MEK7-dependent Activation of p38 MAP Kinase in Keratinocytes*

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Previous studies suggest that a PKC/Ras/MEKK1 cascade regulates involucrin (hINV) gene expression in human epidermal keratinocytes. MEK7, which is expressed in epidermis, has been identified as a member of this cascade (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). However, the kinase that functions downstream of MEK7 has not been identified. Our present studies show that MEK7 expression in keratinocytes markedly activates p38α and modestly activates JNK. Activation of p38 MAPK by MEK7 is a novel finding, as previous reports have assigned MEK7 as a JNK regulator. We also demonstrate that this regulation is physiologically important, as the p38α- and JNK-dependent activities regulate hINV promoter activity and expression of the endogenous hINV gene.

Mitogen-activated protein kinase signal transduction pathways are three kinase modules that include MAPK1 kinase kinase (MEKK), MAPK kinase (MEK), and MAPK (1). MAPK activation requires dual phosphorylation at threonine and tyrosine residues separated by a single amino acid (1, 2). Activated MAPKs, in turn, translocate to the nucleus and phosphorylate nuclear transcription factors such as Elk-1 and AP1 (3, 4). These factors then modulate gene expression by binding to DNA elements (5–7). Seven different mammalian MEKs have been described (8). MEK1 and MEK2 activate ERK1 and ERK2, MEK3 and MEK6 activate p38 kinase, MEK4 activates p38 and the SAPK/JNK pathways, and MEK5 and MEK6 regulate ERK5/BMK1 (8). MEK7 is a recently described MAPK kinase (9). Studies in various cell types indicate that MEK7 activates JNK/SAPK but not ERK or p38 (9, 10).

Previous studies from our laboratory indicate that MAPK cascades have a central role in the regulation of keratinocyte differentiation, as measured by the effects of activation of this cascade on the expression of a marker of keratinocyte differentiation, involucrin (6, 7). This cascade, which includes the novel PKC isoforms, Ras, and MEKK1, activates involucrin expression, by a mechanism that requires MEK1, MEK3, and MEK7 activity (6). MEK3 appears to regulate hINV gene expression via activation of p38 MAPK (6); however, the path of signal transmission following activation of MEK1 and MEK7 is not known. In the present experiments, we address the role of MEK7 as an activator of hINV gene expression. These studies suggest that MEK7 increases hINV gene expression via activation of p38α.

MATERIALS AND METHODS

Tissue Culture, Cell Transfection, and Adenovirus Infection—Third passage normal human epidermal keratinocytes were maintained in 35-mm dishes in keratinocyte serum-free medium (KSFM). For transfection studies, 50% confluent cells were incubated with 2 μg of plasmid mixed with 4 μl of FUGENE-6 reagent and 96 μl of KSFM, incubated for 24–48 h, washed with phosphate-buffered saline, dissolved in 140 μl of cell lysis reagent (Promega), and harvested by scraping. Luciferase activity was assayed immediately using a Promega Luciferase assay kit and a Berthold luminometer. All assays were performed in triplicate, and each experiment was repeated at least three times. Luciferase activity was normalized per μg of protein as previously described. We used a green fluorescent protein-encoding expression plasmid to normalize transfection efficiency (11, 12).

ERK1/2, p38, and JNK Kinase Activity Assays—p38, ERK1/2, and JNK activity were assayed using nonradioactive assay methods. Lysates were prepared in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μM leupeptin, 1 μM phenylmethylsulfonyl fluoride), and pH 7.4; 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 25 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 10 mM Na3VO4, 1 μg/ml leupeptin, 1 μg/ml phenylmethylsulfonyl fluoride, and pH 7.5; 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, and 10 mM MgCl2). To assay total p38 activity, a monoclonal antibody (New England BioLabs 9219) that binds to the phosphorylated form (Thr180/ Tyr182) of all four p38 isoforms was used to immunoprecipitate active p38 kinase from cell lysates. The precipitated p38 was then assayed for activity based on its ability to phosphorylate ATF-2. ATF-2 phosphorylation (at Thr72) was detected by immunoblot using a rabbit anti-phospho-Thr72/ATF-2-specific antibody (New England BioLabs 9221S). ERK1/2 activity was monitored by immunoprecipitation of phosphorylated ERK1/2 using a monoclonal antibody (New England BioLabs 9109) that binds to Thr180/Tyr182-phosphorylated ERK1/2. Activity of the precipitated kinase was assayed based on its ability to phosphorylate ELK-1. Phosphorylated ELK-1 (at Ser383) was detected by immunoblot using a rabbit anti-phospho-ELK1 antibody (New England BioLabs 9181S). To measure JNK/SAPK activity, endogenously activated JNK was precipitated using c-Jun fusion protein beads (New England BioLabs 9811, Ref. 13). Activity of the precipitated JNK was monitored by using c-Jun as a substrate. Phosphorylated c-Jun was detected by immunoblot using anti-phospho-c-Jun (New England BioLabs 9810).

Western Blot Analysis—Cell monolayers were rinsed with phosphate-buffered saline and lysed in Laemml sample buffer, and an equivalent amount of protein (10 μg) was electrophoresed on 8% acrylamide gels.
MEK7 Activates p38

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, and band intensity was estimated by densitometry.

Adenoviruses and Plasmids—Adenoviruses and/or plasmids encoding wild-type (wt) constitutively active (ca), and dominant-negative (dn) kinases were used in these studies. These include wtMEK7, caMEK7 (14); and FLAG-tagged p38β, p38γ, and p38δ; adenoviruses (14, 15); and plasmids encoding dnJNK and the corresponding empty control plasmid (13, 16). An empty adenovirus was generated by recombining pC3 plasmid with the pM17 adenovirus backbone in 293 cells. This virus was purified and used as an empty control adenovirus. The green fluorescent protein-encoding virus, CMV-GFP, was used to define the optimal adenovirus infection multiplicity. Recombinant adenoviruses were propagated in 293 cells and purified by cesium chloride centrifugation. pINV-2473, a plasmid encoding the human involucrin gene promoter fused to luciferase, was constructed as previously described (5).

Detection of mRNA—Total keratinocyte poly(A)+ RNA was isolated using the Oligotex Direct mRNA protocol (Qiagen). Equivalent quantities of mRNA were assayed by real time RT-PCR using an RNA amplification kit (SYBR Green I, Roche Molecular Biochemicals). Melting curve analysis of the PCR products distinguished the higher melting-specific PCR product from non-specific products. For quantification, following the elongation step in each PCR cycle and just prior to the fluorescence reading, the reaction temperature was stepped up to the temperature required to melt nonspecific PCR products. Primers amplifying a 330-bp fragment from the hINV gene, primers amplifying individual p38 isoforms, and primers amplifying a 600-bp fragment of the human GAPDH gene were used in parallel. hINV mRNA quantities were normalized based on GAPDH mRNA levels. The GAPDH primers were 5′-CCACCCATGGCAATTTGCAGCA and 5′-CCACCTGAGCT- GGACGCCAGATCT and the hINV primers were 5′-CTCCACCAAGGCCTCTGC and 5′-CTGCTTAAAGTCTGTGCT. Primers for detection of p38α, p38β, p38δ, and p38γ were as previously described (17). MEK7 mRNA was detected by real time RT-PCR using MEK7-specific primers (5′-ATCCCGAGCGCTATTGTGG, and 5′-TGATCGTGCCCGCGTGC- TC). These primers amplify a 120-nucleotide segment of human MEK7.

RESULTS

MEK7 Increases p38 MAPK Activity—To determine which MAPK is activated in the presence of MEK7, we delivered wild-type or constitutively active MEK7 to keratinocytes using adenovirus. As shown in Fig. 1A, p38 kinase activity is not detected in mock-infected cells or cells infected with empty adenoviral vector; however, the presence of wt or caMEK7 results in activation of endogenous p38 MAPK. To compare the relative activity of wtMEK7 and caMEK7, we measured the level of each protein in infected cells by immunoblot (Fig. 1B). When corrected for level of expression, wt and caMEK7 appeared to be equally effective activators of p38 activity. We further tested caMEK7 as a regulator of JNK and ERK activity. In addition to the marked increase in p38 MAPK activity, caMEK7 also produced a modest increase in JNK activity (3-fold), but did not regulate ERK activity (Fig. 1C). In addition, as shown in Fig. 1D, MEK7 did not alter p38, JNK, or ERK protein level, suggesting that the changes in p38 and JNK activity are caused by increased activity of pre-existing enzymes.

To identify the specific p38 isoforms that are regulated by MEK7, we performed three types of experiments. First, we used real time RT-PCR to measure the level of mRNA encoding each individual p38 isoform. The results indicate that keratinocytes express all p38 isoforms except p38γ (Fig. 2A). p38α and p38δ are the most abundant, followed by p38ε. To measure the activity of individual p38 isoforms, we infected cells with adenoviruses encoding FLAG-tagged forms of each p38 isoform in the presence or absence of caMEK7. After 48 h, individual p38 isoforms were precipitated using anti-FLAG antibody, and the activity of the precipitated antibody was monitored based on ability to phosphorylate ATF-2. As shown in Fig. 2B, in the absence of MEK7, the only p38 isoform displaying substantial activity is p38α. A similar profile is observed in the presence of caMEK7, except that p38α activity is slightly increased. As expected, no activity is observed in cells not infected with FLAG-p38 expression virus. The results shown in panels A and B suggest that p38β, p38δ, and p38ε are not involved in mediating the MEK7-dependent activation. To further examine the role of p38α, we mock-infected cells, or infected cells with empty virus or caMEK7-encoding virus. After 48 h, we precipitated endogenous p38α using a specific antibody, and monitored the level of activated enzyme based on its ability to phosphorylate ATF-2. As shown in Fig. 2C, the presence of caMEK7 increases endogenous p38α activity. In addition, although not shown, a smaller increase in activity was also detected for cells infected with wtMEK7-encoding adenovirus. To confirm the specificity of the p38α antibody, we infected cells with empty vector or a virus encoding p38α and then detected expression of p38α by immunoblot using anti-p38α. As shown in Fig. 2D, the antibody detects a single band in cells infected with empty vector. This band corresponds to the migration of expressed FLAG-p38α, when the migration is adjusted for the contribution of the FLAG tag (lane α). The antibody also nonspecifically cross-reacts with overexpressed FLAG-p38β but does not detect p38δ.

MEK7 Induces hINV Gene Expression—Our previous studies suggest that a PKC-activated cascade can act via MEK1, MEK3, and MEK7 to regulate hINV gene expression (6, 7). To directly examine the role of MEK7 as a regulator of hINV gene expression, we transfected keratinocytes with the hINV gene
promoter reporter plasmid, pINV-2473 (5), followed by infection with empty or MEK7-encoding adenoviruses. As shown in Fig. 3A, wtMEK7 and caMEK7 increase promoter activity by 2- and 8-fold, respectively. This regulation appears to be physiological, because, as shown in Fig. 3B, real-time RT-PCR shows that hINV mRNA is increased by 4-fold in the presence of caMEK7. hINV protein levels also increase. hINV protein is not readily detected in mock- or empty vector-infected keratinocytes (Fig. 3C); however, the level is increased in the presence of caMEK7. Moreover, SB203580, an agent that inhibits p38α and p38β activity at concentrations less than 1–2 μM (18), inhibits the caMEK7-dependent activation at a concentration of 0.5 μM (19, 20).

MEK7 Activates p38 MAPK isoforms in keratinocytes. A, the relative level of the p38 isoforms in keratinocytes were compared using real-time RT-PCR and p38 isoform-specific primers (17). B, to measure the enzymatic activity of individual p38 isoforms in response to MEK7, keratinocytes were coinfected with empty vector (EV) or caMEK7, and FLAG-p38α, -β, -γ, or -δ. After 48 h, the p38 isoforms were immunoprecipitated using mouse monoclonal anti-FLAG antibody (Sigma F3165) followed by 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 as described in the legend to Fig. 1. C, endogenous p38α activity was measured by mock-infecting keratinocytes, infecting with empty (EV) adenovirus, or infecting with adenovirus encoding wtMEK7 or caMEK7. After 48 h, the cells were harvested, and endogenous p38α was immunoprecipitated using rabbit anti-p38α (1:5000). p38α activity was monitored based on ability to phosphorylate ATF-2 as described in Fig. 1. D, keratinocytes were infected with adenoviruses encoding FLAG-p38α, -β, -δ, or -γ. After 48 h, lysates were prepared for immunoblot using rabbit anti-p38α (1:5000). Binding of the primary antibody was detected using peroxidase-conjugated donkey anti-rabbit IgG (1:10,000). Each of these experiments was repeated three times.

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MEK7 Regulates hINV Promoter Activity via p38- and JNK-dependent Pathways—MEK7 has been reported to activate JNK (9). Consistent with this observation, MEK7 increases
JNK activity in keratinocytes (Fig. 1C). To test the importance of this regulation, we monitored the effects of JNK inactivation on hINV promoter activity. Keratinocytes were transfected with pINV-2473 and in the presence or absence of plasmid-encoding dnJNK followed 24 h later by MEK7-encoding adenovirus. As shown in Fig. 4A, inactivation of JNK by dnJNK results in an increase in wtMEK7- and caMEK7-associated promoter activity. This result suggests that JNK is a negative regulator of hINV promoter activity.

**DISCUSSION**

The MAPK kinase cascades provide an important mode of transferring regulatory information from the cell surface to the nucleus (1). These cascades include a 3-kinase module that includes a MAPK kinase kinase (MEKK), a MAPK kinase (MEK), and a MAPK. Four major mitogen-activated protein kinases (MAPK) have been identified including ERK1/2, ERK5, p38 MAPK, and the c-Jun NH2-terminal kinase (JNK, Refs. 8, 21–23). Dual phosphorylation of the regulatory loop of these kinases by MEKs results in MAPK activation (8). The MEKs play a central role in maintaining both basal and regulated gene expression (6, 7, 11, 12, 29). Using dominant-negative kinases and pharmacologic agents to identify important regulatory enzymes has a preferred downstream target. For example, expression of the dominant-negative form of p38a completely inactivates normal regulation (6). An exploration of other candidate enzymes in these cascades suggest that the novel protein kinase C isoforms are involved, as are Ras and MEKK1 (7). Other enzymes, including Raf1, do not appear to play a role. MEKK1, in turn, appears to target several MEKs, including MEK1, MEK3, and MEK7, but not MEK4 (6). Expression of dominant-negative forms of MEK1, MEK3, or MEK7 eliminates MEKK1-dependent regulation. Downstream targets of this pathway include C/EBP and AP1 transcription factors that, in turn, bind to specific DNA elements that have been identified in the hINV promoter proximal and distal regulatory regions to regulate transcription (5–7, 12, 29, 30).

Taken together these results suggest that the MEKs transmit the regulatory signal via the p38 MAPKs. MEK3 is a known activator of p38 MAPK isoforms (19, 20, 31), and so our results suggest that one major pathway that mediates regulation includes MEKK1, MEK3, and p38. However, the downstream target of MEK7 was not identified. Previous studies suggest that MEK7 is a specific activator of JNK kinase (9, 32). However, because JNK does not appear to mediate the regulation in keratinocytes (6, 7), it appeared unlikely that JNK could be the downstream target. We therefore examined the role of MEK7 as an activator of p38 MAPKs. The present studies show that adenovirus-mediated expression of caMEK7 or wtMEK7 in keratinocytes results in an increase in total p38 activity. Interestingly, both wild-type and constitutively active MEK7 were able to active p38, a result that is consistent with the ability of the wild-type form of other signaling enzymes (e.g. MEKK1) to activate this cascade (6). In addition, MEK7 produced a modest activation of JNK (3-fold) but did not regulate ERK activity.

Among the p38 family members, p38α and p38β appear to be ubiquitously expressed (23). In contrast, p38γ is expressed in muscle where it plays a role in differentiation regulation (33, 34), and p38δ is enriched in lung, kidney, testis, pancreas, and intestine (19). To identify the p38 family members involved in the regulation, we analyzed p38 mRNA levels in keratinocytes using quantitative real time RT-PCR (17). Our studies show that p38α and p38β are the major forms present in keratinocytes with p38δ also present at significant levels. p38γ is absent, thus eliminating it as a possible MEK7 downstream target. Three types of functional studies support a role of p38α MAPK as a mediator of MEK7-dependent signaling. First, total p38 activity is minimally detected in cells infected with empty adenovirus and markedly increased in cells infected with MEK7-encoding adenovirus. Second, infection of cells with individual FLAG-tagged p38 isoform reveal that p38β, p38δ, and p38γ are inactive in the presence or absence of MEK7. Third, a specific role for p38α was confirmed by immunoprecipitation of increased levels of active p38α from cells expressing MEK7.

One puzzling feature of these data is the high level of p38α activity in cells expressing FLAG-tagged p38α (Fig. 2B). We would have expected, based on the other results presented in the manuscript, that this activity would be low. However, similar results have been reported in other systems (14, 35). For example, Alonso et al. (35) showed that recombinant p38α exhibits significant activity in the absence of MEK kinase, and that this activity markedly increased with a slight increase in MEK levels. In contrast, p38β, p38γ, and p38δ were substantially less sensitive. Thus it is conceivable that when present at high levels, p38α may become activated by the endogenous MEKs. Despite this, p38α activity did increase in the presence of MEK7. Taken together with the involucrin gene regulatory data, these results suggest that MEK7 is a physiologic regulator of keratinocyte differentiation; it is expressed at high levels.
in epidermis (9, 10), and it regulates hINV promoter activity and expression of the endogenous involucrin gene. Real time RT-PCR also confirms that MEK7 mRNA is present in cultured keratinocytes (not shown).

MEK7 is 47 and 43% homologous to MEK6 and MEK3, respectively, proteins that have been previously shown to regulate p38 MAP kinase (9). As noted above, MEK7 has been reported to be a specific activator of JNK in a variety of cell types. For example, previous reports show that MEK7 activates JNK in 293T cells (10), in cultured neonatal rat cardiomyocytes (14), and in CHO cells (9). The present results argue that MEK7 activates both JNK and p38 in keratinocytes, and that the p38 activation appears to be more substantial. To our knowledge, this is among the first reports suggesting that p38 MAPK is a MEK7 target. A recent report indicates that MEK7, along with MEK3, 4 and 6, can activate p38 in 293T cells (36). The outcome of our experiments suggest that MEK7-dependent regulation of downstream kinase activation may be tissue- or perhaps cell-type specific with respect to MEK7 targets.

An additional point of interest in the present study, is the observation that JNK appears to suppress hINV promoter activity. This is consistent with a recent report indicating that JNK inhibits expression of the SPRR1B gene, another gene that, like involucrin, is regulated in a differentiation-dependent manner in squamous epithelia (37). Our results suggest that coincident regulation by MEK7 of p38 and JNK activity may function as a mechanism to determine the overall level of hINV gene expression, with p38o functioning to increase and JNK function to decrease expression (Fig. 4B). This is likely to be part of a more complex mechanism of signal integration that defines the impact of p38 versus the JNK signals.

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