitored using c-jun as a substrate. Phospho-c-jun was detected by immunoblot using anti-phospho-c-jun (New England Biolabs number 9811).

Western Blot Analysis—Cells were rinsed with phosphate-buffered saline, lysed in Laemmli buffer (11), and an equivalent amount of protein (10 μg) was electrophoresed on 8% acrylamide gels. The blots were incubated with the primary antibody, washed, and exposed to horseradish peroxidase-conjugated secondary antibody. Specific antibody binding was visualized using chemiluminescence detection reagents.

Antibodies—Peroxidase-conjugated monoconal anti-FLAG (number A8592, diluted 1:1000), anti-FLAG monoconal (number F3165, diluted to 10 μg/ml), rabbit anti-p38α (number M0800, diluted 1:5000 for immunoblot and at 5 μg/immunoprecipitation), rabbit anti-JNK1/2 (number J4500, diluted 1:2000), rabbit anti-ERK1/2 (number M5670 diluted 1:5000), and mouse monoclonal anti-β-actin (number A5441, diluted 1:10,000) were obtained from Sigma. Goat anti-MEK6 (sc-6073, diluted 1:1000), goat anti-SAPK4 (p38α, sc-7585 used at 5 μg/immunoprecipitation), and peroxidase-conjugated donkey anti-goat IgG (sc-2020, diluted 1:10,000) were purchased from Santa Cruz. Mouse anti-p38α (number 35-5700 used at 5 μg/immunoprecipitation), peroxidase-conjugated donkey anti-rabbit IgG (NA934, diluted 1:10,000), and peroxidase-conjugated sheep anti-mouse IgG (NA931, diluted 1:10,000) was purchased from Amersham Pharmacia Biotech. Protein G/Ag-agarose (IP05, 30 μl/reaction) was from Oncogene, and rabbit anti-hINV was previously described (12).

Adenoviruses and Plasmids—Adenoviruses and/or plasmids encoding wild type, constitutively active (ca) and dominant-negative (dn) kinases were used in these studies. These include caMEK6 (13, 14), and FLAG-tagged p38α, β, δ, and γ, adenoviruses (13, 15), and plasmids encoding dnp38α and the corresponding empty control plasmid (10, 16). An “empty” adenovirus was generated by recombining pCA3 plasmid with the pJM17 adenovirus backbone in 293 cells (EV). The green fluorescent protein-encoding virus, CMV-GFP, was used to define the optimal adenovirus infection multiplicity. pINV-2473, a plasmid encoding the human involucrin gene promoter fused to luciferase, was previously described (12).

Detection of MEK6 mRNA—Poly(A+) RNA was isolated from cultured keratinocytes using the Oligotex Direct mRNA System (Qiagen). For real time RT-PCR, the RNA amplification kit (SYBR Green I, Roche Molecular Biochemicals) used 20 ng of mRNA and 0.5 μM of each MEK6 primer (5′-AGC GGA TCC GAG CCA CAG TAA ATA-3′ and 5′-CCC GAA ACA GTG CGC CAT AAA AG-3′) in a total reaction volume of 20 μl. The denaturation, annealing, and elongation steps were 95 °C for 1 s, 55 °C for 10 s, and 72 °C for 20 s, respectively. The primers were designed using the DNASTAR computer program based on the published MEK6 sequence (17) to amplify a 115-nucleotide segment of MEK6. Control reactions to evaluate primer specificity included no template or 0.1 ng of plasmid encoding MEK3, MEK6, or MEK7.

RESULTS

MEK6 Regulates hINV Promoter Activity—To evaluate the role of MEK6 as a regulator of hINV gene expression, keratinocytes were transfected with the pINV-2473 reporter plasmid and then infected with empty adenovirus (EV) or adenovirus encoding constitutively active MEK6. As shown in Fig. 1A, mock-infected and EV-infected cells display basal hINV promoter activity. In contrast, caMEK6 increases hINV promoter activity by 2.7-fold. To determine whether MEK6 similarly regulates endogenous hINV expression, cells were infected with MEK6-encoding virus, and endogenous hINV protein levels were assayed by immunoblot (2). Basal involucrin protein levels were detectable in mock-infected and EV-infected cells (Fig. 1B). In contrast, caMEK6 produced a 3- to 4-fold increase in endogenous involucrin level. To confirm expression of the exogenously delivered protein, we measured the level of adenovirus-expressed MEK6 proteins by immunoblot. As shown in Fig. 1C, caMEK6 is clearly expressed.

SB203580 Inhibits MEK6-dependent hINV Gene Expression—To determine whether MEK6 acts via p38, we tested the ability of SB203580, an inhibitor of p38α and β, to block the caMEK6-dependent increase in hINV protein levels. SB203580 at a concentration of 1 μM inhibits p38α and β but not p38γ or
MEK6 activates p38 MAPK activity. Normal human keratinocyte were mock-infected, infected with empty adenovirus (EV), or infected with adenovirus encoding constitutively active MEK6. After 48 h, the cells were lysed, and extracts were prepared. A, activated ERK1/2, p38, and JNK MAPKs were immunoprecipitated, respectively, using agarose-conjugated mouse monoclonal anti-phospho-ERK1/2 (New England Biolabs number 9181), agarose-conjugated mouse monoclonal anti-phospho-p38, β, δ, γ (New England Biolabs number 9219), and c-jun fusion beads (New England Biolabs number 9811). The immunoprecipitated material was then measured for ability to phosphorylate the appropriate substrate. Substrate phosphorylation was monitored by immunoblot using rabbit anti-phospho-ATF-2 (Sigma number M0800), mouse anti-p38-β-δ-γ (New England Biolabs number M5670 diluted 1:5000), and goat anti-p38-βδ-γ (Amersham Pharmacia Biotech number 33–8700), each at 5 μg/ml Polybrene®. The media was replaced every 12 h with fresh keratinocyte-serum free medium containing fresh SB203580-HCl. At 48 h after infection, the cells were lysed, and hINV protein level was assayed by immunoblot (12). Identical results were observed in each of three experiments. β-actin was monitored as an internal control to confirm appropriate protein loading.

To determine whether MEK6 acts via p38 to activate hINV gene expression, cells were mock-infected, or infected for 48 h. The cells were then harvested, and an in vitro kinase assay was performed by selectively immunoprecipitating the activated form of all p38 isoforms using anti-phospho-p38. Kinase activity was then measured based on the ability of the precipitated enzyme to phosphorylate ATP-2. As shown in Fig. 3A, caMEK6 robustly increases endogenous p38 kinase activity in cultured keratinocytes. However, caMEK6 does not regulate ERK or JNK activity. To assure that the observed response is due to a change in activity and not p38 kinase level, we measured endogenous p38 levels in cells infected with constitutively active MEK6. As shown in Fig. 3B, treatment with MEK6 does not change p38 protein level or the level of ERK or JNK.

**Fig. 3.** MEK6 activates p38 MAPK activity. A, to measure the enzymatic activity of individual p38 isoforms in response to caMEK6, keratinocytes were co-infected with empty vector (Control (EV)), or caMEK6 and FLAG-p38α, β, γ, or δ. After 48 h, the p38 isoforms were immunoprecipitated using mouse monoclonal anti-FLAG antibody M2 (Sigma number F3165, diluted to 5 μg/ml) and 30 μl of protein G/A-agarose (Oncogene IP05). p38 activity was monitored based on ability of the precipitated kinase to phosphorylate ATF-2. Phosphorylated ATF-2 was detected by immunoblot at 35, 42, and 48 kDa. This result was confirmed in three separate experiments. B, p38 kinase activity was measured by mock infecting keratinocytes, infecting with empty (EV) adenovirus, or infecting with adenovirus encoding caMEK6. After 48 h, the cells were harvested and endogenous p38α, β, δ, and γ were immunoprecipitated, respectively, using rabbit anti-p38α (Sigma number M0800), mouse anti-p38-βδ-γ (Amersham Pharmacia Biotech number 33–8700), and goat anti-p38-βδ-γ (Santa Cruz sc-7585), each at 5 μg/ml precipitation. p38 kinase activity was monitored based on the ability to phosphorylate ATF-2. Phosphorylated ATF-2 was detected by immunoblot as in Fig. 3. This result was confirmed in three separate experiments.

**Fig. 4.** Regulation of p38 MAPK isoforms in keratinocytes. A, to investigate the efficiency and specificity of activation of the p38 isoforms by MEK6, we expressed FLAG-tagged p38α, β, γ, or δ in cells infected with caMEK6. After 48 h, the cells were lysed, and the p38 isoforms were immunoprecipitated using anti-FLAG antibody and assayed for p38 kinase activity. Fig. 4A shows that adenovirus-expressed recombinant p38α is active in the presence of absence of caMEK6. caMEK6 strongly activates p38δ and γ, and slightly increases the already high level of p38α activity. Validity of this assay requires that each p38
isoform be expressed at a comparable level in keratinocytes. This was confirmed by immunoblot using an anti-FLAG antibody (Fig. 4B). As previously reported, overexpressed p38α is active in control cells (21, 22). Therefore, to determine whether caMEK6 activates p38α, endogenous p38α was precipitated using an antibody that specifically detects p38α, and the precipitated p38α enzyme was assayed for ability to phosphorylate ATF-2. As shown in Fig. 4C, endogenous p38α activity is substantially increased in the presence of caMEK6. Similarly, the endogenous p38β and p38δ isoforms were precipitated using specific antibodies and assayed for the ability to phosphorylate ATF-2. Fig. 4C also shows that caMEK6 does not increase endogenous p38β kinase activity; however, endogenous p38δ kinase activity is increased.

**p38 Isoforms Differentially Regulate caMEK6-dependent hINV Promoter Activation**—If p38α is an activator of hINV gene expression, we would expect that dominant-negative p38α would inhibit and wild type p38α would stimulate the caMEK6-dependent promoter activation. As shown in Fig. 5A, caMEK6-dependent hINV promoter activity is inhibited by expression of dnp38α. In addition, caMEK6 and p38α each increase promoter activity, and coexpression of these kinases results in a slightly greater increase (Fig. 5B). In contrast, as shown in Fig. 6, expression of wild type p38δ inhibits caMEK6-dependent promoter activity.

**p38α and δ Differentially Regulate Endogenous hINV Gene Expression**—To determine whether p38α regulates endogenous hINV gene expression, cells were infected with empty adenovirus or adenovirus encoding p38α or caMEK6. After 48 h, cells were lysed and assayed for hINV protein expression. Fig. 7A shows that p38α and caMEK6 increase endogenous hINV protein levels. We next measured the ability of p38δ to regulate both the caMEK6- and the TPA-dependent increase in endog-
enos hINV gene expression. TPA is a keratinocyte differentiating agent that is known to increase hINV levels (2, 6). Treatment with TPA, caMEK6, or TPA/caMEK6 increases hINV protein level (Fig. 7B), and expression of p38α inhibits the increase. It is important to note that in one-half of the experiments co-treatment with caMEK6 and TPA results in enhanced levels of hINV compared with each individual treatment (not shown). In each case, the presence of p38α inhibits the increase.

MEK6 Is Expressed in Keratinocytes—For the present studies to be physiologically relevant, it is important to show that MEK6 is expressed in keratinocytes. To test this, we used real time RT-PCR to measure MEK6 mRNA levels. As shown in Fig. 8A, nonspecific background signal was detected in RT-PCR reactions programmed without template (blank), or 0.1 ng of MEK3-, MEK6-, or MEK7-encoding plasmid. Fluorescence intensity is directly correlated with accumulation of PCR product. B, to detect endogenous MEK6 protein expression, a total cell extract was prepared, and 100 μg of extract was electrophoresed on a 6% polyacrylamide gel. An extract, prepared from cells infected with MEK6-encoding adenovirus, was electrophoresed in parallel as a positive control for antibody activity (expressed). MEK6 was detected by immunoblot using rabbit anti-MEK6 (Chemicon International, AB3185) diluted 1:700. Binding of the primary antibody was visualized using peroxidase-conjugated donkey anti-rabbit IgG (Amer- sham Pharmacia Biotech NA934, diluted 1:10,000), and the signal was visualized by chemiluminescence.
The MAP kinases are ubiquitously expressed enzymes that transfer signals from the cell surface to the nucleus (23). These enzymes are organized as part of three-kinase modules that include a MAPK kinase kinase (MEKK), a MAPK kinase (MEK), and a MAPK (24). Four major mitogen-activated protein kinases (MAPK) have been identified including ERK1/2, ERK5, p38 MAPK, and the c-jun NH2-terminal kinase (JNK) (24–27). MEK-dependent dual phosphorylation of the regulatory loop of the MAPK results in kinase activation (24). MEKs are known to differentially activate specific MAPKs. For example, MEK1/2 activates ERK1/2, MEK5 activates ERK5/BMK1, MEK4 activates p38 and JNK, MEK7 activates JNK and p38, and MEK3 and MEK6 activate p38 (23, 24, 28–30). Thus, an understanding of signal transduction requires an evaluation of how each MEK regulates MAPK activity in each particular cell type.

Involucrin is a marker of keratinocyte differentiation that is specifically expressed only in differentiated keratinocytes. Involucrin expression is restricted to the suprabasal layers in stratifying squamous surface epithelia (31, 32). Involucrin expression in cultured cells is increased by agents that enhance keratinocyte differentiation such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (1, 31, 33) and calcium (2, 6). Previous studies have shown that the MAPK cascades play a central role in maintaining both basal and regulated expression (2, 3, 8, 9, 34). Studies using dominant-negative kinases and pharmacological agents indicate that p38 kinase activity is required for activation of hINV gene expression. This hINV gene regulatory cascade includes the novel protein kinase C isoforms, Ras, and MEKK1 (2, 3). MEKK1, in turn, targets several MEKs, including MEK1, MEK3, and MEK7 but not MEK4 (3, 22). Downstream targets of this pathway include the C/EBP and AP1 transcription factors (6–8). These transcriptional regulators, in turn, bind to specific elements in the hINV promoter proximal and distal regulatory regions to regulate transcription (2, 3, 6, 9, 34, and 35).

Although MEK3 and MEK7 are known to function as regulators of hINV gene expression (2, 3, 22), the role of MEK6 has not been evaluated. MEK6 is a potential regulator, as it has been shown to regulate p38 MAPK in other systems (36–38). Our recent report using quantitative real time RT-PCR to measure p38 isoform mRNA levels, showed that p38γ is not expressed in cultured keratinocytes (22). p38γ activity is also increased by caMEK6. However, we observed that the MEK6-dependent increase in hINV gene expression is reduced by p38δ, suggesting that this isoform is an inhibitor of expression. In addition, p38δ inhibits the TPA-associated increase in hINV gene expression. As TPA is known to enhance hINV gene expression via a nPKC, Ras, MEK1, MEK, p38 cascade (2, 3), this result further suggests the potential physiological importance of p38δ as an inhibitor of hINV gene expression. In addition, dominant-negative p38α strongly inhibits MEK6-mediated activation of hINV promoter activity. Thus, the most likely scenario is that the MEK6 stimulus is carried downstream by p38α. An interesting observation is the high level of p38α activity observed in p38α overexpressing cells in the presence of upstream stimulus (Fig. 4). We would have expected, based on the other results presented in the manuscript, that this activity would be low. However, increased p38α activity has been previously reported in cells expressing high levels of p38α. It has been suggested that this is due to the tendency of this p38 isoform to become selectively activated by low levels of MEK6 (21, 22). This effect is not observed for the other p38 MAPKs (21). Thus, when p38α is present at high levels, mass action may drive its activation. Our previous report shows that p38α is more abundant in keratinocytes than p38δ (22). In fact, the tendency of p38α to become active at lower MEK6 concentrations may explain why the net effect of MEK6 activation is to increase hINV gene expression, despite the fact that it can also activate a MAPK, p38δ, which functions to decrease hINV gene expression.

We propose that the effect of MEK6 on hINV gene expression is determined by the balance between MEK6-dependent activation of p38α and δ and that in the conditions used in the present experiments, the p38α-dependent activation of gene expression is the predominant response.

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