Mitogen-activated protein kinases (MAPK), including p38, ERK1/2, and JNK1/2, are important regulators of cell function and activity without reducing ERK1/2 level. Thus, p38 may directly suppress ERK1/2 activity. Additional studies show that p38 activates p38 but not other p38 isoforms. p38 activation is increased as early as 0.5 h after OA addition, and activity is maximal at 8 and 24 h. ERK1 and ERK2 activity are reduced on an identical time course. We show that p38 forms a complex with ERK1/2, and overexpression of p38 inhibits ERK1/2 activity without reducing ERK1/2 level. Thus, p38 may directly suppress ERK1/2 activity. Additional studies show that p38 is expressed in the epidermis, suggesting a role for p38 in regulating differentiation. To evaluate its function, we show that increased p38 activity is associated with increased levels of API and CAAT enhancer binding protein factors, increased binding of these factors to the involucrin (hINV) promoter, and increased expression. Moreover, these responses are maintained in the presence of SB203580, an agent that inhibits p38α and p38β, further suggesting a central role for the p38 isoform. Dominant-negative p38 also inhibits these responses. These unique observations suggest that p38 is the major p38 isoform driving suprabasal hINV gene expression and that p38 directly regulates ERK1/2 activity via formation of a p38-ERK1/2 complex.

This paper is available on line at http://www.jbc.org

**MATERIALS AND METHODS**

**Reagents**—Keratinocyte serum-free medium, gentamicin, trypsin, and Hank’s balanced salt solution were purchased from Invitrogen. OA and SB203580 were obtained from Calbiochem. Phorbol ester (12-O-tetradecanoylphorbol-13-acetate (TPA)) and dimethyl sulfoxide were purchased from Sigma. The pGL2-Basic plasmid and the chemiluminescent luciferase assay system were purchased from Promega. [γ-32P]ATP was obtained from PerkinElmer Life Sciences. The human involucrin-specific polyclonal antibody was generated by injecting rabbits with recombinant human involucrin (4). Rabbit polyclonal antibodies specific for AP1 transcription factor family members c-Jun (SC-1694X), JunB (SC-46X), JunD (SC-74X), Fra-1 (SC-605X), Fra-2 (SC-171-X), c-Fos, and Pan-Fos (SC-253X), as well as rabbit polyclonal antibodies selective for C/EBP transcription factor family members C/EBPα (SC14AAX) and C/EBPβ (SC-7585) were obtained from Santa Cruz Biotechnology. Goat polyclonal antibody specific for p38α (SAPK4) (SC-7585) was obtained from Santa Cruz Biotechnology. Anti-FLAG M2 mouse monoclonal antibody (F-3165) was purchased.
from Sigma. Protein AG/agarose was obtained from Santa Cruz Biotechnology.

**Plasmids**—The structure of the hINV promoter construct pINV-241, which consists of nucleotides −241/−7 of the hINV promoter, linked to the luciferase reporter gene in pGL2-Basic, has been previously described (13). All of the positions are defined relative to the hINV gene transcription start site (13, 14). Dominant-negative p38 MAPK and dominant-negative MEK3 were generously provided by Dr. Roger Davis (15, 16).

**Recombinant Adenoviruses**—Adenoviral constructs encoding wild type FLAG epitope-tagged p38 isoforms α, β, γ, and δ were kindly provided by Dr. Y. Wang (17). Empty control adenovirus, Ad5-EV, was previously described (4). Recombinant adenoviruses were propagated in 293 cells and purified by cesium chloride centrifugation. Green fluorescent protein-encoding adenovirus, Ad5-GFP, was utilized to determine the optimal multiplicity of infection (MOI). Adenoviruses were administered at 15 MOI in the presence of polybrene.

**Immunoblot Analysis**—Total cell extracts or nuclear extracts were prepared from cultured human epidermal keratinocytes as described (18, 19). Protein concentration was determined using Bradford Bio-Rad protein assay. Equal quantities of protein were electrophoresed on a 10% denaturing SDS-polyacrylamide gels and transferred to nitrocellulose. The blots were blocked and then incubated with an indicated primary antibody, washed, and exposed to an appropriate horseradish peroxidase-conjugated secondary antibody. Secondary antibody binding was detected using a chemiluminescent detection system (Amersham Biosciences).

**In Vitro Kinase Assays**—The activities of MAP kinases were measured using nonisotopic p44/p42 (ERK1/2), p38 MAPK, and JNK/SAPK assay systems (New England BioLabs) (4, 19, 20). Briefly, keratinocyte total cell lysates were prepared under nondenaturing conditions. Protein concentration was determined, and equal amounts of total protein were used per each in vitro kinase assay sample. Active (phosphorylated) ERK1/2 and p38 kinases were selectively immunoprecipitated from cell lysates using immobilized, dual phospho-specific monoclonal antibodies. JNK/SAPK activity was selectively precipitated from cell lysates using c-Jun fusion protein glutathione-Sepharose beads. Precipitated kinases were then allowed to phosphorylate their major substrate proteins (Erk1 for ERK1/2; ATF-2 for p38, and c-Jun for JNK/SAPK) in a kinase reaction performed in the presence of ATP. Phosphorylation of the substrate proteins was measured by immunoblot using the corresponding phospho-specific antibody. The activity of adenovirus transduced FLAG epitope-tagged p38 MAPK isoforms was assessed in the similar fashion using anti-FLAG M2 mouse monoclonal antibody (Sigma; F3165) to immunoprecipitate specific p38 isoforms. The expression of individual p38 MAPK isoforms at a comparable level was confirmed by immunoblot using anti-FLAG antibody. To measure the activity of the endogenous p38 isoform, p38, p38- specific antibody (SC-7585) was used to selectively immunoprecipitate this kinase followed by a kinase assay performed as described above.

**Tissue Culture, Transient Transfection, and Luciferase Assay**—Third passage normal human foreskin keratinocytes were transfected when 50% confluent with 2 μg of involucrin promoter–luciferase reporter construct, pINV-241, in the presence of 6 μg of FuGen6 6 transfection reagent/dish (Roche Applied Science), according to the manufacturer’s instructions. At 24 h post-transfection, the keratinocytes were treated with keratinocyte serum-free medium in the presence or absence of TPA (100 nM) and C/EBP (5 μg/ml), OA (10 nM), or the two reagents combined. For co-transfection experiments, the cells were transfected with 2 μg of hINV promoter reporter plasmids and 2 μg of empty control vector or vector encoding dominant-negative p38 in the presence of 12 μg of FuGen6 6/dish. At 24 h, the keratinocytes were treated with 10 μM OA. After an additional 24 h of incubation, the cells were harvested, and the luciferase activity was measured as previously described, using Promega luciferase assay kit and a Berthold luminometer (13, 19). All of the assays were performed in triplicate, and each experiment was repeated three times. The luciferase activity was normalized per μg of protein. The transfection efficiency was monitored using a green fluorescent protein expression plasmid (Clontech) (21).

**Gel Mobility Shift Analysis**—The gel mobility shift assay was carried out as previously published (13, 18), using double-stranded, 32P-labeled oligonucleotides encoding the hINV promoter API−1 site (−5′-TTGGTGGAGAACAGGAGCTT-3′, API1 sequence in bold type) and the CEBP site (−5′-GGTTTTCTGCTTAAAGTGCTT-3′) (13, 18). Oligonucleotides encoding mutated API−1 (−5′-TTGGTGGAGAACAGGAGCTT-3′) and CEBP (−5′-GGTTTTCTGCTTAAAGTGCTT-3′) were used in competition experiments. The altered nucleotides are underlined. The Sp1 oligonucleotide sequence is

![FIG. 1. Okadaic acid activates p38 MAPK and JNK1/2 and inhibits ERK1/2 in keratinocytes. A, kinase activity of p38, JNK1/2, and ERK1/2. Cultured human epidermal keratinocytes were treated with 100 nM OA for 24 h. The cells were then lysed, total cell extracts were prepared, and active forms of p38, JNK, or ERK1/2 MAP kinases were immunoprecipitated from samples containing equal amount (200 μg) of protein, using immobilized monoclonal phospho-p38 antibody, c-Jun fusion protein glutathione-Sepharose beads, or immobilized monoclonal phospho-ERK1/2 antibody, respectively. The ability of each immunoprecipitated kinase to phosphorylate the appropriate substrate was analyzed by immunoblot using antibodies specific to phospho-ATP-2, phospho-c-Jun, and phospho-ERK1, respectively. B, p38, JNK, and ERK1/2 total protein levels were measured by immunoblot using specific antibodies. The β-actin blot is included as an indicator of equal protein loading. C, kinetics of OA-induced changes in activity of p38, JNK1/2, and ERK1/2 in keratinocytes. Cultured cells were treated with OA for the indicated time periods. After that the cells were lysed, and total cell extracts were prepared. p38 and JNK1/2 activities were measured exactly as described for A. ERK1/2 activity was assessed by immunoblot using phospho-specific ERK1/2 antibody that recognizes only phosphorylated activated form of ERK1/2 protein. In a parallel experiment, ERK1/2 activity was measured by kinase assay with similar results (not shown). The total ERK1/2 protein level was assayed by immunoblot. These experiments were repeated a minimum of three times with similar results.](image-url)
p388 Regulates Keratinocyte Differentiation

OA Regulation of MAPK Activity—We treated keratinocytes with 100 nM OA for 24 h and then monitored the effects on MAPK activity. To monitor p38 MAPK activity, active (phosphorylated) p38 MAPK was immunoprecipitated using an antibody that reacts with the phosphorylated form of all p38 MAPK isoforms (α, β, γ, and δ). The ability of the precipitated activated p38 MAPK to phosphorylate its substrate protein, ATF-2, was then examined by kinase assay. As shown in Fig. 1A, OA treatment markedly increases p38 MAPK activity. Activity of JNK1/2 showed a slight increase, ERK1/2 activity is transiently increased at 4 h and then returns to baseline. To differentiate the effects of OA on ERK1 versus ERK2 activity, we prepared extracts and directly measured p38, JNK1/2, and ERK1/2 levels by immunoblot. Fig. 1B demonstrates that the levels of these enzymes are not changed by OA treatment.

Because the extent and rate of MAPK activation is thought to influence downstream events, we monitored the time course of kinase activity following OA treatment. Keratinocytes were treated with 100 nM OA for 4 h and then monitored the effects on MAPK activity. To measure p38 activity, keratinocytes were harvested at each time point, and phospho-p38 was precipitated. We then monitored the ability of the precipitated kinase to phosphorylate ATF-2. As shown in Fig. 1C (top panel), p38 activity is detected by 4 h and is maximally increased by 8 h. To differentiate the effects of OA on ERK1 versus ERK2 activity, we prepared extracts and assayed for the presence of phosphorylated (active) ERK1/2. The upper ERK1/2 panel in Fig. 1C shows that P-ERK levels decrease beginning at 4 h and that both ERK1 and ERK2 are inhibited. The lower panel shows that there are no changes in total ERK1 or ERK2 concentrations during the time course. The JNK1/2 panel in Fig. 1C shows that JNK1/2 activity is transiently increased at 4 h and then returns to a level that is slightly elevated over untreated cells.

Differential Regulation of p38 Isoforms by OA—The above study indicates that p38 activity is increased by OA treatment. Protein expression was analyzed by immunoblot using p388-specific antibody (p388). The β-actin blot is included as a control to assure equal loading. D, SB203580 does not suppress OA-induced activation of p38 MAPK. Keratinocytes were pretreated with or without 2 μM SB203580 for 30 min prior to administration of 100 nM OA for 24 h. The cells were then lysed, and p388 activity was measured using kinase assay with ATP-2 as a substrate. The β-actin level was monitored as a loading control. E, keratinocytes were infected with 15 MOI of adenovirus encoding p388. After 48 h, the cells were harvested and assayed for ERK1/2 levels and activity as described for Fig. 1C. The β-actin levels were monitored to normalize gel loading.

RESULTS

OA Regulation of MAPK Activity—We treated keratinocytes with 100 nM OA for 24 h and then monitored the effects on MAPK activity. To monitor p38 MAPK activity, active (phosphorylated) p38 MAPK was immunoprecipitated using an antibody that reacts with the phosphorylated form of all p38 MAPK isoforms (α, β, γ, and δ). The ability of the precipitated activated p38 MAPK to phosphorylate its substrate protein, ATF-2, was then examined by kinase assay. As shown in Fig. 1A, OA treatment markedly increases p38 MAPK activity. Activity of JNK1/2 showed a slight increase, ERK1/2 activity is transiently increased at 4 h and then returns to baseline. To differentiate the effects of OA on ERK1 versus ERK2 activity, we prepared extracts and directly measured p38, JNK1/2, and ERK1/2 levels by immunoblot. Fig. 1B demonstrates that the levels of these enzymes are not changed by OA treatment.

Because the extent and rate of MAPK activation is thought to influence downstream events, we monitored the time course of kinase activity following OA treatment. Keratinocytes were treated with 100 nM OA for times ranging from 0 min to 24 h. To measure p38 activity, keratinocytes were harvested at each time point, and phospho-p38 was precipitated. We then monitored the ability of the precipitated kinase to phosphorylate ATF-2. As shown in Fig. 1C (top panel), p38 activity is detected by 4 h and is maximally increased by 8 h. To differentiate the effects of OA on ERK1 versus ERK2 activity, we prepared extracts and assayed for the presence of phosphorylated (active) ERK1/2. The upper ERK1/2 panel in Fig. 1C shows that P-ERK levels decrease beginning at 4 h and that both ERK1 and ERK2 are inhibited. The lower panel shows that there are no changes in total ERK1 or ERK2 concentrations during the time course. The JNK1/2 panel in Fig. 1C shows that JNK1/2 activity is transiently increased at 4 h and then returns to a level that is slightly elevated over untreated cells.

Differential Regulation of p38 Isoforms by OA—The above study indicates that p38 activity is increased by OA treatment. Protein expression was analyzed by immunoblot using p388-specific antibody (p388). The β-actin blot is included as a control to assure equal loading. D, SB203580 does not suppress OA-induced activation of p38 MAPK. Keratinocytes were pretreated with or without 2 μM SB203580 for 30 min prior to administration of 100 nM OA for 24 h. The cells were then lysed, and p388 activity was measured using kinase assay with ATP-2 as a substrate. The β-actin level was monitored as a loading control. E, keratinocytes were infected with 15 MOI of adenovirus encoding p388. After 48 h, the cells were harvested and assayed for ERK1/2 levels and activity as described for Fig. 1C. The β-actin levels were monitored to normalize gel loading.
However, p38 is expressed as four isozenes, p38α, β, δ, and γ (22–26), that can produce different responses in cells. To identify the responsive p38 isoform, the cells were infected with adenoviruses encoding FLAG-tagged p38α, β, δ, or γ and equilibrated for 24 h prior to the start of treatment with 100 nM OA. The cell extracts were prepared at time points from 0 min to 24 h after the start of OA treatment, and each adenovirus-delivered p38 isoform was immunoprecipitated using an anti-FLAG antibody. The precipitated kinase was then assayed for the ability to phosphorylate ATF-2. As shown in Fig. 2A, p38α activity is modestly increased at 0.5–24 h, and p38γ activity is elevated at 24 h. In contrast, p38β activity is not regulated. The p38 isoform exhibits the most dramatic increase in activity. Activity begins to increase at 30 min and is maximal at 8–24 h. This result suggests that p38β is the major OA-responsive p38 isoform. Fig. 2B confirms that expression of each FLAG-tagged p38 isoform is stable over the 24-h OA treatment time course. This was monitored by isolating FLAG-p38α, β, δ, or γ-expressing cells at the beginning of OA treatment (t = 0) and at t = 24 h and measuring the level by anti-FLAG immunoblot. β-Actin levels were monitored to assure equal loading.

The above results suggest that p38 is the major OA-responsive p38 isoform. To confirm that the endogenous p38 isoform is activated, keratinocytes were treated with OA for 0, 6, 16, and 24 h. Endogenous p38 was precipitated using a p38-specific antibody, and the precipitated kinase was evaluated for the ability to phosphorylate ATF-2. As shown in Fig. 2C (top panel), the above results suggest that p38 is activated by OA treatment. The middle panel displays an immunoblot showing that the increase in p38 activity is not due to an increase on p38 levels. A β-actin blot is included to normalize for differences in sample loading.

SB203580 is an inhibitor of the p38α and p38β isoforms but does not inhibit p38γ (25, 27). As an additional method of confirming that p38γ is the isoform that transmits the OA signal downstream, we treated keratinocytes with OA in the presence or absence of 2 μM SB203580. After 24 h, the extracts were prepared, total activated p38 (phosphorylated p38α, β, δ, and γ) was precipitated using anti-phospho-p38, and the ability of the precipitated p38 kinase to phosphorylate ATF-2 was determined. This was monitored by isolating FLAG-p38α, β, δ, or γ-expressing cells at the beginning of OA treatment (t = 0) and at t = 24 h and measuring the level by anti-FLAG immunoblot. β-Actin levels were monitored to assure equal loading. The above results suggest that p38γ is the major OA-responsive p38 isoform. To confirm that the endogenous p38 isoform is activated, keratinocytes were treated with 100 nM OA for 0, 6, 16, and 24 h. Endogenous p38 was precipitated using a p38-specific antibody, and the precipitated kinase was evaluated for the ability to phosphorylate ATF-2. As shown in Fig. 2C (top panel), the above results suggest that p38 is activated by OA treatment. The middle panel displays an immunoblot showing that the increase in p38 activity is not due to an increase on p38 levels. A β-actin blot is included to normalize for differences in sample loading.

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The above results were obtained using expressed MAPK
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**Fig. 4. p388 is expressed in adult human epidermis.** A, extracts prepared from human epidermis (EPI) were electrophoresed on a denaturing and reducing 8% acrylamide gel, transferred to nitrocellulose, and blotted with p388-specific antibody. As a control, extract prepared from keratinocytes infected for 48 h with FLAG-p388-encoding adenovirus was electrophoresed in the adjacent lane (KER). β-Actin was detected on a parallel blot as a control. Binding of the primary antibodies was detected using an appropriate secondary antibody. The epidermis was indicated by the brackets in each panel.

**Fig. 5. AP1 and C/EBP transcription factor expression is regulated by OA.** A, keratinocytes were treated with 100 nM OA for 24 h (AP1 factors) or 8 h (C/EBP factors). Nuclear extracts were then prepared and assayed for AP1 and C/EBP protein expression by immunoblot using antibodies against specific AP1 and C/EBP factors. Gel scanning of longer film exposures that show the bands in the untreated lanes. B, time course of OA-dependent activation of C/EBP and AP1 factor expression. The cells were treated with 100 nM OA for the specified time periods followed by nuclear extract preparation and immunoblot analysis using antibodies against individual AP1 and C/EBP proteins. C, SB203580 does not suppress OA-dependent increase in AP1 and C/EBP expression. Keratinocytes were pretreated with or without 2 μM SB203580 for 30 min prior to administration of 100 nM OA for 24 h. Nuclear extracts were prepared, and immunoblot was performed using antibodies against Fra-1 and C/EBPβ proteins.

**p388 Expression in Human Epidermis**—The above results suggest that p388 is functionally important in cultured keratinocytes. However, although p388 has been detected in developing mouse epidermis (28), evidence demonstrating that p388 is expressed in human epidermis has not been presented. We therefore prepared extracts of adult human epidermis for detection of p388 by immunoblot. Fig. 4A compares p388 obtained from epidermal extracts with authentic p388 obtained from cultured keratinocytes infected with adenovirus producing authentic p388. This experiment clearly shows that p388 is expressed in epidermis. Fig. 4B confirms expression in all epidermal layers.

**OA Regulation of AP1 and C/EBP Transcription Factor Expression**—The above studies suggest that p388 is activated by OA and also suggests that it may have a physiologic role, because it is expressed in human epidermis. Our next effort was to identify downstream responses associated with p388 activation. C/EBP and AP1 transcription factors are known to be important in regulation of keratinocyte differentiation (13, 18, 19, 29–36). We therefore examined the effects of OA treatment on AP1 and C/EBP factor expression. The cells were treated with 100 nM OA for 24 h, and the nuclear extracts were prepared for immunoblot with AP1 and C/EBP factor-specific antibodies. Fig. 5A shows that OA treatment increases c-Jun, JunB, JunD, Fra-1, and Fra-2 levels and also increases C/EBPα and C/EBPβ levels. We next examined the time course of the OA-dependent increase. The cells were treated for 0.5–24 h with 100 nM OA, and C/EBPα, C/EBPβ, and Fra-1 levels were monitored by immunoblot. As shown in Fig. 5B, the C/EBPα, C/EBPβ, and Fra-1 levels are optimally increased by 8 h after initiation of treatment. Thus, the time course of increase is remarkably consistent with that observed for p388 activation as shown in Fig. 2. To confirm that the response was not mediated by p388α or p388β, we treated cells with OA in the presence or absence of SB203580 for 24 h and then assayed C/EBPβ and Fra-1 levels by immunoblot. As shown in Fig. 5C, SB203580 does not inhibit the response, a result that is consistent with the regulation being p388-dependent. It is important to note that additional exposure of the film reveals expression of each of the AP1 and C/EBP factors in untreated cells. These exposures are not shown, because they result in overexposure of the OA-treated lanes.

**OA Regulates Differentiation-associated Gene Expression**—Involucrin is a marker of differentiation that is regulated in an AP1-and C/EBP transcription factor-dependent manner (13, 18, 19, 36). We have used it as an end point of OA and p388 action. Keratinocytes were transfected with hINV-241, a plasmid that encodes the proximal involucrin promoter linked to luciferase (13). Fig. 6A shows that both OA and TPA treatment increases hINV promoter activity and that the activation is...
enhanced by OA/TPA co-treatment. This regulation is mediated via an AP1-dependent mechanism, because mutation of the functionally important AP1–1 site (13), in construct pINV-241(AP1–1m), results in a loss of the regulation. In addition, the OA-dependent regulation is lost when the functionally important C/EBP site (18) is inactivated by mutation. To determine whether OA treatment and the concomitant p38/ H9254 activation translates into enhanced binding of AP1 and C/EBP factors to their respective binding sites on the hINV promoter, we performed gel mobility shift studies using oligonucleotides encoding the hINV promoter AP1 and C/EBP sites (13, 18).
were treated in the absence (+) or presence (−) of 100 nM OA for 24 h. The total cell extracts were then prepared and assayed for hINV protein expression by immunoblot. In parallel cultures, mRNA was prepared, and the hINV mRNA level was monitored by reverse transcription-PCR. Ribosomal 18 S RNA level was monitored in parallel to assure equal gel loading. B, OA-dependent increase in hINV protein expression is not blocked by pretreatment with SB203580. Keratinocytes were pretreated with or without the indicated concentrations of SB203580 for 30 min prior to administration of 100 nM OA for 24 h. The nuclear extracts were prepared, and immunoblot was performed using antibody against hINV. β-Actin level was monitored as a loading control. C, dominant-negative p38 (dnp38) and dominant-negative MEK3 (dnMEK3) inhibit the OA-dependent activation of gene expression. The keratinocytes were transfected with 2 μg of pINV-241 and hINV promoter reporter plasmid (13) in the presence of 2 μg of EV or vector encoding dnp38 or dnMEK3 and treated with 10 nM OA for 24 h. After an additional 24 h, the cells were harvested, and the extracts were assayed for luciferase activity.

compared with the band that is observed in untreated extracts. As shown in the right panel of Fig. 6B, this band can be competed by addition of a 50-fold excess of the homologous DNA fragment but not by a fragment containing an AP1 site mutation or a double-stranded oligonucleotides encoding and Sp1 site. Fig. 6C characterizes individual AP1 factor binding to the AP1–1 region using extracts prepared from OA-treated cells. Antibodies specific for Fra-1, Fra-2, JunB, and JunD produce supershifted bands, and a Fos antibody, Pan-Fos, which detects all Fos factors, efficiently reduces binding.

In addition, as shown in Fig. 6D (left panel), C/EBP factor binding to the C/EBP site is markedly enhanced by OA treatment. Moreover, this binding is specific because it is competed by homologous radioinert C/EBP oligonucleotides but not by the same nucleotide in which the C/EBP site is mutated (C/EBPm). In addition, some C/EBPα is present in this complex, as evidenced by the C/EBPα antibody-dependent supershift.

**OA Regulation of Endogenous hINV Gene Expression**—To be physiologically relevant, OA-dependent p38 inhibition should regulate expression of the endogenous hINV gene. To test this, we treated keratinocytes for 24 h with 100 nM OA and prepared total cell extracts for immunodetection of hINV (12). Fig. 7A (left panel) shows that OA treatment increases hINV protein level. A β-actin blot is included as an indicator to normalize protein loading. The right panel in Fig. 7A shows that hINV mRNA levels change in parallel. Gel scanning reveal that hINV level increase by 4–6-fold in several experiments. To provide additional evidence for a p38 role in this regulation, we treated with SB203580, a pharmacological agent that inhibits p38α and β but does not inhibit p38δ (25, 37). Fig. 7B shows that the increase in endogenous hINV gene expression is not inhibited by this agent, suggesting that the regulation is mediated by p38δ. To confirm this, Fig. 7C shows that expression of dominant-negative p38 inhibits the OA-dependent increase in promoter activity, confirming that the targeted knockout of p38 activity can blunt the response. In addition, dominant-negative MEK3 also antagonized the OA-dependent increase.

**DISCUSSION**

**Regulation of p38 MAPK Activity in Keratinocytes**—The cells respond to extracellular signals by generating intracellular instructions that coordinate cellular responses. The MAPK cascades are among the best characterized of these intracellular signaling pathways (2). These cascades consist of a three-kinase module that includes a MEK kinase that activates a MEK and ERK1/2 or interaction via an intermediary protein(s). ERK1/2 inhibition reduces the ability of the cell to survive and proliferate, p38δ triggers downstream signals that increase transcription factor expression. The transcription factors in turn interact with the hINV promoter to increase hINV promoter activity.
There are four known p38 MAPK isozymes: α, β, δ, and γ (22–25). Three of these isoforms are expressed in keratinocytes (α, β, and δ) (4), and recent studies suggest they function as regulators of keratinocyte differentiation (4, 19, 20). However, little information is available regarding the role of individual p38 isoforms. In the present study, okadaic acid, a phosphatase inhibitor, is used to activate MAPK signaling and study p38 function. Our studies show that OA increases p38 kinase and JNK kinase activity and decreases ERK1/2 activity.

The increase in JNK1/2 activity is transient and was not studied in the present report. In contrast, the increase in total p38 activity is substantial and sustained; activity increases at 4 h, and maximal levels are observed at 8 and 24 h. A detailed examination of individual p38 isoforms reveals that only p38δ is activated by OA stimulation. Parallel studies using pharmacologic inhibitors and dominant-negative p38 kinase further suggest that p38δ mediates the OA-dependent regulation. These findings are consistent with reports in other systems indicating that p38 isoforms are selectively activated in response to particular stimuli (40–44). We also evaluated the ability of the MEK kinase, MEK3, to regulate OA- and p38δ-dependent responses. This kinase has been reported to modulate p38 activity in other systems (22, 40). Studies using dnMEK3 indicate that MEK3 activity is required for OA-dependent regulation, suggesting that MEK3 is the kinase that functions immediately upstream of p38δ (Fig. 8). This result suggests that okadaic acid activates a cascade that includes MEK3 and p38δ. Additional studies (not shown) indicate that protein kinase C activity is not required for the OA-dependent regulation. This is expected, because OA, which increases the level of active phosphorylated kinase, activates the signaling cascade downstream of protein kinase C.

A p38δ–ERK1/2 Complex in Keratinocytes—The kinetics of increase in p38 activity is associated with a corresponding change in ERK1/2 activity. An important feature of this regulation is the inverse relationship between p38 and ERK1/2 activity. An important question is the mechanism that achieves this inverse regulation. Our results show that p38δ and ERK1/2 form a complex in keratinocytes. The presence of this complex was confirmed by reciprocal co-immunoprecipitation using expressed p38 MAPK as bait and also by demonstrating an interaction between endogenous ERK1/2 and p38δ. Moreover, when p38δ was overexpressed, a decrease in ERK1/2 activity was observed in the absence of any change in ERK1/2 level. These are the first findings suggesting an interaction between ERK1/2 and p38δ in keratinocytes or any other cell type. This unique finding suggests that p38δ, by association with ERK1/2, may directly influence ERK kinase activity. Whether this involves direct interaction or an intermediary protein will require further study. The only other known example of this type of interaction is a p38α–ERK1/2 complex that has been described in HeLa cells. This complex forms upon treatment with stimulating agent (11). This interaction appears to block ERK1/2 phosphorylation/activation by upstream MEK1/2 kinase (11). The complex we describe differs in two ways. First, it involves p38δ and not p38α, and second, ERK1/2 and p38δ appear to be constitutively associated (i.e. association is not stimulus-dependent). Our study and the study by Zhang et al. (11) suggest that ERK and p38 MAPKs may have motifs designed to facilitate interaction (40). Additional studies will be necessary to identify the interaction motifs. Based on these results, we propose a simple model in which OA activates MEK3, and MEK3 activates p38δ. Then p38δ, either by covalently modifying ERK1/2, by making it inaccessible to upstream kinases (MEKs), or by causing a conformation change in ERK1/2, causes its inactivation. It should be emphasized that this influence may be conferred by direct interaction or via interaction with another protein or proteins. Additional studies show that p38δ activation increases transcription factor level and activity, which in turn increases hINV gene expression (Fig. 7). This includes an increase in Fra-1, Fra-2, JunB, and JunD binding to the hINV AP1 site and increased C/EBPα binding to the C/EBP site. Although these studies provide some clues regarding the composition of these OA-stimulated transcriptional complexes, additional studies will be required to identify other participants. More studies are also required to understand the mechanism whereby p38δ activation alters nuclear transcription factor level and activity. It is well established that MAPKs can translocate to the nucleus and directly or indirectly regulate transcription factors (45). This regulation could alter the AP1 and C/EBP factor level (46) and/or the phosphorylation state (47, 48). Additional studies will be needed to provide this information.

Expression of p38δ in Human Epidermis and Regulation of Downstream Responses—The above results suggest that p38δ can be activated by OA in cultured keratinocytes. However, it was not clear that this enzyme has a physiological role in vivo, because it has not been demonstrated in human epidermis. We show by immunoblot of epidermal extracts and immunohistochemical staining of skin sections that p38δ is expressed in skin. Moreover, staining was observed throughout the epidermis. At present, we do not know whether the enzyme is differentially activated (phosphorylated) in specific layers. It is possible that p38δ activation is controlled by the presence of its activator. For example, protein kinase Cη, which plays a key role in regulation of terminal keratinocyte differentiation, is expressed in the suprabasal epidermal layers (49) and activates p38δ (50). Hence, the differentiation-specific expression of upstream activators may ensure that p38δ is activated only in the suprabasal layers. However, the availability of downstream targets may also be a point of regulation. For example, C/EBPα expression is restricted to the upper suprabasal epidermal layers (31, 33), and AP1 factor expression is also differentiation-dependent (51). Thus, p38δ may be active throughout the epidermal layers, but differentiation-associated responses may only be engaged when the appropriate downstream target is present.

In cultured keratinocytes p38δ clearly activates involucrin gene expression. Involucrin is a differentiation marker that is expressed in the spinous and granular epidermal layers in vivo (52, 53). Involucrin gene expression is regulated by AP1 and C/EBP transcription factors (13, 18, 19, 36, 38). Our results demonstrate that OA-dependent p38δ activation is associated with increased AP1 and C/EBP transcription factor level, increased binding of AP1 and C/EBP factors to the appropriate sites on the hINV promoter, and increased hINV promoter activity. These responses are not inhibited by SB203580, an inhibitor of α and β isoforms of p38 MAPK. Thus, these studies suggest that p38δ activates hINV gene expression. Moreover, this regulation requires the presence of intact AP1 and C/EBP sites within the hINV promoter, suggesting that the OA- and p38δ-associated increase in AP1 and C/EBP activity is responsible for the regulation. Moreover, expression of the endogenous hINV gene is also increased, suggesting that the promoter response is physiologically meaningful. Based on these studies, we propose that OA activates differentiation via a signaling cascade that includes MEK3 and p38δ. This cascade in turn regulates AP1 and C/EBP transcription factor function to regulate hINV gene expression. A novel component of this response involves a p38δ-dependent decrease in ERK1/2 activity.
