BMK1 (ERK5) Regulates Squamous Differentiation Marker *SPRR1B* Transcription in Clara-like H441 Cells

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Various toxicants and carcinogens upregulate the expression of small proline-rich protein 1B (*SPRR1B*), a squamous differentiation marker, in bronchial epithelial cells both *in vivo* and *in vitro*. We have recently shown that phorbol 13-myristate 12-acetate (PMA)–stimulated *SPRR1B* transcription in Clara-like H441 cells is mainly mediated by activator protein–1 (AP-1) and *c-Jun* N-terminal kinase-1 (JNK1). Though mitogen-activated protein kinase (MAPK) kinase (MEK)-1/2 pathway inhibitors strongly suppressed both basal and PMA-inducible *SPRR1B* transcription, overexpression of dominant negative (dn) forms of extracellular signal-regulated kinase (ERK)-1 and/or -2 did not have any significant effect indicating the involvement of another ERK-like MAPK in this pathway. Here, we report for the first time the involvement of ERK5 in PMA-inducible *SPRR1B* transcription in H441 cells. PMA significantly induced ERK5 activation in H441 cells. Overexpression of dn-ERK5 strongly suppressed both basal and PMA-inducible *SPRR1B* transcription, whereas wild-type ERK5 upregulated it. Consistent with this, a mutant form of MEK-5, an upstream activator of ERK5, strongly suppressed PMA-inducible promoter activity. However, coexpression of *c-Jun* restored promoter activation suppressed by dn-ERK5. Thus, in addition to JNK1, the activation of MEK5-ERK5 MAPK pathway probably plays a pivotal role in transcriptional regulation of AP-1–mediated *SPRR1B* expression in the distal bronchiolar region.

In response to injury caused by various pollutants, toxicants, and carcinogens, proximal and distal airway epithelial cells lose their normal secretory functions and express squamous and keratinizing properties (1–3). This phenomenon, which is thought to be a protective response against toxicants and pollutants, may lead to epithelial cell transformation and bronchial carcinogenesis if not properly restored (1, 2). Although much progress has been made in defining the regulatory mechanisms of terminal differentiation in keratinocytes, the signal transduction pathways and downstream effectors governing induction of the abnormal squamous differentiation in airway epithelium remain enigmatic. Therefore, delineating the molecular and cellular mechanisms involved in the induction of squamous differentiation will enable a better understanding of toxicant-induced epithelial injury-repair and differentiation processes, and the development of respiratory pathogenesis.

(*Received in original form January 16, 2002 and in revised form March 8, 2002*)

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Abbreviations: activator protein-1, AP-1; dimethyl sulfoxide, DMSO; dominant negative, dn; *c-Jun* N-terminal kinase-1, JNK-1; extracellular signal-regulated kinase, ERK; mitogen-activated protein kinase, MAPK; MAPK kinase, MEK; phorbol 13-myristate 12-acetate, PMA; small proline-rich protein 1B, *SPRR1B*.

Am. J. Respir. Cell Mol. Biol. Vol. 27, pp. 64–70, 2002
Internet address: www.atsjournals.org

Clara cells, which are located in the distal bronchiolar region, are the major targets for pulmonary toxicity induced by various environmental pollutants and toxicants (3). Clara cells secrete various proteins, such as surfactants and a 10-kD Clara cell protein, CC10 (3), which play an important role in respiratory function. However, upon exposure to toxicants and pollutants, such as phorbol 13-myristate 12-acetate (PMA), tobacco smoke, naphthalene, and other cytotoxicants, Clara cells loose their differentiation markers and express squamous properties both *in vivo* and *in vitro* (4–8). Small proline-rich proteins (*SPRR*s) are highly abundant in various squamous tissues; however, the expression patterns are distinct. Their expression in general is high in epithelia of oral tissues, such as tongue, esophagus, and stomach, in contrast to external dry epithelia, such as skin (9). SPRRs belong to a multi-gene family consisting of two *SPRR1* (1A and 1B) genes, seven *SPRR2* (2A–2F) genes, and one each of *SPRR3* and *SPRR4* (10). At their N- and C-terminal regions SPRRs contain lysine and glutamine residues, which are cross-linked to themselves and/or other proteins by transglutaminase during formation of cornified cell envelope. The cornified cell envelope is a terminal phenotype of squamous cell and acts as a barrier to various external and internal epithelia.

In contrast to squamous epithelia, the expression of *SPRR*s is low or undetectable in airway epithelium, which normally express mucous properties (11). Among the genes studied, *SPRR1B* was originally identified as vitamin A–suppressed gene in airway epithelial cells (11). However, *SPRR1B* expression is highly inducible in airway epithelium both *in vitro* and *in vivo* by a variety of agents, which potently induce airway squamous cell metaplasia, such as PMA, carcinogens, and tobacco smoke (12–14). Recently, we have demonstrated that PMA, a stable functional analog of diacylglycerol that is produced *in vivo* by hydrolysis of phospholipids, selectively upregulates the expression of *SPRRs* in human Clara-like H441 cells (15). The induction of *SPRR1B* expression in proximal (BEAS-2B clone S-6) and distal (H441) airway epithelial cells is primarily regulated at the transcriptional level, mainly by the AP-1 protein complex composed of Jun/Fra-1 (15, 16). Further studies revealed that extracellular signal-regulated kinase (ERK)-like kinase(s) mediate PMA-inducible *SPRR1B* expression in S-6 and H441 cells (15, 17). Surprisingly, ERK1 and ERK2 do not participate in this process in both cell types.

Recent studies indicated that, in addition to ERK1 and ERK2, ERK5 (also known as big mitogen-activated kinase 1, BMK1), plays a regulatory role in proliferation and differentiation in various cell types (18). However, little information is available about the role of ERK5 in growth factor and/or toxicant-induced injury-repair and differen-

tiation processes in airway epithelium. We present here, for the first time, evidence for the involvement of ERK5 in PMA-inducible *SPRR1B* transcription in Clara-like H441 cells. PMA significantly induced ERK5 activation in H441 cells, whereas overexpression of dominant-negative (dn) form of ERK5 strongly suppressed both basal and PMA-inducible *SPRR1B* transcription. Consistent with this, overexpression of dn mutant of MEK5, an upstream activator of ERK5, significantly blocked PMA-inducible promoter activation. Thus, the activation of MEK-ERK5 pathway probably plays a regulatory role in AP-1–dependent gene expression in the distal bronchiolar region.

Materials and Methods

Cell Culture and Reverse Transcriptase–Polymerase Chain Reaction Analysis

NCI-H441 (Clara-like bronchiolar pulmonary adenocarcinoma) cell line was obtained from the ATCC and maintained in RPMI culture medium supplemented with 5% serum, 1% streptomycin and penicillin, gentamicin (250 ng/ml) and fungizone (125 ng/ml). Human primary bronchial epithelial cultures and cell lines, HBE-1, BEAS-2B clone S-6, and A549 cells were propagated as described elsewhere (16). For reverse transcriptase–polymerase chain reaction (RT-PCR) analysis, total RNA (750 ng) isolated using Trizol reagent was reverse transcribed into cDNA. PCR amplification was performed at 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min with an aliquot of cDNA using ERK5 (F: 5′-CCTCTGA AAGCCTTGAGGAG-3′; R: 5′-GCAGCCC-ACAGACCAGAG GTC-3′) or β-actin (F: 5′-GAGAAAATCTGGCACCACAAC-3′; R: 5′-TACCCCTCGTAGATG-GGCAC-3′) gene-specific forward (F) and reverse (R) primer pairs. The amplified ERK5 and β-actin cDNA fragments, 780 and 201 bp, respectively, were separated on 1.2% agarose gel and photographed using the Gel Doc 2,000 System (Bio-Rad, Hercules, CA).

Expression Vectors, Reporter Plasmids, and Transient Transfections

The wild-type (wt) and dn (AEF; mutation of Thr218 and Tyr 220 with Ala and Phe) ERK5 cDNAs cloned in pcDNA3 expression vectors (19) were kindly provided by Jiing-Dwan Lee (The Scripps Research Institute, La Jolla, CA). The dn-MEK5 (MEK5DD; mutation of Ser311 and Thr315 to Asp) cDNA cloned in pCEFL expression vector (20) was generously provided by Silivio Gutkind (National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD). The constitutive active c-Raf-1 (Raf-BXB) expression vector was kindly provided by Stephen Ludwig (Institut für Medizinische Strahlenkunde und Zellforschung, Würzburg, Germany). *c-Jun* dn-Ras (Ras-N17) and ca-Ras (Ras-V12) were generated as previously described (15). The promoter of –150 to +12 bp *SPRR1B* fused to luciferase (Luc) gene was described elsewhere (15). AP-1–Luc reporter plasmid (Stratagene, La Jolla, CA) containing seven copies of AP-1–responsive elements (TGACTAA), henceforth referred to as AP-1–Luc. The −165 to +19 bp promoter of human IL-8, which contains the functional motifs such as nuclear factor κB (21), subcloned into CAT3 plasmid vector was kindly provided by Reen Wu (University of California at Davis). IL-8 promoter was PCR amplified and directionally subcloned into pGL-3 vector (Promega, Madison, WI) at the restriction sites of *Sac*I and *Nhe*I. The orientation and sequence of the promoter was verified by DNA sequencing. DNA transfections were performed using a Fugene transfection reagent (Roche Applied Science, Indianapolis, IN). Cells were grown on 48-well plates at 70–80% confluence and then transfected with 100 ng of promoter reporter construct, 50 ng of cytomegalovirus–β-galactosidase (β-gal) DNA, and 50–200 ng of parental empty or expression vectors as indicated. Approximately 24 h after transfection, cells were treated with either dimethyl sulfoxide (DMSO) or PMA (20 ng/ml) for 5–12 h. Cells were lysed and Luc activity was measured using a commercially available kit (Promega). Luc activity of individual samples was normalized against β-gal activity and/or total protein as described previously (17). Promoter activity for every construct was analyzed in duplicates and all experiments were repeated two to three times.

Immunoprecipitation and *In Vitro* ERK5 Kinase Assays

H441 cells were cultured to 80–90% confluence and then treated with PMA (20 ng/ml) for the indicated time periods. Cells were washed three times with chilled phosphate-buffered saline containing 1 mM Na_3VO_4 (sodium orthovanadate) and scraped into a lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM ethylene diamine tetraacetic acid, 1 mM ethyleneglycol-*bis*-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid, 1% Triton, 2.5 mM sodium pyrophospate, 1 mM Na_3VO_4, 5 mM β-glycerolphosphate, and 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 BCA reagent (Pierce, Rockford, IL). An equal amount of cellular protein (200 μg) was immunoprecipitated with anti-ERK5 antibodies (0.4 μg, Cat # E1523; Sigma, St. Louis, MO) and washed extensively. Half of the immunoprecipitate was used for analyzing kinase activity, and the other half was used for immunoblot analysis to determine ERK5 content. ERK5 kinase activities of various samples were determined using 2.5 μg myelin basic protein as substrate in a reaction mixture containing 10 μCi γ-^{32}P (adenosine triphosphate), 25 mM HEPES (pH 7.4), 15 mM $MgCl_2$, and 1 mM dithiothreitol (17). After 15 min incubation, the *in vitro* kinase assays were terminated with 2× sodium dodecyl sulfate loading buffer. The reaction products were separated on sodium dodecyl sulfate/polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and substrate phosphorylation was determined by PhosphorImager analysis. Total ERK5 content of the immunoprecipitates was analyzed by the Western blot analysis using ERK-5 specific antibodies. ERK5 activity was normalized against total ERK5 content of the respective samples. The value of the untreated sample was considered as one unit.

Statistical Analysis

Data are expressed as the mean ± standard error. The StatView program was used to perform analysis of variance between different samples. Statistical significance was accepted at $P < 0.05$. All assay samples were performed in duplicates and each experiment was repeated at least two times.

Results

PMA Activates ERK5 in H441 Cells

To examine a role for ERK5, H441 cells were treated with PMA, which induces epithelial injury and repair process in distal bronchiolar region (22), for different time periods as indicated. Whole cell extracts were immunoprecipitated with anti-ERK5 antibodies (developed against amino acid 789–802 of the c-terminus region of human ERK5) and *in vitro* kinase assays were performed using myelin basic protein as a substrate as described previously (19). As shown in Figure 1, following PMA treatment the ERK5 kinase activity rapidly increased within 5 min (> 3-fold) and was maximal at 30 min (> 4-fold). ERK5 activation remained elevated for 3 h. The increase in kinase activity following PMA treatment is nearly comparable to that observed with

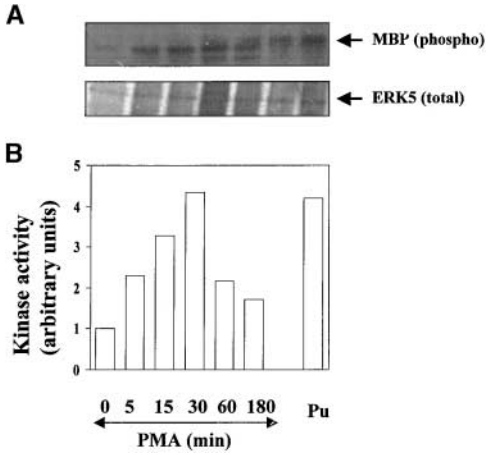

Figure 1. PMA stimulates ERK5 activity in H441 cells. Cells were grown to 70–80% confluence and treated with vehicle (DMSO) or PMA (20 ng/ml) for different time periods as indicated. Cellular extracts (200 μg) were immunoprecipitated with 0.4 μg of anti-ERK5 antibodies. (*A*) The kinase activity of the immunoprecipitates was analyzed using myelin basic protein as substrate (*top*). As a positive control for ERK5 activation, H441 cells were treated with puromycin (Pu, 2 μg/ml for 30 min). The autoradiogram (*bottom*) depicts the Western blot analysis of total ERK5 content in the immunoprecipitates used for *in vitro* kinase assay. (*B*) The ERK5 kinase activity was normalized against total ERK5 content. The value of untreated samples was designated as one.

exposure to puromycin, which stimulates ERK5 activity in various cell types (23).

Expression of ERK5 in Airway Epithelial Cells

To confirm the expression of ERK5 in airway epithelium, total RNA was isolated from various human airway epithelial cells, and the message levels were amplified using ERK5 gene-specific primers. As shown in Figure 2, the mRNA expression of ERK5 is detectable in proximal (HBE-1 and S-6) and distal (Clara-like H441 and alveolar-like type II epithelial cell line, A549) airway epithelial cell lines, indicating a broad expression pattern of ERK5. Moreover, ERK5 mRNA expression was also detectable in primary cultures derived from tracheobronchial epithelium (*lanes 8* and *9*). The amplified PCR product was purified and digested with *Eco*RI to yield 415 and 365 bp DNA fragments (GenBank Accession # U25278) confirming the specificity of ERK5 cDNA amplification (data not shown).

ERK5 Regulates PMA-Inducible *SPRR1B* Transcription in H441 Cells

Recently, we have shown that PMA-stimulated *SPRR1B* mRNA expression (data not shown) and promoter activation (15) in H441 cells is strongly inhibited by PD98059, which prevents the activation of MKK1/2 (24). Surprisingly, overexpression of dn forms of ERK1 and/or ERK2, which are downstream effectors of MKK1/2, did not suppress PMA-stimulated *SPRR1B* transcription. A similar observation was also made in BEAS-2B clone S-6 cells, indicating the involvement of a different ERK-like kinase in this pathway (17). It was reported that PD98059 and U0126 compounds also inhibit ERK5 activation (25, 26). Therefore, we examined whether ERK5 mediates PMA-inducible *SPRR1B* transcription in H441 cells. H441 cells were exposed to 2.5–100 ng/ml of PMA, and *SPRR1B* promoter–driven luciferase expression was analyzed as described in MATERIALS AND METHODS. PMA increased *SPRR1B* promoter activity in a dose-dependent manner, with the maximal response seen at 20 ng/ml (Figure 3A). Although higher doses of PMA (50–100 ng/ml) caused significant stimulation of the promoter compared with the untreated controls, these PMA

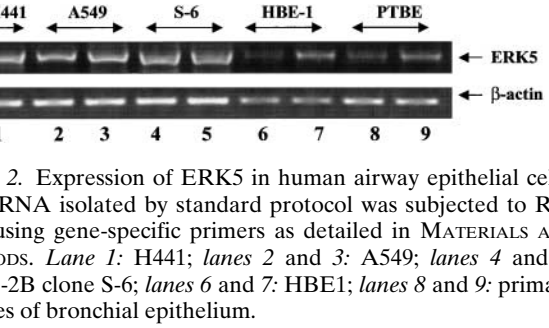

Figure 2. Expression of ERK5 in human airway epithelial cells. Total RNA isolated by standard protocol was subjected to RT-PCR using gene-specific primers as detailed in MATERIALS AND METHODS. *Lane 1:* H441; *lanes 2* and *3:* A549; *lanes 4* and *5:* BEAS-2B clone S-6; *lanes 6* and *7:* HBE1; *lanes 8* and *9:* primary cultures of bronchial epithelium.

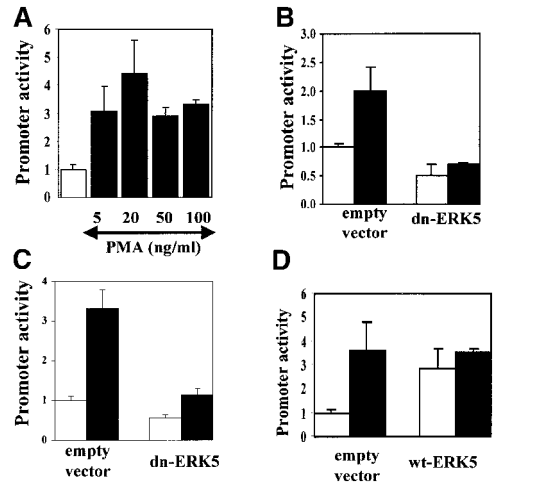

Figure 3. ERK5 regulates PMA-inducible *SPRR1B* transcription in H441 cells. Upon reaching 70–80% confluence, cells were cotransfected with 100 ng of –150 to +12 bp *SPRR1B* promoter–driven Luc reporter construct and pCMV-β-gal (50 ng). After ~ 24 h transfection, cells were treated either with vehicle (control) or with different doses of PMA (5–100 ng/ml; *A*) or with indolactam (Indo, 5 nM; *B*). Cells were transfected with *SPRR1B* promoter reporter construct and pCMV-β-gal vector in the presence of 100 ng either empty parental, dominant-negative ERK5 mutant (dn-ERK5; *C*), or wild-type ERK5 (wt-ERK5; *D*) expression plasmids. After ~ 24 h transfection, cells were treated either with vehicle (control) or PMA (20 ng/ml) in a fresh culture medium. Reporter expression was analyzed as described in MATERIALS AND METHODS. Promoter activity was calculated using the values of parental empty vector transfected cells as one. *A*, *B*, and *D: open bars*, control; *solid bars*, PMA. *C: open bars*, control; *solid bars*, indolactam.

doses caused a slight reduction of the promoter activity compared with cells treated with 20 ng/ml PMA. Therefore, we have used the dose of 20 ng/ml of PMA throughout the study. Moreover, we have previously shown that over a comparable range PMA stimulates *SPRR1B* expression in H441 cells (15). In support of a role for ERK5 in *SPRR1B* regulation, overexpression of dn ERK5 strongly suppressed PMA-stimulated reporter gene expression (Figure 3B). Similarly, dn-ERK5 also suppressed PKC activator indolactam-stimulated *SPRR1B* transcription (Figure 3C). Consistent with this, overexpression of wild-type ERK5 significantly increased *SPRR1B* promoter activation, which could be augmented with PMA treatment (Figure 3D).

MEK5 Regulates PMA-Inducible *SPRR1B* Transcription in H441 Cells

We next examined the role of MEK5, an upstream activator of ERK5, in the regulation of *SPRR1B* expression. Cells were cotransfected with dn-MEK5 expression vector along with *SPRR1B* promoter reporter construct. After overnight incubation, cells were treated with DMSO or PMA and reporter gene expression was analyzed. Overexpression of dn-MEK5 significantly suppressed PMA-stimulated *SPRR1B* transcription (Figure 4). Thus, these results indicate that the MEK5-ERK5 pathway mediates PMA-stimulated *SPRR1B* transcription in H441 cells.

ERK5 Mutant Suppresses Ras- and Raf-Inducible *SPRR1B* Transcription in H441 Cells

Recently, we have shown that Ras regulates PMA-inducible *SPRR1B* transcription in H441 cells (15). To determine whether ERK5 mediates Ras-inducible gene expression, cells were cotransfected with constitutively active (ca-Ras) expression vector along with *SPRR1B* promoter construct in the presence or absence of dn-ERK5 plasmid DNA. Consistent with our previous results, overexpression of dn-Ras strongly suppressed PMA-stimulated activity nearly to the basal level (data not shown). In contrast, overexpression of ca-Ras significantly stimulated promoter activity ($\sim$ 2.5-fold) compared with an empty vector–transfected control (Figure 5). However, coexpression of dn-ERK5 mutant significantly suppressed such activation. Together, these results indicate that Ras inducible *SPRR1B* transcription in H441 cells is mediated in part by ERK5.

We next tested whether downstream effectors of Ras, which include Raf-1 and MEKK1 (27), were involved in PMA-induced *SPRR1B* expression. Although MEKK1 does not appear to activate MEK5 (28), recent studies showed the involvement of Raf-1 in ERK-5–mediated gene regulation (28). The involvement of c-Raf-1 in ERK-5–mediated PMA-inducible *SPRR1B* expression was demonstrated as overexpression of constitutively active form of c-Raf-1 (ca-Raf-1) markedly upregulated *SPRR1B* transcription in H441 cells (Figure 6A). In support of ERK5 as downstream target of Raf-1, cotransfection of an equal amount of dn-ERK5 strongly suppressed active c-Raf-1–enhanced *SPRR1B* promoter activity. In contrast, cotransfection of dn-MEK5 did not suppress c-Raf-1–inducible *SPRR1B* promoter activation (Figure 6B). These data suggest that c-Raf-1 inducible promoter activity is mediated via ERK5, whereas MEK5 does not appear to participate in it.

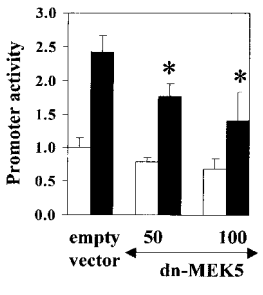

Figure 4. MEK5 regulates *SPRR1B* transcription in H441 cells. Cells were cotransfected with *SPRR1B*-Luc reporter and β-gal constructs in the presence of empty (pCEFL, 100 ng) or dominant-negative MEK5 (dn-MEK5) expression vector and treated with vehicle (*open bars*) or PMA (*solid bars*). The total DNA amount was kept constant by adding empty vector. *$P < 0.01$ compared with PMA-treated empty vector–transfected group.

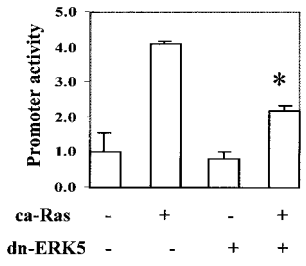

Figure 5. ERK5 mutant suppresses Ras-inducible *SPRR1B* promoter activation. Cells were transiently cotransfected with 150-*SPRR1B*-Luc (100 ng) and β-gal (50 ng) vectors along with 100 ng of either empty parental or constitutively active Ras (ca-Ras) expression vectors in the presence or absence of an equal amount of dn-ERK5 plasmid DNA. *$P < 0.01$ compared with ca-Ras–transfected group.

Overexpression of *c-Jun* Restores Basal and PMA-Stimulated *SPRR1B* Transcription Suppressed by ERK5 Mutant

We have previously shown that *c-Jun*, an immediate-early gene, strongly upregulates *SPRR1B* transcription in BE cells. Because ERK5 regulates PMA-inducible *SPRR1B* transcription and activates *c-Jun* transcription (29), H441 cells were cotransfected with *SPRR1B* promoter construct along with dn-ERK5 in the presence or absence of *c-Jun* expression vector. Cells were treated with DMSO or PMA and reporter gene expression was analyzed. As shown in Figure 7, overexpression of dn-ERK5 strongly suppressed

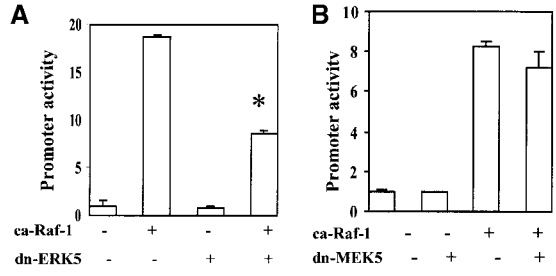

Figure 6. ERK5, but not MEK5, mutant suppresses c-Raf-1–inducible *SPRR1B* transcription. (*A*) Cells were transiently cotransfected with SPRR1B-Luc and β-gal vectors along with 100 ng of either empty parental or constitutively active c-Raf-1 (ca-Raf-1) in the presence or absence of an equal amount of dn-ERK5 (*A*) or dn-MEK5 (*B*) plasmid DNA. *$P < 0.001$ compared with ca-Raf-1–transfected group.

both basal and PMA-stimulated promoter activity. However, no significant effect was noticed when *c-Jun* was coexpressed along with mutant ERK5. In fact, *SPRR1B* transcription remains robustly elevated in the presence of *c-Jun*.

ERK5 Mutant Suppresses PMA-Stimulated AP-1, but Not IL-8, Promoter Transcription

To further determine the specificity of the ERK5-dependent gene expression, we have transfected H441 cells with AP-1-Luc or with human IL-8 promoter reporter constructs in the presence or absence of dn-ERK5 plasmid DNA. As shown in Figure 8A, coexpression of dn-ERK5 strongly suppressed both basal and PMA-stimulated reporter gene expression driven by the consensus AP-1–responsive elements. Note that at the higher concentration, dn-ERK5 can suppress the PMA-inducible promoter activity near to the basal levels. In contrast, dn-ERK5 did not have any significant effect on PMA-inducible IL-8 gene transcription, which appears to be mainly regulated by NF-κB (Figure 8B). These results suggest that the ERK5 MAPK pathway probably plays a major role in AP-1–dependent gene expression in bronchiolar cells.

Discussion

The present study clearly establishes a novel role for the MEK5-ERK5 MAPK pathway in PMA-inducible transcriptional stimulation of *SPRR1B* expression in Clara-like H441 cells. ERK5 is a novel member of the MAPK family (18). Like ERK1/2, ERK5 shares the Thr-X-Tyr sequence and is activated by dual phosphorylation within a TXY motif. However, unlike other MAPKs, such as ERK1/2, p38, and JNKs, ERK5 contains a distinctly larger (by 400 amino acids) C-terminal region (thus named as a Big MAPK) as well as a unique 12-domain loop containing two proline-rich regions (18). The unique C-terminal region of ERK5 can serve as a localization and/or regulatory domain. For example, ERK5 can be localized in the nucleus (18). Also, it was recently shown that C-terminal region of ERK5 contains potent transcriptional activation and interacting domains, which are required for coactivation of myocyte enhancer-binding factor-2D (MEF-2D) during calcium signaling in T cells (30). The loop 12-domain contains several small Pro-Ala and Pro-Thr repeats, similar to that of myosin light chain kinase, which apparently play an important role in cytoskeletal organization (31). Indeed, a recent study showed a role for MEK5-ERK5 pathway in the serial assembly of sarcomeres (32). Therefore, it appears that ERK5 has functions distinct from those of ERK1/2, p38, and JNK MAPKs.

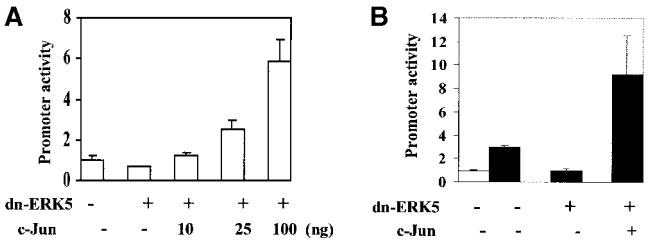

Figure 7. ERK5 mutant does not suppress *c-Jun*–enhanced *SPRR1B* transcription. (*A*) Cells were transfected with *SPRR1B* promoter reporter construct along with varying amounts of *c-Jun* expression vector in the presence or absence of 100 ng of dn-ERK5 plasmid. DNA concentration was kept equal in all samples by adding appropriate amount of pcDNA3 vector. (*B*) Cells were cotransfected with *SPRR1B* promoter and 100 ng Jun expression vector in the presence or absence of 200 ng of dn-ERK5. Cells were treated with DMSO (*open bars*) or PMA (*solid bars*) and Luc expression was determined. The value of the empty vector–transfected untreated samples was taken as one.

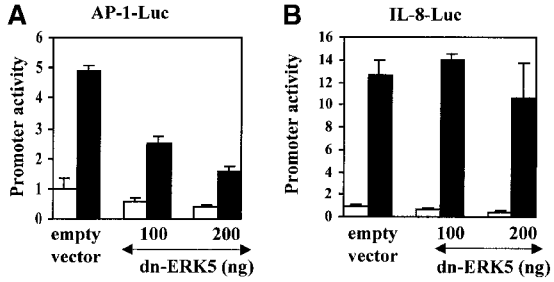

Figure 8. ERK5 mutant suppresses AP-1– but not NF-κB–dependent gene transcription. Cells were transfected with AP-1–Luc (*A*) or IL-8–Luc (*B*) reporter plasmid (*see* MATERIALS AND METHODS) in the presence or absence of dn-ERK5 expression vector as indicated. After 24 h incubation, cells were treated with DMSO (*open bars*) or PMA (20 ng/ml; *solid bars*) for 5 h. Luc expression was determined as in Figure 3.

ERK5 activation is induced more pronouncedly by oxidative stress signals, such as H_2O_2 and hyperosmolarity, than by growth factors such as epidermal growth factor and serum (23). Various agents that potently induce ERK1/2, such as TNF α, PMA, and PDGF, are apparently weak activators of ERK5 (23). Though much is being learned in other cell types, the mode(s) of activation and functional significance of MEK5/ERK5 pathway in airway epithelium, a major target of various toxicants and pollutants, are not known. By RT-PCR analysis, we were able to detect ERK5 message levels in both primary bronchial epithelial cultures and various cell lines that represent both upper and distal region of the lung (Figure 2). More importantly, *in vitro* kinase assays of the H441 cellular extracts immunoprecipitated with anti–ERK-5 antibodies revealed stimulation of ERK5 activation in H441 cells by PMA (Figure 1) and H_2O_2 (data not shown). Furthermore, the dn form of ERK5 suppresses PMA-stimulated reporter gene expression, driven by the *SPRR1B* promoter harboring two functional PMA-responsive elements (or AP-1 sites) or by the consensus AP-1 sites. This suggests a role for ERK5 in the regulation of AP-1–dependent gene expression in the distal bronchiolar region.

The downstream effector(s) that mediate ERK5-regulated *SRRR1B* transcription in H441 cells are not known. The best-known targets that are phosphorylated and activated by ERK5 include MEF2 family transcription factors (18). Though mainly involved in muscle cell differentiation, the MEF2 family of proteins, MEF2A–D, are also expressed in different cell types, suggesting other regulatory functions for these transcription factors (29). It has been

shown that ERK5 regulates *c-Jun* expression possibly through the activation of MEF2D (29), which bind at the *c-Jun* promoter to stimulate its transcription (33). Consistent with this, PMA-inducible *SPRR1B* transcription is mainly mediated by two functional AP-1 sites. Furthermore, PMA induces mRNA expression as well as DNA-binding activity of AP-1 proteins, which form strong protein–DNA complexes at consensus AP-1 sites as well as at the *SPRR1B* promoter (16, 34). The protein complex at the latter is preferentially occupied by Jun/Fra-1. Moreover, overexpression of *c-Jun* or Fra-1 robustly upregulates *SPRR1B* transcription in both H441 and S-6 cells. Therefore it is plausible that overexpression of dn-ERK5 suppresses *c-Jun* transcription, which results in downregulation of *SPRR1B* expression. Indeed, in support of this view, overexpression of mutant form of ERK5 did not suppress *c-Jun*–enhanced *SPRR1B* transcription (Figure 7A). Furthermore, coexpression of *c-Jun* blocks the suppressive effects of mutant ERK5 on PMA-inducible *SPRR1B* promoter activation (Figure 7B). Because ERK5 interacts and coactivates transcription factors (30), we cannot rule out the possibility of potential transcription activation and/or interaction of ERK5 with other proteins, such as ETS family member ESE-1, which also upregulates *SPRR1B* transcription in S-6 and H441 cells (unpublished data). Indeed, phosphorylation of Sap1a, an ETS family member, by ERK5 has been documented (25).

It is well established that MEK5 is the upstream activator of ERK5. Though overexpression of mutant MEK5 significantly suppressed PMA-inducible *SPRR1B* transcription, it did not completely suppress promoter activation, which suggests the involvement of other pathways. Indeed, our previous study demonstrated the involvement of Ras/MEKK1/JNK1 pathway in PMA-inducible *SPRR1B* transcription (17). Coexpression of ERK5 mutant also suppressed both constitutively active Ras^{-} and its downstream effector c-Raf-1–inducible promoter activation. However, overexpression of MEK5 mutant did not have any effect on c-Raf-1–inducible promoter activation. Consistent with this, previous studies have shown that Raf-1 and MEKK1, which are downstream effectors of Ras, did not activate MEK5 (28). Interestingly, Raf-1–dependent and –independent pathways have been shown to regulate ERK5-mediated gene regulation (35). For example, it was shown that c-Raf-1 binds *in vivo* with ERK5, but not with ERK2 or JNK1, suggesting specific protein–protein interactions between MAPKs also play a role in cell proliferation and transformation (28). Although the present study does not rule out such possibilities, a detailed investigation is warranted to further delineate upstream kinase modules that regulate MEK5-ERK5–mediated PMA-inducible *SPRR1B* transcription in H441 cells.

In summary, we have shown that PMA stimulates ERK5 activation. Furthermore, we have shown that PMA-inducible *SPRR1B* transcription in H441 cells is mediated in part by MEK5-ERK5 MAPK module probably through *c-Jun*. Based on this information and on our previous study (15), it appears that a coordinated complex network of MAPK modules, rather than a straightforward linear module, regulates the induction of squamous cell functions in the distal bronchiolar region (*see* Figure 9). Though regulation of AP-1–dependent gene expression by MEK5-ERK5 MAPK pathway adds a new dimension to the biology of airway epithelium, further investigation(s) is necessary to delineate other downstream effectors, including transcription factors and target genes, as well as upstream activating MAPK modules. This will provide additional insight to the mechanisms of toxicant-induced epithelial injury repair and transformation.

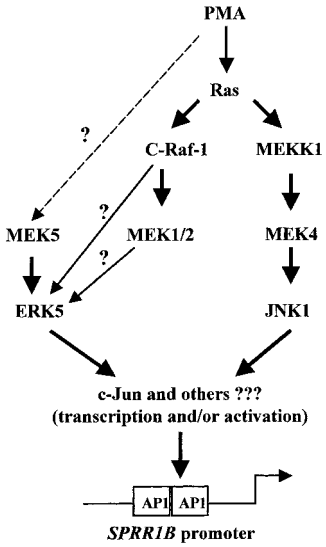

Figure 9. The proposed model depicts the multiple MAPK signaling modules that regulate PMA-stimulated SPRR1B expression in Clara-like H441 cells. *Question marks* indicate unknowns. The *broken arrow* indicates upstream MAPK module(s) that activate MEK5 and AP1, AP-1 binding sites.

Acknowledgments: This work was supported by NIH Grant HL58122, by Johns Hopkins Urban Asthma Center's Young Investigator Award, and by the funds EPA-R82672401 and NIEHS-ES09606 to S.R. The authors thank Dhan Kalvakolanu and Anne Jedlicka for critically reading the manuscript. They acknowledge the Johns Hopkins Urban Environmental Health Center for use of its core facilities supported by Grant ES30819.

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