Phorbol Ester-induced Expression of Airway Squamous Cell Differentiation Marker, SPRR1B, Is Regulated by Protein Kinase Cα/Ras/MEKK1/MKK1-dependent/AP-1 Signal Transduction Pathway*

Hue Vuong‡, Tricia Patterson‡, Paul Shapiro‡, Dhananjaya V. Kalvakolanu†, Reen Wu, Wei-Ya Ma**, Zigang Dong**, Steven R. Kleeberger‡, and Sekhar P. M. Reddy‡‡

From the ‡Department of Environmental Health Sciences, The Johns Hopkins University School of Public Health, Baltimore, Maryland 21205, †University of Maryland School of Pharmacy, Baltimore, Maryland 21201, *Greenbaum Cancer Center, University of Maryland School of Medicine, Baltimore, Maryland 21201, the ‡Department of Internal Medicine, School of Medicine, University of California, Davis, California 95616, and **The Hormel Institute, University of Minnesota, Minneapolis, Minnesota 55912

The transcriptional induction of SPRR1B by phorbol 12-myristate 13-acetate (PMA) is mainly mediated by the first -152-base pair 5′-flanking region containing two functional AP-1 sites. In this study, we have analyzed the signaling pathways that mediate the induction in tracheobronchial epithelial cells. PKC inhibitor ablated PMA-stimulated expression of endogenous SPRR1B and reporter gene expression driven by SPRR1B promoter. PKC activator promoted the transcription. The dominant negative protein kinase Cα (dn-PKCα) and rottlerin (PKCζ inhibitor) completely suppressed PMA-stimulated promoter activity. dn-Ras or dn-MEKK1 inhibited PMA-stimulated promoter activity, while their corresponding constitutively active mutants augmented it. dn-c-Raf-1 did not have any effect on reporter gene expression. Since MEKK1 activates multiple parallel pathways, we examined involvement of JNK/SAPK, p38, and MKK1 in promoter regulation. Co-expression of the dominant negative forms of MKK4, MKK7, JNK/SAPK, MKK3, MKK6, or p38ζ did not suppress PMA-stimulated reporter gene expression. However, MKK1 inhibitors U0126 and PD98059 suppressed gene expression. Consistent with this, expression of dn-MKK1 strongly suppressed PMA-stimulated promoter activity, while the constitutively active MKK1 augmented it. However, MKK1-mediated induction of SPRR1B probably does not depend on extracellular signal-regulated kinases 1 and 2, suggesting the requirement of another kinase(s). dn-c-Jun mutants abolished PMA-stimulated expression supporting an important role for AP-1 proteins in SPRR1B expression. Together, these results suggest that a PKCα/MEKK1-dependent/1B-pathway regulates the PMA-inducible expression of the SPRR1B in tracheobronchial epithelial cells.

The expression of squamous cell function in the respiratory tract epithelium is a phenomenon that is frequently associated with injury caused by various environmental pollutants, such as phorbol ester PMA,1 tobacco smoke, and carcinogens (1, 2). Our studies and others have demonstrated a close relationship between early induction of human small proline-rich protein type I (SPRR1) and squamous cell differentiation in airway epithelium (for a review, see Ref. 3). SPRR1 (now referred to as SPRR1B or cornifin) was originally identified as a vitamin A-suppressed gene from TBE cells (4). Vitamin A, which plays an important role in maintaining mucociliary cell differentiation, suppresses induction of squamous cell differentiation in TBE cells (5). In contrast to squamous tissues, such as esophagus, tongue, and skin, which contain higher levels of SPRR1B message, the presence of SPRR1B message level is very low in respiratory tract epithelia that normally express mucociliary functions (3, 4). However, a variety of agents that promote squamous differentiation of TBE cells, such as phorbol ester PMA, vitamin A deprivation, tobacco smoke, and carcinogens rapidly induce SPRR1B message levels (3). Previously, we have demonstrated a rapid increase of SPRR1B products (mRNA and protein) in cultured human and monkey TBE cells (4). This increase can be reduced, mainly at a post-transcriptional level, by supplementing the culture medium with vitamin A or its synthetic retinoids (6, 7). On the other hand, PMA (4) and tobacco smoke (8), which potently induce airway squamous differentiation, induce SPRR1B expression mainly at the transcription level. However, the molecular and cellular pathways regulating expression of SPRR1B in airway epithelial cells are not clearly understood.

SPRR1B belongs to a multigene family consisting of two SPRR1 genes (SPRR1A and -1B), seven SPRR2 genes (SPRR2A to -2F), and one SPRR3 gene, which is located on

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† To whom all correspondence should be addressed: Dept. of Environmental Health Sciences, The Johns Hopkins University, Division of Physiology, Rm. W7006, 615 N. Wolfe St., Baltimore, MD 21205. Tel.: 410-614-5442; Fax: 410-955-0299; E-mail: sreddy@jhsp.h.edu.

1 The abbreviations used are: PMA, phorbol 12-myristate 13-acetate; ca, constitutively active; CAT, chloramphenicol acetyltransferase; dn, dominant negative; ERK, extracellular regulated kinase; Ets, E-26 transformation specific; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; TBE, tracheobronchial epithelial; BIM, bisisdolylmaleimide I; IND, indolactam V; pp, base pair(s); PKC, protein kinase C; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MKK, mitogen-activated protein kinase kinase; TAD, transactivation domain; DD, DNA-binding domain; LZD, leucine zipper domain; GST, glutathione S-transferase; CE, cornified envelope.

2 S. P. M. Reddy and R. Wu, unpublished data.
chromosome 1q21, now termed as an epidermal differentiation locus (9). These genes encode small molecular weight proteins exceptionally rich in proline, cysteine, and glutamate and were first identified as inducible gene products in human keratinocyte cultures after UV irradiation and PMA treatment (10, 11). SPRRs are differentially expressed in the suprabasal epithelial cell layer of various squamous tissues (3). SPRRs are cross-linked to themselves and/or to other cornified envelope (CE) precursor proteins such as loricin and involucrin and play an important role in modulation of biochemical properties of squamous tissues, depending upon their physical requirements and function (12). The terminal phenotype of squamous differentiation is the formation of a CE catalyzed by transglutaminase to form an insoluble mesh, which plays an important role in barrier function (13). Recently, we have demonstrated the actual participation of SPRR1B in the cornification of TBE cells (14). However, the exact functional role(s) of SPRR1B in the induction of terminal squamous differentiation of TBE cells is unclear. SPRR1B is also expressed in other nonsquamous cells, such as mammary epithelium (15), Chinese hamster ovary (16), and smooth-muscle cells (17), suggesting that it might play yet unidentified role(s) besides its involvement in cell cornification.

SPRRs have two exons separated by a single intron (10). The first exon of SPRRs contains the 5′-untranslated region, while the second exon contains the complete coding region and the 3′-untranslated region in a manner similar to that found in other genes such as involucrin and loricin (18). The first 152-bp 5′-flanking region of the SPRR1B reveals overall ~50% identity to the mouse counterpart (19, 20). However, there is a high degree of identity (>75%) in the promoter sequences and positions at the TATA box, Ets binding site, and AP-1 sites. Recently, Sark et al. (21) isolated the genomic clone of human SPRRIA and demonstrated that the first 152-bp 5′-flanking region contains functional motifs, such as a TATA box, Ets, and an AP-1 site, in identical locations as found in the human SPRR1B promoter. Moreover, both Ets and AP-1 sites are critical for PMA-stimulated SPRRIA gene regulation in human keratinocytes (21). We have also observed that PMA stimulates SPRRIA mRNA levels and promoter activity in human TBE cells (data not shown).

Previously, we have demonstrated that the treatment of TBE cells with PMA stimulates the expression of the SPRR1B mainly at the transcriptional level (19). By in vitro footprinting, deletion, and site-directed mutagenesis, we have demonstrated that the ~152 to ~12 bp promoter region contains two functional AP-1 sites that are required for both basal and PMA-enhanced SPRR1B promoter regulation (22). Moreover, AP-1 proteins, such as c-Jun, bind to these sites (22). In the present study, we have analyzed the cellular signaling pathways that regulate PMA-stimulated SPRR1B expression in TBE cells. We show that PMA-stimulated SPRR1B expression and promoter regulation is mainly mediated by PKCα/Ras/MEKK1/MEKK1-dependent/AP-1 signal transduction pathway. Interestingly, ERK1/2 are not required for this process.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Bisindolylmaleimide I (BIM), indolactam V (IND), genistein, PD98059, SB202190, Go6986, and PMA were obtained from Calbiochem (San Diego, CA). U0126 was purchased from Promega (Madison, WI).

**Expression Vectors and Reporter Constructs**—Dominant negative Ras (Ras-N17) was generated by introducing a point mutation at the 17-position of the Hs-Ras and then cloned into kRSPA vector with a Rous sarcoma virus promoter. Constitutively active Ras (Hs-Ras-V12) was generated as described previously (23). Expression vectors of dominant negative MEKK1 (367MEKK1-KR), constitutively active MEKK1 (367MEKK1), dominant negative MKK1 (218 and 222 serine residues converted to alanines), constitutively active MKK1 (218 and 222 serine residues converted to glutamates), and dominant negative SEKI1/MK4 (SEKI1-AL, serine 220, and threonine 224 mutated to alanine and leucine, respectively) all cloned in pECEMV were generously provided by Dr. Dennis Templeton (24, 25). Dominant negative MKK7 mutant (F.MKK7), dominant negative JNK1 mutant (AFF), and dominant negative MKK6 mutant (ala) all cloned in pCDNA5; dominant negative p38 mutant cloned in pCMV was generously provided by Dr. Syunichi Hirai (30). Dominant negative ERK1 (Lys1→Arg) and ERK2 (Lys22→Arg) mutants each cloned in pCEP4 vector were generously provided by Dr. Melanie Cooper (26, 27). The panel of dominant negative c-Jun mutants (with five inactivating mutations in the transactivation domain (c-Jun-TAD), DNA binding domain (c-Jun-BDB), and leucine zipper domain (c-Jun-LZD) were kindly provided by Dr. Stephan Ludwig (33). The dominant negative ERK5 (also known as big MAP kinase, BMK1) mutant (BMK1AEF, Thr34 and Tyr38) and Try26 amino acids are replaced by alanine and phenylalanine) was kindly provided by Dr. J. D. Lee (34).

**Human SPRR1B promoter** and its mutants fused to chloramphenicol acetyltransferase (CAT) gene had been described previously (22). We have used the ~152 to ~12 bp SPRR1B promoter cloned into CAT reporter vector and abbreviated as 152-SPRR1B-CAT3 throughout. The normal human TBE cell line, BEAS-2B (subclone S6), immortalized by SV40-T antigen was obtained from J. F. Lechner. This cell line (passages between 22 and 30) was maintained at 37 °C, 5% CO2 in a serum-free hormone-supplemented medium as described previously (22). Briefly, cells were cultured in F-12 medium (Life Technologies, Inc.) supplemented with the following growth factors: transferrin (5 μg/ml; Sigma), insulin (5 μg/ml; Sigma), cholester (20 ng/ml; Lister, Cambridge, CA), bovine pituitary extract (20 μg/ml; Pel-Freeze Biologicals, Arkansas), hydrocortisone (0.5 μg/ml; Sigma), and epithelial growth factor (5 μg/ml; Upstate Biotechnology, Inc.). The medium was also supplemented with 10 μM HEPES buffer (pH 7.4), penicillin and streptomycin (60 units/ml), gentamycin (12.5 μg/ml), and fungizone (60 μg/ml).

**Transient Transfections and Reporter Gene Assays**—DNA transfections were performed using a Fugene transfection reagent according to the manufacturer’s recommendations (Roche Molecular Biochemicals). Cells were grown on 12-well plates at 70–80% confluence and then transfected with 0.4 μg of promoter construct, 0.1 μg of CMV-β-galactosidase (β-gal) DNA, and 0.1–0.8 μg of empty or expression plasmid vectors. After 18–20 h post-transfection, cells were treated with either Me2SO (vehicle control) or PMA (100 ng/ml) for 24 h. Where indicated, cells were treated for 30 min with appropriate kinase inhibitors prior to PMA. Cells were lysed, and CAT expression was measured using an enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals). The β-galactosidase activity was monitored as described previously (22). CAT activity of individual samples was normalized against β-galactosidase activity.

**Isolation and Northern Blot Hybridization**—For Northern blots, equal amounts of total RNA (20 μg/lane) were subjected to electrophoresis, transblotted onto Nytran membranes, and hybridized with 32P-labeled SPRR1B cDNA probe as described previously (4). Hybridized filters were washed and exposed to Eastman Kodak Co. x-ray film. Membranes were stripped and rehybridized with 32P-labeled 18 S RNA cDNA probe.

**Immunoprecipitations and in Vitro Kinase Assays**—TBE cells were cultured to 70–80% confluence and treated with PMA (100 ng/ml) for 0–24 h and washed three times with chilled PBS containing 1 mM Na2VO4 (sodium orthovanadate). Cells were then lysed in 750 μl of lysis buffer containing 20 μl Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton, 2.5 mM sodium pyrophosphate, 1 mM Na3VO4, 5 mM β-glycerophosphate, 1 μg/ml leupeptin. Lysates were sonicated for 15 s and centrifuged for 10 min at 10,000 × g at 4 °C to remove cellular debris. Protein concentration in the lysates was determined using the DC protein assay kit (Bio-Rad). Approximately 200 μg of protein was used for immunoprecipitation analysis using 0.4 μg of antibodies derived against ERK2 (C-14), JNK1 (C-17), or p38 (C-20) kinases (all obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) that were used at 1:1,000 dilution in TBS (10 mM Tris, 150 mM NaCl, 0.5% Tween 20, 1% BSA, 0.1% sodium deoxycholate; Bio-tech). Immunoprecipitates were washed extensively with 25 μl of protein A-Sepharose beads. Immunoprecipitates were washed extensively with 25 μl of protein A-Sepharose beads. Intracellular pools of GTP (3H)- bound proteins were determined using 2.5 μg of myelin basic protein, GST-Jun (aminos acids 1–79), and GST-ATF-2, as substrates, respectively, in kinase buffer containing 10 μCi of [γ-32P]ATP, 25 mM HEPES (pH 7.4), 15 mM MgCl2,
then treated with either Go6976 (5 μM) or rottlerin (1–5 μM) for 30 min. The cells were lysed, and CAT expression was measured. PMA significantly increased CAT expression compared with the vehicle control (compare lane 5 and lane 2).

RESULTS

PKC Regulates PMA-stimulated SPRR1B mRNA Levels and Promoter Activity—As shown in Fig. 1A, treatment of TBE cells with PMA (100 ng/ml) significantly enhanced SPRR1B mRNA levels (lanes 1 and 2). This response was inhibited by treatment with BIM, a specific inhibitor of PKC (lanes 3 and 4). In contrast, IND, a PKC activator, significantly enhanced SPRR1B mRNA levels, similar to PMA (Fig. 1A, compare lane 5 and lane 2). Treatment of cells with genistein, a tyrosine kinase inhibitor, also suppressed PMA-stimulated SPRR1B mRNA levels (Fig. 1B). We next analyzed by transient transfection analysis whether PKC regulates PMA-stimulated SPRR1B promoter activity. Cells were transiently transfected with a 152-bp SPRR1B-CAT3 chimeric construct, and transfected cells were treated with either BIM, IND, or vehicle (Me2SO) for 30 min prior to PMA treatment. Cells were lysed, and CAT expression was measured. PMA significantly stimulated (~4–5-fold) SPRR1B promoter activity when compared with the vehicle (Fig. 1C). However, pretreatment of cells with BIM totally suppressed PMA-stimulated CAT gene expression, while it had little or no effect on the basal expression. On the other hand, IND significantly enhanced the CAT expression compared with that seen with PMA (Fig. 1D). Protein-tyrosine kinase inhibitor, genistein, also completely suppressed PMA-stimulated SPRR1B gene expression (Fig. 1E). Together, these results suggest that both the serine/threonine and tyrosine kinases regulate SPRR1B promoter.

PKCδ Regulates SPRR1B Promoter Activity—Human TBE cells mainly express PKC isoenzymes such as α, βII, δ, and ε, but not βI, γ, and η (36). To identify specific PKC isoenzymes involved in SPRR1B promoter regulation, we have used Go6976 and rottlerin, which were shown to specifically inhibit PKCα (37) and PKCδ (38) isoenzymes, respectively. Cells were transiently transfected with 152-bp SPRR1B-CAT3 reporter and then treated with either Go6976 (5 μM) or rottlerin (1–5 μM) for 30 min. Subsequently, cells were cotransfected with either PMA (100 ng/ml) or Me2SO (vehicle) in the presence of inhibitors. Treatment of cells with rottlerin strongly suppressed both basal and PMA-stimulated CAT gene expression (Fig. 2A). In contrast, Go6976 did not suppress PMA-stimulated gene expression. On the contrary, it stimulated a nearly 1-fold increase in basal expression (Fig. 2B). Based on this observation, we used mutants of PKCα and PKCδ to study their effect on gene expression. The dn-PKCδ suppressed both the basal and PMA-stimulated promoter activities (Fig. 2C). In contrast, dn-PKCα did not suppress PMA-stimulated promoter activity (data not shown). Together, these results indicate involvement of PKCδ in PMA-dependent SPRR1B promoter activation.

Ras but Not Raf-1 Regulates SPRR1B Promoter—Having demonstrated the involvement of PKCδ in PMA-dependent SPRR1B gene expression, we next investigated whether Ras, a downstream target of PKCδ (39, 40), is also involved in the signaling cascade. Cells were co-transfected with active (Ras-V12) or mutant (Ras-N17) Ras expression vector along with SPRR1B-CAT3 construct. Following overnight incubation, cells were treated with either PMA or Me2SO, and the CAT expression was measured. Expression of Ras-N17 completely suppressed PMA-stimulated activity nearly to the basal level (compare bars 2 and 4 with bar 1, Fig. 3A). dn-Ras slightly reduced the basal CAT gene expression (Fig. 3A). Coexpression of Ras-V12 significantly stimulated promoter activity (~2.5-fold) when compared with vector-transfected controls (compare bars 1 and 2, Fig. 3B). Furthermore, treatment of cells with PKCδ inhibitor rottlerin (5 μM) did not suppress Ras-V12-enhanced activity (compare bars 3 and 2, Fig. 3B). Together these results indicate that PKCδ activates Ras, which in turn regulates SPRR1B promoter activation. Ras activates multiple parallel pathways that involve Raf, MEK, and ERK kinases (39), so we therefore investigated the involvement of Raf-1 in SPRR1B expression. As shown in Fig. 3C, dominant negative c-Raf-1 (dn-c-Raf-1) did not suppress PMA-stimulated SPRR1B promoter activity. Coexpression of dn-c-Raf-1 along with Ras-V12 did not suppress the Ras-enhanced SPRR1B promoter activity.

Airway Squamous Signaling Pathways

![Image](329x462 to 533x656)

![Image](329x668 to 533x729)

![Image](329x322 to 533x425)
constitutively active form of M KK1 (ca-MKK1) significantly enhanced the promoter activity. PMA treatment further augmented the ca-MKK1 dependent promoter activity. PD98059 significantly suppresses PMA-stimulated SPRR1B mRNA levels (Fig. 5C). Furthermore, expression of dn-MKK1 suppressed Ras-enhanced SPRR1B promoter activity (compare bar 2 with bar 3, Fig. 5D), while dn-ERK1/2 did not have any effect (compare bar 2 with bar 4, Fig. 5D; see below). Taken together, these results indicate that MKK1/2 regulates both basal and PMA-stimulated expression of SPRR1B.

ERK1 and ERK2 Do Not Regulate PMA-stimulated SPRR1B Expression—We next examined the role of ERK1 and ERK2 MAP kinases, the well known downstream targets of MKK1/2, in SPRR1B expression. As shown in Fig. 6A, co-expression of a dominant negative form of ERK1 or ERK2 either alone or in combination did not suppress PMA-stimulated CAT gene expression. The dominant negative effect of dn-ERK1 was demonstrated using a different CAT reporter construct that is driven by AP-1 sites (Fig. 6B). Expression of dn-ERK1 and dn-ERK2 mutant proteins was seen in transfected cells, and they inhibited phosphorylation of endogenous ERK1/2 proteins (data not shown). We next analyzed whether PMA activates the phosphorylation of ERK1/2 proteins in TBE cells. Cells were treated with PMA (100 ng/ml) for various time periods and phosphorylation of ERK1/2 was analyzed by using a phosphospecific ERK1/2 antibody. The ERK2 phosphorylation increased 2–3-fold rapidly (within 5 min) following PMA treatment (Fig. 6C, compare bar 1 with bar 2). However, we did not observe a persistent increase in the ERK1/2 phosphorylation at later time points (Fig. 6C, compare bars 4–6 with bar 1). We have also analyzed the kinase activity of the samples that were immunoprecipitated with ERK2 (Fig. 6D). Although PMA treatment slightly enhanced ERK2 activity at 5 min, no persistent activation was observed at later time points (from 1 to 22 h). In fact, we observed a decrease in the ERK2 activity. Taken together, these results suggest lack of involvement of either ERK1 and/or ERK2 in PMA-stimulated SPRR1B promoter regulation.

ERK5 Does Not Regulate PMA-stimulated SPRR1B Expression—Recently, it was demonstrated that MKK1/2 inhibitor PD98059 also inhibits the activation of ERK5 (43). Since PMA-stimulated activity is not inhibited by the expression of dn-ERK1 and/or dn-ERK2, we investigated the role of ERK5 in SPRR1B promoter regulation. As shown in Fig. 7, co-transfection of the dominant negative mutant of ERK5 (AEF) along with SPRR1B-CAT reporter construct into TBE cells did not suppress the PMA-stimulated CAT gene expression compared with the control group transfected with empty vector alone. In fact, we noticed a slight increase in the PMA-stimulated CAT expression. These results indicate that ERK5 or BMK1 pathway may not be involved in SPRR1B promoter activation.

SEK1-JNK/SAPK Pathway Is Not Involved in SPRR1B Promoter Regulation—Besides MKK1, MKK1 also activates multiple downstream targets, which include MKK4, MKK7 (also known as SEK1), MKK3, and MKK6. MKK1/2 then activates ERK, and MKK4 activates JNK/SAPK, while MKK3 and MKK6 activate p38 MAP kinases (41). Therefore, we have examined the involvement of these three MAPK kinase pathways in SPRR1B regulation. PD98059, a pharmacological inhibitor of MKK1/2, prevents the activation of MKK1/2, thereby inhibiting phosphorylation of downstream ERK1/2 kinases (42). Treatment of cells with PD98059 (30 μM) inhibited both basal and PMA-stimulated CAT expression. Both the basal (nearly 40%) and PMA-stimulated (~100%) CAT activity was suppressed by PD98059 (Fig. 5A). These results were further confirmed using U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene), which specifically inhibits the function of activated MKK1/2, thereby inhibiting the activation of ERK1/2 proteins (42). U0126 suppressed both the basal (40%) and PMA-stimulated (~100%) activity at lower (2.5 μM) concentration (Fig. 5A). Consistent with this, expression of a dominant negative MKK1 (dn-MKK1) robustly suppressed PMA-stimulated CAT gene expression (Fig. 5B). Conversely, a
FIG. 3. Ras but not c-Raf regulates promoter activity. A, TBE cells were transiently transfected with 152-SPRR1B-CAT3 and β-galactosidase vectors along with either empty parental or dn-Ras mutant vector. B, cells were transfected with SPRR1B promoter construct along with 0.4 μg of empty vector (bar 1), ca-Ras (bars 2 and 3), or ca-Ras plus 0.4 μg of dn-c-Raf1 (bar 4). Various amounts of parental vector were added to keep the total amount of transfected DNA equal in all samples. After overnight incubation, cells were treated with Me2SO (bars 1, 2, and 4) or 5 μM rottlerin (bar 3). C, cells were co-transfected with promoter vector along with empty parental vector or dn-c-Raf expression vector. Cells were then treated with either Me2SO (light shaded bars) or PMA (100 ng/ml; dark shaded bars) for 24 h and harvested, and CAT expression was analyzed as in Fig. 1. Bars show the mean and S.E. of relative CAT activity.

(F).MKK7) did not significantly reduce PMA-stimulated CAT gene expression (Fig. 6B). We have also analyzed the kinase activity of the PMA-treated cellular extracts that were immunoprecipitated with JNK1 antibodies (Fig. 6C). PMA treatment slightly enhanced JNK1 kinase activity at 5–30 min, and no persistent activation was observed at later time points (3–22 h). Together, these results indicate that the MKK4/7-JNK/SAPK pathway is not involved in PMA-stimulated expression of the SPRR1B in TBE cells.

MKK3/5/MKK6/p38 Pathway Does Not Regulate SPRR1B Promoter—MKK1 also activates p38 kinases, which belong to the stress-activated MAPK family. They are induced in response to UV irradiation, inflammatory cytokines, and various environmental stresses (44). MKK3 and MKK6 activate p38 MAPKs (41). To examine the role of the p38 pathway in SPRR1B expression, we have used a chemical inhibitor, SB202190 (45). Treatment of cells with SB202190 (10 μM) did not inhibit significantly either the basal or PMA-stimulated CAT gene expression (Fig. 9A). Furthermore, expression of dominant negative forms of p38, MKK3, and MKK6 also did not suppress PMA-stimulated CAT gene expression (Fig. 9, B–D). Analysis of p38 kinase activity in the immunoprecipitates of PMA-treated cells did not reveal a significant activation of p38α (Fig. 9E). Thus, the p38 MAPK pathway is not necessary for regulating PMA-stimulated SPRR1B expression.

AP-1 Proteins Regulate SPRR1B Promoter Activity—Previously, we have shown that two AP-1 sites are critical for SPRR1B promoter regulation and are bound by AP-1 proteins, such as c-Jun (22). Therefore, we have used a panel of dominant negative c-Jun constructs with mutations in the transactivation domain (TAD), DNA binding domain (DBD), or leucine zipper domain (LZD) (32) to determine the role of AP-1 proteins in promoter regulation. Cells were transiently transfected with 152-SPRR1B-CAT3 reporter along with either the wild type or the dn-c-Jun mutant expression vectors, and CAT expression was analyzed. As shown in Fig. 10, expression of wild type c-Jun robustly enhanced the promoter activity, comparable with that observed with PMA treatment. Moreover, treatment of c-Jun-transfected cells with PMA synergistically induced CAT expression. The c-Jun-TAD mutant strongly inhibited PMA-stimulated activity of SPRR1B promoter. Expression of the c-Jun-DBD mutant also had a similar but more profound effect on gene expression. Although the c-Jun-LZD mutant significantly suppressed both basal and PMA-stimulated promoter activities, it was not as effective as c-Jun-TAD and c-Jun-DBD mutants as previously reported (32).

DISCUSSION

The plasticity of cell differentiation is the major biological thrust of airway epithelium, maintaining mucociliary function under normal conditions and expressing squamous and keratinizing properties after injury or vitamin A deficiency (1, 2). This is accompanied by a multistep process in which cells undergo terminal cell division followed by the expression of CE precursor proteins such as involucrin, loricrin, SPRR1, etc. and finally formation of cornified envelope (46, 47). It has been suggested that a similar “squamous cell differentiation” is involved in the preneoplastic lesion of bronchogenic cancer development (48, 49). However, the signaling pathways regulating the induction of squamous differentiation in nonsquamous airway epithelium are not clearly understood. PMA, a stable analog that mimics the effects of diacylglycerol, potently induces squamous differentiation in TBE cells (50, 51) and keratinocytes (47, 52). PMA activates PKC, which in turn initiates a signaling cascade to stimulate expression of genes involved in cell growth and differentiation in different cell types including...
Cells were co-transfected with various constructs as in A (3–6) squamous cell differentiation. PKC

Consistent with this, PKC induces expression and the activity of transglutaminase I (55). SPRR1B

in ERK1/2 (39). Our data (Fig. 3) of Raf (c-Raf-1, A-Raf, and B-Raf), which in turn activates

p38, which stimulate activity of various transcription factors

response to growth factors and hormones (39). Activated Ras in

promoters. PKC6 inhibits expression in TBE cells (46) and keratinocytes (53). We have previously

demonstrated an important role for PKC in the induction of keratinocyte differentiation (55). Overexpression of PKC in keratinocytes increases cell size, induces growth arrest, and induces expression and the activity of transglutaminase I (55).

Consistent with this, PKC regulates PMA-enhanced hINV promoter activity in keratinocytes (56), another marker for squamous cell differentiation. PKC regulates also in PMA-induced myeloid differentiation (57), the regulation of growth arrest in fibroblasts (30), and cell cycle progression in Chinese hamster ovary cells (58), suggesting an important role for this isoform in the differentiation process in other cell types. Moreover, it was shown that PKC6 activates AP-1/Jun through Ras-dependent signal transduction pathways in NIH 3T3 cells (30, 59).

To our knowledge, ours is the first study to report the involvement of PKC in regulation of PMA-inducible SPRR1B expression in airway epithelial cells. Consistent with this view, treatment of TBE cells with PMA enhances accumulation of PKC in particulate fractions, indicating activation of this isoenzyme (36). Thus, activation of PKC may be one of the important factors for commitment of airway epithelial cells to squamous differentiation.

Ras, a well known downstream target of PKC, induces cell proliferation, differentiation, and morphological changes in response to growth factors and hormones (39). Activated Ras in turn stimulates three distinct MAPK cascades, ERK, JNK, and p38, which stimulate activity of various transcription factors (39, 40). Ras mediates its effects in part through the activation of Raf (c-Raf-1, A-Raf, and B-Raf), which in turn activates ERK1/2 (39). Our data (Fig. 3A) clearly support a role for Ras in SPRR1B regulation. Furthermore, PKC6 inhibitor did not suppress constitutively active Ras-enhanced promoter activation (Fig. 3B), indicating that Ras is a downstream target of PKC6 in SPRR1B promoter regulation. However, this process does not require c-Raf, since expression of a dominant negative c-Raf-1 mutant did not have an effect either on PMA-stimulated (Fig. 3C) or Ras-enhanced (Fig. 3B) SPRR1B promoter activity. In keratinocytes, Ras mediates PMA-stimulated hINV regulation (60), where it has the opposite effect on the SPRR2A promoter (61). Together, these data suggest a differential role for Ras in regulation of cornified envelope precursor gene expression. Mutations in the Ras oncogene are thought to be involved in the initiation of the squamous cell carcinomas (40, 62). SPRR1B is expressed in normal keratinocytes and TBE cells but not in malignant squamous cell carcinomas (63–66). Therefore, it would be interesting to see whether mutations altering Ras activity lead to the suppression of SPRR1B expression in malignant cells.

Besides c-Raf, Ras was also shown to activate MEKK1 (67). The fact that dn-MEKK1 suppresses PMA-induced SPRR1B gene expression (Fig. 4), indicates that Ras mediates its effects through activation of MEKK1, but not by c-Raf. MEKK1 phosphorylates MKK1/2, which then activates two downstream kinases, ERK1 and ERK2, to stimulate the binding of transcription factors to regulate target gene expression (68). Indeed, chemical inhibitors of MKK1/2, PD98059 and UO126, or a dominant negative MKK1 mutant robustly suppressed PMA-stimulated SPRR1B expression (Fig. 5, A and C). Interestingly, dominant negative forms of ERK1 and ERK2 either alone or in combination did not suppress PMA-stimulated promoter activity (Fig. 6A), ruling out a role for ERK1/2 in SPRR1B expression. The activation of ERK1 and ERK2 by PMA through the PKC-Ras-MEKK1 pathway has been observed in many cell types (59, 69). However, suppression of PMA-stimulated SPRR1B expression by MKK1-specific inhibitors PD98059 and UO126, but not by ERK1, ERK2, or ERK5 mutants, strongly suggests the presence of an unidentified ERK-like kinase that

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Fig. 5. MKK1/2 pathway regulates SPRR1B expression. A, upon reaching 70–80% confluence, cells were co-transfected with 152-SPRR1B-CAT reporter and β-galactosidase vectors as in Fig. 1. After transfection, cells were treated with either vehicle (Me2SO, bars 1 and 2), UO126 (bars 3–6), or PD98059 (bars 7 and 8) prior to PMA treatment. B shows the effect of dn-MKK1 and ca-MKK1 mutants on SPRR1B promoter regulation. Cells were co-transfected with various constructs as in A either in the absence (bars 1 and 2) or presence of dn-MKK1 (bars 3 and 4) or ca-MKK1 (bars 5 and 6) mutants. After transfection, cells were treated with vehicle (light shaded bars) or PMA (100 ng/ml; dark shaded bars). C shows the effect on MKK1 inhibitor PD98059 on SPRR1B expression. Cells were grown to 80% confluence and subsequently treated without (bars 1 and 2) or with 30 µg PD98059 (bars 3 and 4) for 30 min prior to PMA treatment. Northern blot was carried out as described under “Experimental Procedures.” D shows the effect of dn-MEKK1 and dn-ERK1/2 mutants on Ras-enhanced SPRR1B promoter activity. Cells were transfected with SPRR1B promoter construct along with 0.4 µg of either empty (bar 1), ca-Ras (bar 2), ca-Ras plus 0.4 µg of dn-MKK1 (bar 3), or ca-Ras plus 0.4 µg of dn-ERK1/2 (bar 4) expression vectors. CAT activity was analyzed as described under “Experimental Procedures.”
regulates SPRR1B expression. This is the first demonstration that expression of the airway squamous differentiation marker is regulated by a divergent and ERK1/2-independent mechanism. Consistent with this, it was shown that MEKK1 activates MKK1 without altering the ERK1 and ERK2 activity, suggesting the existence of other ERK-related kinase(s) that might regulate gene expression depending upon cellular and promoter context (70). In a similar manner, results were obtained with PMA-stimulated \textit{hINV} promoter regulation in keratinocytes (60). Expression of Fra-2, a FOS family member, down-regulates PMA-stimulated SPRR1B promoter activity in TBE cells (data not shown). ERK2 phosphorylates Fra-2, thereby converting it from a nonfunctional transcriptional activator to an active one (71). This would explain why expression of a dominant negative mutant of ERK2 significantly augments the basal level activity of SPRR1B promoter (Fig. 6A). ERK5 (also known as BMK1), a member of the MAP kinase superfamily, is also activated by growth factors, oxidants, and osmotic stress (43). However, expression of dn-ERK5 did not suppress the promoter activity (Fig. 7), indicating that it may not be required for PMA-stimulated SPRR1B expression in TBE cells. Taken together, it seems likely that Ras-MEKK1-MKK1-mediated SPRR1B expression is probably mediated by a kinase other than ERK1, ERK2, and ERK5. The nature of

![Fig. 6. ERK1 and ERK2 do not regulate PMA-stimulated SPRR1B promoter activity.](image)

![Fig. 7. ERK5 pathway does not regulate PMA-stimulated SPRR1B promoter regulation.](image)
this putative MAP kinase remains to be investigated.

Besides activating MKK1, MEKK1 also activates the JNK/ SAPK and p38 kinase pathway (72). JNK and p38 kinases are activated by cellular stress, such as UV and γ-irradiation, osmotic stress, heat shock, and inflammatory cytokines (72). In turn, these kinases activate transcription factors, such as c-Jun.
and ATF-2, to modulate gene transcription. However, neither of these pathways seems to be important for regulating PMA-stimulated SPRR1B expression (Figs. 8 and 9). This is in contrast to another study that demonstrated the involvement of the PKC-Ras-MEK1-MKK1-MKK3/6-p38 pathway in hINV gene expression in keratinocytes (60). Taken together, these results suggest that the activation of cornified envelope precursor gene expression, although initiated by a common signaling cascade at the upstream levels, deviated into different downstream modules. Alternatively, the induction of squamous differentiation in nonsquamous airway epithelium is mediated by a different type of transcription factor(s) than the one that regulates differentiation in squamous tissues such as skin. For example, it was demonstrated that the expression of dominant negative form of c-Jun up-regulates SPRR2A promoter activity in keratinocytes (61, 73), whereas it completely blocks both basal and PMA-stimulated SPRR1B expression in TBE cells (Fig. 10; see below). In fact, inhibitors of JNK/SAPK and p38 pathways, moderately up-regulate SPRR1B promoter activity, suggesting that these pathways may be negative regulators of PMA-stimulated SPRR1B expression in TBE cells.

AP-1 proteins (Jun/Fos) induce transcription of a variety of genes that are involved in cell growth and differentiation. The JUN family proteins c-Jun, Jun B, and Jun D can either homodimerize or heterodimerize with the FOS family proteins, c-Fos, Fos-B, Fra-1, and Fra-2, which then bind to the AP-1 site and regulate gene expression in response to PMA depending on cellular and promoter context (74). Several studies have established a key role for AP-1 proteins in the modulation of PMA-inducible cornified envelope precursor gene expression in keratinocytes (75). Previously, we demonstrated that PMA-stimulated SPRR1B promoter activity is mediated by c-Jun (22). Here we show that the expression of dominant negative c-Jun mutant ablated PMA-stimulated promoter activity, supporting an important role for AP-1 proteins in SPRR1B expression in TBE cells (Fig. 10). We have also observed the stimulation of promoter activity by Jun B and Jun D, but these are less potent than c-Jun. In contrast, expression of Fra-2 had an opposite effect indicating a differential regulation of SPRR1B expression by the AP-1 family members (data not shown).

In summary, we have demonstrated that activation of PKC, especially PKCδ, is required for PMA-dependent SPRR1B gene expression. Second, Ras mediates its effects through activation of MEKK1, but not by c-Raf. Third, MKK1/2 regulates SPRR1B in response to PMA but does not require ERK1/2. Finally, we showed that AP-1 proteins play an important role in SPRR1B promoter regulation. Therefore, we conclude that PMA-stimulated squamous differentiation marker, SPRR1B, expression in airway epithelium is mainly mediated by the PKCδ-Ras-MEK1-MKK1-2-dependent/AP-1 signal transduction pathway.

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