Regulation of Human Involucrin Promoter Activity by a Protein Kinase C, Ras, MEKK1, MEK3, p38/RK, AP1 Signal Transduction Pathway*

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Involucrin is a marker of keratinocyte terminal differentiation. Our previous studies show that involucrin mRNA levels are increased by the keratinocyte differentiating agent, 12-O-tetradecanoylphorbol-13-acetate (TPA) (Welter, J. F., Crish, J. F., Agarwal, C., and Eckert, R. L. (1995) J. Biol. Chem. 270, 12614–12622). We now study the signaling cascade responsible for this regulation. Protein kinase C and tyrosine kinase inhibitors inhibit both the TPA-dependent mRNA increase and the TPA-dependent increase in hINV promoter activity. The relevant response element is located within the promoter proximal regulatory region and includes an AP1 site, AP1-1. Co-transfection of the hINV promoter with dominant negative forms of Ras, MEKK1, MEK1, MEK7, MEK3, p38/RK, and c-Jun inhibit the TPA-dependent increase. Wild type MEKK1 enhances promoter activity and the activity can be inhibited by dominant negative MEKK1, MEK1, MEK7, MEK3, p38/RK, and c-Jun. In contrast, wild type Raf-1, ERK1, ERK2, MEK4, or JNK1 produced no change in activity and the dominant negative forms of these kinases failed to suppress TPA-dependent transcription. Treatment with an S6 kinase (S6K) inhibitor, or transfection with constitutively active S6K produced relatively minor changes in promoter activity, ruling out a regulatory role for S6K. These results suggest that activation of involucrin transcription involves a pathway that includes protein kinase C, Ras, MEKK1, MEK1, MEK3, and p38/RK. Additional pathways that transfer MEKK1 activation via MEK1 and MEK7 also may function, but the downstream targets of these kinases need to be identified. AP1 transcription factors appear to be the ultimate target of this regulation.

Epidermis is a layered tissue. The proliferating cells are located in the basal layer. As the cells leave this layer they undergo extensive biochemical and morphological remodeling. Ultimately these cells turn into corneocytes (1, 2). In vitro, cultured keratinocytes mimic this process. In this regard phorbol ester is a useful tool for the study of keratinocyte biology. Treatment of cultured keratinocytes with 12-O-tetradecanoylphorbol-13-acetate (TPA) increases morphological differentiation, a change that is accompanied by an increase in the expression of keratinocyte-specific differentiation marker proteins (1). Previous studies have shown that involucrin (hINV) mRNA levels are increased by treating cells with TPA. Involucrin is a precursor of the keratinocyte cornified envelope that is selectively expressed in the suprabasal epidermal layers (3–5). The hINV promoter consists of 2500 base pairs upstream of the transcriptional start site. Several sequence elements in this upstream regulatory region are important for gene regulation (6–13). PKC appears to be a major regulator of hINV gene expression (6, 14). Previous studies have described transcriptional activator elements within the distal regulator element, which encompasses the −2473/−2088 DNA segment (6, 7, 9). A region closer to the transcription start site, spanning nucleotides −241/−7 and called the proximal regulator region, is also required for optimal transcription (6). However, this region remains relatively uncharacterized and the signal transduction pathway that mediates the regulation has not been examined.

Cells respond to extracellular signals by transmitting intracellular instructions to coordinate fundamental cellular responses. The mitogen-activate protein kinase (MAPK) cascades are among the best characterized of these intracellular signaling pathways. These cascades consist of a three-kinase module that includes a MEK kinase (MEKK) which activates a MAPK/ERK kinase (MEK) which activates a MAPK (15). Three distinct groups of MAP kinases have been identified in mammalian cells, including mitogen-responsive ERKs (extracellular signal regulated kinases), and the stress-responsive JNK/SAPKs (c-Jun N-terminal kinase/stress activated protein kinases) and p38 MAP kinases. Phosphorylation on both threonine and tyrosine residues at the Thr-X-Tyr dual phosphorylation motif is required for MAPK activation (15, 16). Activated MAPKs translocate to the nucleus where they phosphorylate and activate transcription factors and other target proteins (15, 16). The activated transcription factors, in turn, bind to DNA elements and modulate gene expression (17). Our knowledge of these signaling pathways is limited with respect to regulation of specific genes in epidermal keratinocytes. In the present study we use the human involucrin gene as a model to study the regulation of differentiation-dependent keratinocyte gene expression by the MAPK cascades. Our evidence

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1 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; ERK, extracellular regulated kinase; S6K, S6 kinase; RSK1, ribosomal S6 kinase/mitogen-activated protein kinase; AP-1, activator protein-1; hINV, human involucrin gene; dn, dominant negative; EBS, ets factor-binding site; GST, glutathione S-transferase.
suggests that hINV promoter activity is regulated via a MAPK pathway that includes PKC, Ras, MEKK1, MEK3, p38/RK, and AP1. Alternate pathways may also exist. We also demonstrate that activity in this pathway is directed to a single AP1 site, or glyceraldehyde-3-phosphate dehydrogenase (31). The probes were labeled by random priming in the presence of [α-32P]dCTP. Signal intensity was determined by densitometry.

In Vitro Kinase Assays—The JNK kinase assay was performed as described (32). Briefly, keratinocytes were grown in 100-mm diameter dishes in KSFM. Cells were then treated with the desired agents, rinsed twice with ice-cold phosphate-buffered saline, and harvested by scraping in the lysis buffer containing 25 mM HEPES, pH 7.7, 300 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 mM Na3VO4, 2 μg/ml leupeptin, and 100 μg/ml PMSF. JNK was precipitated by addition of excess GST-c-Jun fusion protein linked to GSH-agarose beads. The beads were extensively washed with binding buffer (20 mM HEPES, pH 7.7, 30 mM NaCl, 2.5 mg/ml MgCl2, 0.1 mM EDTA, 0.05% Triton X-100, 0.1 mM Na3VO4, 2 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride), and resuspended in 30 μl of kinase buffer (20 mM HEPES, pH 7.6, 20 mM MgCl2, 20 mM β-glycerophosphate, 20 mM p-nitrophenyl phosphate, 0.1 mM Na3VO4, 2 mM dithiothreitol, 20 μM ATP, and 5 μCi of [γ-32P]ATP). After 15 min at 30 °C, the kinase reactions were terminated by boiling in Laemmli sample buffer. The samples were electrophoresed on a 12% polyacrylamide gel, stained with Coomasie Blue, and dried. The presence of labeled GST-c-Jun was detected by autoradiography.

RESULTS

Inhibition of the TPA-dependent Increase in hINV mRNA by Inhibitors of PKC and MEK—We have shown that treatment of keratinocytes with TPA results in an increase in involucrin mRNA levels (6). In Fig. 1 we extend these results and show that this response can be inhibited by simultaneous treatment of cultures with BIS-IM, a specific inhibitor of PKC, or genistein, a tyrosine kinase inhibitor. Thus, PKC (serine/threonine kinase), and tyrosine kinases (perhaps MEKs, threonine/tyrosine duality specific kinases), regulate hINV gene expression.

Effects of Inhibitors on hINV Promoter Activity and Location of Response Element(s)—To determine how these agents regulate hINV gene expression, we evaluated the effects of each agent on the activity of the full-length promoter construct, pINV-2473 (6). As shown in Fig. 2, both genistein and BIS-IM produce a concentration-dependent inhibition of promoter activity. Identical results were observed with a shorter promoter segment, pINV-241 (not shown); we therefore focused on the -241/-1 segment in subsequent experiments. To locate the responsive DNA element, we tested activity of the hINV promoter truncation series shown in Fig. 3A. The activity of pINV-241 and pINV-128 are increased approximately 10-fold in TPA-treated cultures (Fig. 4A). In contrast, although 250 μM genistein, when given alone, did not regulate activity, genistein co-treatment completely inhibited the TPA-dependent increase.

Materials and Methods

Chemicals and Reagents—Keratinocyte serum-free medium (KSFM), gentamicin, trypsin, Hank’s balanced salt solution, and Lipofectin were obtained from Life Technologies, Inc. Genistein, bis-indolylmaleimide (BIS-IM), PDE5659, SB203580, and rapamycin were from Calbiochem. Phorbol ester (TPA), anisomycin, and dimethyl sulfoxide were purchased from Sigma. Diapase was obtained from Boehringer Mannheim.

[α-32P]dCTP was obtained from NEN Life Science Products. The pGL2-Basic plasmid, AP1 consensus oligonucleotide (AP1c), and the chemiluminescent luciferase assay system were obtained from Promega. Chemiluminescence was measured using a Berthold luminometer. Oligonucleotides for gel shifts and construction of mutant promoter sequences were synthesized using an Applied Biosystems DNA synthesizer.

Plasmid Construction—We have previously published the structure of the hINV promoter constructs, pINV-2473 and pINV-241, which initiate transcription at -2473/-7 and contain a functional proximal regulatory region of the hINV promoter, linked to the luciferase reporter gene in pGL2-Basic (6). All positions are defined relative to the hINV gene transcription start site (6, 18). The plasmids containing mutated activator protein-1 (AP1) and AP1 binding sites (EBS) were constructed by replacing the Apal/PstI fragment of pINV-241 with a double-stranded oligonucleotide encoding the respective mutated binding sites.

pRSV-TAM encodes a transdominant negative form of c-Jun (19). Wild type ERK1 and ERK2, and dominant negative ERK1 (K71R) and ERK2 (K22R), each cloned in pCEP4, were kindly provided by Dr. Melanie Cobb (20). Dominant negative H-Ras (S17N), cloned in pSRα, and wild type Raf-1 and dominant negative Raf-1 (K375W), each cloned in pRSV, were kindly provided by Dr. Michael Karin (21). Constitutively active Ras mutant (Ras12V), cloned in pZipNeoSVX1, was kindly provided by Dr. Michael Simonson (22). Wild type MEKK1, dominant negative MEK1, dominant negative SEK1 (S220A, T224L), wild type S6 kinase (S6K), and constitutively active S6 kinase (AN/77/6K), each cloned in PECEMV, were obtained from Dr. Dennis Templeton (23–25). Dominant negative MEKK1 (K432M), cloned in pSRα, was obtained from Dr. Michael Karin (21). Kinase negative MEK7, (M32K, K325K), cloned in pSRa, was a gift from Dr. Eisuke Nishida (26). Dominant negative MKK3 (MKK3 Ala), cloned in pRSV, and dominant negative p38 MAPK (p38 AGF), cloned in pCMV5, and dominant negative JNK1 (JNK1 APF), cloned in pCDNA3, were generously provided by Dr. Roger Davis (27–29). GST-c-Jun (5–73 amino acids) expression vector was obtained from Dr. James Woodgett. Transformation of the GST-c-Jun fusion protein expression vector into Escherichia coli cells, protein induction, and purification were performed according to standard procedures.

Tissue Culture, Cell Transfection, and Luciferase Assay—Normal human foreskin keratinocytes were cultured as described previously (6, 8). Third passage keratinocytes were transfected in 60-mm diameter dishes when approximately 60% confluent. For transfection experiments, 16 μg of Lipofectin reagent and 4.5 μg of involucrin promoter reporter plasmid were mixed, added to cells in 3 ml of KSFM and incubated for 5 h at 37 °C. For co-transfection experiments, 2.5 μg of involucrin promoter reporter plasmid and 0–2 μg of a second plasmid were transfected. The final DNA concentration in all groups was adjusted to 4.5 μg of DNA per 16 μg of Lipofectin/60-mm dish by addition of empty expression vector. At 5 h, 3 ml of KSFM was added and the incubation continued for 19 h. The cells were then given fresh medium, allowed to recover for 24 h, and treated with KSFM in the presence or absence of TPA and/or the indicated inhibitor. After an additional 24 h, the cells were washed with phosphate-buffered saline, dissolved in 250 μl of cell culture lysis reagent (Promega), and harvested by scraping. Luciferase activity was assayed immediately using Promega luciferase assay kit and a Berthold luminometer. All assays were performed in triplicate. Each experiment was repeated at least three times. Luciferase activity was normalized per micrograms of protein as previously described (7, 8).

RNA Isolation and Analysis—Polyadenylated RNA was prepared, electrophoresed on a formaldehyde-containing agarose gel, and transferred to Biodyne A membranes as described previously (30). The membranes were hybridized with cDNA probes encoding human involucrin or involucrin promoter activity.
TPA increases activity of the native pINV-241 construct, and this activity is inhibited by genistein and BIS-IM (Fig. 4, C and D). Mutation of the EBS-2 site increases the overall activity of the promoter, but does not effect the regulation by TPA or the kinase inhibitors. In contrast, mutation of the AP1-1 site reduces overall activity, and eliminates response to TPA, suggesting that TPA and the inhibitors effect a common pathway that terminates at the AP1-1 site.

**Ras Activity Is Required for hINV Promoter Activity**—The above studies show that the TPA-dependent hINV gene regul-
latory pathway terminates on the hINV AP1-1 site. We next designed experiments to identify individual steps in the signaling cascade. Ras is a downstream target of PKC (33, 34); we therefore determined whether Ras activity is required for promoter activation. Keratinocytes were co-transfected with pINV-241 and a dominant interfering form of Ras, Ras-N17 (dnRas) (35). As shown in Fig. 5, dnRas inhibits both basal and TPA-dependent activity. Moreover, in the absence of TPA treatment, constitutively active RasG12V increases promoter activity (Fig. 5).

Raf-1 Is Not Required for Promoter Activity—Ras activates multiple downstream targets, including Raf-1 (36, 37) and MEKK1 (38, 39). Raf-1, in turn, activates the ERK1 and ERK2 MAPks through MEK1 and MEK2 (40–42). To evaluate the role of the Raf-1/MEK/ERK cascade, we transfected keratinocytes with pINV-241 and the wild type and dominant negative forms of Raf-1, MEK1, ERK1, and ERK2. Wild type Raf-1 did not change basal promoter activity (Fig. 6A), and dnRaf-1 did not suppress basal activity or the TPA-dependent increase in activity. Dominant negative MEK1, however, suppressed basal and TPA-dependent promoter activity (Fig. 6B). Wild type MEKK1 increased promoter activity as efficiently as TPA, and dnMEKK1 also inhibited this activation, suggesting that MEK1 may relay the MEKK1-dependent signal. A similar suppression was observed using a specific MEK1/2 inhibitor, PD90859 (43). However, as shown in Fig. 6C, ERK1 and ERK2 do not appear to be important for regulation. Wild type ERK1 did not regulate basal activity, wild type ERK2 increased basal activity a modest 2-fold. The dominant negative forms of these kinases increased basal activity slightly (dnERK1) or had no effect (dnERK2). Moreover, the dnERK forms did not suppress the TPA-dependent increase.

Sustained ERK activation is required for differentiation of several cell types, including neuronal (44, 45), megakaryocytic (46), and muscle (47) cells. TPA is a differentiation-inducing agent in human keratinocytes. We therefore examined if TPA treatment of keratinocytes leads to the sustained ERK activation. ERK1/2 activity, as measured by the ability of immunoprecipitated activated ERK1/2 to phosphorylate its substrate protein GST-Elk1 was increased rapidly in response to 5 min of TPA treatment. However, within 30 min, ERK activity had returned to basal level (Fig. 6D). Thus, TPA stimulation of human keratinocytes results in only transient activation of ERK1/2 MAP kinases.

MEKK1 Is Required for Promoter Activity—In addition to the Raf-1 cascade, Ras also activates the MEKK1 signaling cascade (38, 39). MEKK1 activates MEK4 (also called SEK1 and
**Fig. 6.** MEK1 regulates, but Raf1, ERK1, and ERK2 do not regulate hINV promoter activity. Keratinocytes were transfected with pINV-241 and expression vectors encoding wild type or dominant negative forms of Raf1 (panel A), dominant negative MEK1 (panel B), or wild type or dominant negative ERK1 and ERK2 (panel C). The empty expression vector (vector) was transfected in control groups. The cells were then treated for 24 h without (open bars) or with (solid bars) 50 mg/ml TPA, harvested, and extracts were assayed for luciferase activity. In panel B, the crosshatched bars indicate that pINV-241 transcription was activated by transfection with MEKK1 and without TPA treatment. In the PD98059-treated groups, PD98059 was added at 50 µM for 30 min prior to TPA addition. Panel D shows a kinase assay in which the ability of ERK to phosphorylate Elk1 protein was assayed. Cells were incubated with TPA for various times, extracts were prepared, activated ERK was immunoprecipitated with phospho-ERK-specific antibody, and assayed for ERK activity using GST-Elk1 as a substrate. Phosphorylated Elk1 (P-ELK) is indicated by the arrow. Cells in the control group (C) were not treated with TPA. The molecular weights are given in kilodaltons and the dark band at approximately 54 kilodaltons is the IgG.

JNNK1) which activates JNK/SAPK and p38 MAPKs (21, 24, 27, 48, 49). To evaluate MEKK1 effects, we co-transfected pINV-241 with the MEKK1 dominant negative mutant (21). Co-transfection with dnMEKK1 completely suppressed basal and TPA-dependent promoter activity (Fig. 7A). Moreover, in untreated cells co-transfection with MEKK1 increased basal activity 6-fold (-TPA, crosshatched bar) (Fig. 7B). Constitutively active Ras also increased promoter activity (7-fold). Both MEKK1- and constitutively active Ras-dependent activation was inhibited by dnMEKK1 (Fig. 7B). The fact that Ras-dependent hINV promoter activity is inhibited by dnMEKK1 suggests that MEKK1 is downstream of Ras.

Regulation of hINV Promoter Activity by MEK7, but Not by MEK4 or JNK/SAPK—MEK4 is a known target of MEK1, and JNK/SAPK is activated by MEK4 (27, 48, 49). We therefore determined whether dnMEK4 could suppress hINV promoter activity. As shown in Fig. 8A, dnMEK4 produced a slight increase in basal and TPA-stimulated hINV promoter activity. MEK7 kinase has also been reported to regulate JNK/SAPK activity (26, 50–52). The dominant negative form of MEK7 (dnMEK7) suppressed TPA-dependent pINV-241 promoter activity (Fig. 8A). We also examined the role of JNK/SAPK. Dominant negative JNK1/SAPK (dnJNK1) did not alter basal and slightly increased TPA-stimulated promoter activity (Fig. 8A). A direct assay of JNK activity revealed no increase in response to TPA. As a positive control we show that anisomycin substantially increases JNK activity (Fig. 8B).

p38/RK Suppresses hINV Promoter Activity—p38/RK is a MAP kinase that functions in parallel with ERK and JNK/ SAPK (53–57). As shown in Fig. 9A, dpn38/RK inhibited TPA-dependent hINV promoter activity. MEK3 is a kinase that functions to regulate p38/RK (27, 58). Dominant negative MEK3 (dnMEK3) inhibited both basal and TPA dependent activity (Fig. 9A). The dominant negative forms of p38/RK and MEK3 also inhibited MEKK1- and constitutively active Ras-dependent promoter activity (Fig. 9B).

TAM67 Inhibits the TPA-dependent Response—AP1 transcription factors are frequent targets of MAPK cascades (reviewed in Refs. 59 and 60). To determine whether inactivation of AP1 factor activity abrogates the TPA response, selected hINV reporter plasmids were co-transfected with a c-Jun dominant negative, TAM67 (19, 61). TAM67 inhibits the activity of all Jun and Fos family members (19, 61). Cultured epidermal keratinocytes were co-transfected with hINV reporter constructs and empty expression vector (pRSV2, Control) or vector encoding TAM67 (TAM), and treated with or without TPA. The TPA-dependent activation of pINV-241 is reduced by increasing concentrations of pTAM67 (Fig. 10A). Fig. 10, B and C, confirm that the TAM67 response is mediated by the AP1-1 site situated within the −128/−110 promoter segment.

S6 Kinase Does Not Regulate hINV Promoter Activity—We also tested the effects of agents that regulate the S6 kinase cascade, a pathway that is distinct from the MAP kinase cascades (62). Treatment of cells with rapamycin, a specific inhibitor of S6 kinase (63, 64), or co-transfection of the pINV-241 reporter plasmid with wild type or constitutively active of S6K (25) did not modulate promoter activity (not shown).

**DISCUSSION**

Regulation of hINV Gene Expression by PKC and Ras—Phorbol ester, a diacylglycerol analog and activator of PKC, is a widely used tool for studying keratinocyte function. Administration to cultured keratinocytes induces keratinocyte differentiation and also activates the expression of a variety of differentiation-dependent marker genes. One of these genes, involucrin, is a 68-kDa component of the keratinocyte cornified
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envelope and a marker of suprabasal differentiation in epidermis (65–67). TPA activates PKC in keratinocytes; however, subsequent events in the signal transduction cascade leading to activation of hINV gene expression are not known. In the present study we perform experiments to identify downstream signaling proteins. Our results confirm that TPA increases hINV mRNA levels and hINV promoter activity, and show that this increase is inhibited by BIS-1M, a specific PKC inhibitor (68). Ras is a low molecular weight GTP-binding protein, and a mediator of PKC action (33, 34). To determine if Ras is a regulator of hINV promoter function, we monitored promoter activity in the presence of dominant negative Ras. Dominant negative Ras completely inhibits basal and TPA-stimulated hINV promoter activity. Moreover, constitutively active Ras activates the promoter in the absence of TPA treatment. Thus, Ras activation is important for hINV gene expression.

A Role for MEKK1 and MEK1 but Not Raf-1 or ERK—Ras activates downstream signaling pathways (20, 21, 69), including the Raf-1/MEK/ERK and MEKK1/MEK4/JNK signaling cascades (reviewed in Refs. 15 and 16). To evaluate the role of each pathway, we tested the effects of dominant negative forms of the initial kinase in each cascade, Raf-1 and MEKK1. Dominant negative Raf-1 produced a small increase and native Raf-1 produced a small decrease in basal promoter activity, strongly suggesting that Raf-1 does not regulate hINV promoter activity. In contrast, several lines of evidence suggest that MEKK1 plays a critical role in this regulation. First, the promoter can be activated by MEKK1 in the absence of TPA. Second, dominant negative MEKK1 inhibits basal and TPA-stimulated promoter activity. Third, constitutively active Ras- and MEKK1-dependent promoter activation (in the absence of TPA) are inhibited by dnMEKK1. Our results showing that dnMEKK1 inhibits MEKK1 dependent activity support the assertion that MEKK1 is functionally downstream of MEKK1 in this cascade.

However, experiments with ERK mutants suggest that MEKK1 is not acting via the classical ERK pathway. Dominant negative ERK1 slightly increases basal activity, dominant negative ERK2 and wild type ERK1 produce no change, and wild type ERK2 increases activity 2-fold. In addition, dnRaf-1, dnERK1, and dnERK2 did not suppress the TPA-dependent increase in promoter activity. Direct measurement shows that ERK kinase activity is maximally increased within 5 min after TPA treatment, but that activity returns to the pre-stimulus level within 30 min. The time course of increase in ERK kinase activity correlates with increased levels of phosphorylated ERK as measured by immunoblot (not shown). Thus, although TPA transiently increases ERK kinase activity, experiments with dominant negative mutants suggest that ERK activity it is not required for increased hINV gene expression. Taken together, these observations argue that Raf-1, ERK1, and ERK2 are not involved in regulating hINV promoter activity. This is an interesting observation, as Fra-1 and Fra-2 are part of the AP1 complex that forms on the hINV AP1-1 promoter element (6). In contrast, several lines of evidence suggest that Raf-1 produced a small decrease in basal promoter activity, strongly suggesting that Raf-1 does not regulate hINV promoter activity. In contrast, several lines of evidence suggest that MEKK1 plays a critical role in this regulation. First, the promoter can be activated by MEKK1 in the absence of TPA. Second, dominant negative MEKK1 inhibits basal and TPA-stimulated promoter activity. Third, constitutively active Ras- and MEKK1-dependent promoter activation (in the absence of TPA) are inhibited by dnMEKK1. Our results showing that dnMEKK1 inhibits MEKK1 dependent activity support the assertion that MEKK1 is functionally downstream of MEKK1 in this cascade.

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result is consistent with a report indicating that MEKK1 can phosphorylate and activate MEK1 without affecting ERK activity (71). Thus, in keratinocytes, the kinase immediately downstream of MEK1 is not presently known (Fig. 11).

**MEKK1 Does Not Act via Activation of MEK4 or JNK/SAPK—** In many systems MEKK1 activates MEK4, a dual specificity (threonine/tyrosine) kinase that phosphorylates and activates c-Jun N-terminal kinase (JNK), a member of the MAP kinase superfamily (28, 32). JNK, in turn, phosphorylates c-Jun which dimerizes with c-Fos to activate AP1-dependent transcription (28, 32, 72, 73). A role for MEK4 and JNK/SAPK is suggested by the MEKK1 dependence of promoter activation. However, neither dnMEK4 nor dnJNK influenced the TPA-dependent activation of the hINV promoter, and JNK/SAPK activity was not increased following treatment of keratinocytes with TPA. Previous studies suggest that JunB, JunD, and Fra-1 are involved in regulating hINV gene expression, but that c-Jun does not bind to the hINV AP1 response element (6). JunB is not phosphorylated by the JNKs as its phosphoacceptor region cannot be recognized by these kinases (73). JunD and Fra-1 are involved in regulating hINV gene expression, but that c-Jun does not bind to the hINV AP1 response element (6). JunB is not phosphorylated by the JNKs as its phosphoacceptor region cannot be recognized by these kinases (73). JunD is only weakly phosphorylated by JNK as it lacks an efficient JNK docking site (73). Taken together, these results appear to rule out a role for MEK4 and JNK/SAPK in regulation of hINV gene expression.

**A Role for MEK7—** Recently identified MAPK kinase, MEK7, is a specific activator of JNK/SAPK (26, 50–52), and is a downstream target for MEK1 (50). In our studies, MEK7 appears to have a role as a regulator of hINV gene expression; however, this is not achieved via activation of JNK/SAPK, suggesting that a different downstream target of MEK7 may exist in keratinocytes.

**A Role for MEK3 and p38/RK—** MEKK1 is known to activate MEK4 (27, 48, 49) and p38/RK (74), in vitro. A recent study shows that MEKK1 acting via MEK4 activates p38/RK in vivo (75). It is thus possible that MEKK1 activates hINV gene expression via p38/RK activation. Our studies indicate that dominant negative forms of MEK3 and p38/RK inhibit TPA-, constitutively activated Ras-, and MEKK1-dependent promoter activation. Indeed, among the MAPKs surveyed, p38/RK is the only MAPK required for promoter activity. The p38 MAP kinase family includes p38α (53–57), p38β (76), p38β2 (58, 77), p38γ/SAPK3/ERK6 (77–79), and p38δ/SAPK4 (77, 80, 81). These isoforms are differentially expressed in various human tissues, suggesting distinct physiological functions. p38γ and p38δ differ from the rest of the p38 MAP kinase family regarding sensitivity to TPA stimulation, substrate specificity, and resistance to pyridinyl imidazole inhibitors (53). MEK3 has been reported to phosphorylate and activate p38α and p38γ (27, 29). The pyridinyl imidazole drug, SB203580, efficiently inhibits p38α and p38β2 and only at higher concentrations are p38δ and p38γ inhibited (58, 77, 82). We have observed that the p38 activity involved in regulation of hINV gene expression is relatively insensitive to the presence of SB203580 (i.e., no inhibition of TPA-dependent hINV promoter activity is observed at concentrations up to 20 μM SB203580, not shown). This fact, the fact that p38γ and p38δ are induced by TPA and the fact that MEK3 is essential for hINV gene regulation, suggests that
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Fig. 11. Proposed signal transduction pathway for regulation of hINV gene expression. The kinases indicated in open boxes appear to be involved as regulators of hINV promoter activity. The shaded kinases appear not to be involved. Lines connect individual enzymes in the signal transduction pathways. Dotted lines show those pathways that appear to be important for hINV gene regulation. hINV, human involucrin gene (or promoter). The names of the enzymes are defined in the text. Question marks indicate potential presence of unknown kinases.

The MEK3-dependent activation may be mediated by p38 or p38β. Additional studies are under way to test this hypothesis. Although p38/RK was originally identified as stress-activated kinase, our studies suggest that it may regulate expression of a differentiation-dependent process (i.e. regulation of hINV gene expression). This is consistent with a report suggesting that p38 is involved as a regulator of muscle cell differentiation (79).

The Role of MEK1, MEK3, and MEK7—Our results suggest that the MEK1-dependent regulatory signal is split into at least three distinct ways. The fact that dominant negative forms of MEK1, MEK3, and MEK7 all inhibit the MEK1-dependent promoter activation, suggest that three parallel signal transduction pathways could be involved. It is important to tentatively identify an activation pathway for each of these kinases. MEK3 is very likely acting via regulation of p38/RK (27, 29). MEK7 has been shown to activate JNK/SAPK (26, 50–52), and MEK1 normally activates ERKs (reviewed in Ref. 15). Although we cannot rule out the possibility that MEK1 and MEK7 may be acting via regulation of p38/RK, to our knowledge, there is no evidence in the literature suggesting this pathway of regulation. Our results suggest that both MEK7 and MEK1 have a role in regulating hINV promoter activity; however, these kinases do not appear to act via the traditional downstream targets, indicating that different targets may exist in keratinocytes.

S6 Kinase Does Not Regulate hINV Promoter Function—The p70 S6 kinase pathway is a mitogen-activated pathway distinct in keratinocytes. However, these kinases do not appear to act via the traditional downstream targets, indicating that different targets may exist in keratinocytes.

Role of Jun/Fos Proteins and the AP-1-Binding Site—Gel shift studies identify JunB, JunD, and Fra-1 as regulators of hINV gene expression (6). The present studies confirm the importance of AP1 factors and show that the hINV promoter proximal regulatory region site AP1, is a mediator of the TPA-dependent response. In addition, TAM67, a negative interfering mutant of c-Jun, inhibits promoter activity. TAM67 forms heterodimers with all Jun and Fos factors. The heterodimers bind DNA but are not transcriptionally active (19, 61). These results suggest that AP1 factors are an ultimate target of the TPA-dependent signal transduction cascade.

Summary—In summary, our results suggest (i) that a PKC → Ras → MEKK1 → MEK3 → p38/RK → AP1 pathway is important for regulation of hINV gene expression, (ii) that parallel pathways, possibly involving MEK1 and MEK7 may also be important, and (iii) that these pathways target AP1 factors that bind to an element between –128/100 in the hINV promoter proximal regulatory region.

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