Activation of c-Myb transcription factor is critical for PMA-induced lysozyme expression in airway epithelial cells

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Lysozyme is a major component of airway epithelial secretions, acts as cationic antimicrobial protein for innate immunity. Although lysozyme plays an important role in airway defense and is a key component of airway secretions under inflammatory conditions, little is understood about the regulation of its expression and the associated signaling pathway. We wanted to examine whether Phorbol 12-myristate 13-acetate (PMA), one of PKC activators, treatment of the airway epithelial cell line NCI-H292 increases lysozyme gene expression. In this study, we sought to determine which signal molecules are involved in PMA-induced lysozyme gene expression. We found that PKC and mitogen activating protein/ERK2 kinase are essential for PMA-induced lysozyme expression and also mediate the PMA-induced activation of c-Myb protein. We identified a proximal region of the lysozyme promoter essential for promoter activity containing c-Myb transcription factor binding site. Additionally, by site-directed promoter mutagenesis, we identified that c-Myb preferred the CAA motif of the -85/-73 region of the lysozyme promoter.

Finally, we showed that over-expression of c-Myb without PMA treatment increased the lysozyme promoter activity and protein expression. From these results, we conclude that PMA induces overexpression of lysozyme via ERK1/2 MAP kinase – c-Myb signaling pathways in NCI-H292 cells.

Key words : lysozyme, PMA, ERK MAP kinase, c-Myb

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I. INTRODUCTION

Airway mucosal epithelium presents a critical first line of innate host defense against microbial invader through their secretions which have a number of antimicrobial factors such as lysozyme.[1,2] Lysozyme acts as a cationic antimicrobial protein by degrading the cell wall of microbial cells through hydrolysis of the linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine.[2] In a state of chronic airway inflammation, goblet cell hyperplasia and nasal total secretion correlates with an increase in mucus and lysozyme products,[3,4] The molecular mechanisms of lysozyme expression remain unclear, although several reports have identified stimulators of lysozyme secretion, activity, and gene expression. Studies of macrophages or monocytes have shown that lipopolysaccharide (LPS) and cytokines, including interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and colony-stimulating factor 1 (CSF-1), significantly induce lysozyme activity or gene expression.[5,6,7]

One of the most important molecules that can induce the activation of lysozyme by cytokines and LPS is protein kinase C (PKC). Studies examining the regulation of these stimuli have implicated PKC as an important mediator in inflammation or differentiation signaling. The translocation or activation of PKC isoforms is regulated by IFN-γ,[8] CSF-1,[9] TNF-α,[10,11] and LPS.[12,13] PKC plays critical roles in cellular processes for cytokines, growth factors, and hormones.[14] Moreover, PKC isoforms are pharmacologically activated by phorbol esters such as phorbol 12-myristate 13-acetate (PMA) that can substitute for diacylglycerol, the endogenous PKC activator.[15] PMA reportedly functions as a tumor promoter by modulating diverse cellular responses, including gene transcription, cellular growth and differentiation, programmed cell death, immune response, and receptor desensitization.[16,17] PMA also enhances the release of lysozyme from cytochalasin B-treated human,[18] or rat polymorphonuclear leukocytes (PMNs).[19] Although the regulation of lysozyme activity has previously been explored, the transcriptional mechanisms that underlie lysozyme expression during airway inflammation are not fully understood. We used PMA to induce lysozyme expression in a human airway epithelial cell line in an effort to identify the transcriptional activation of lysozyme by PKC in the human airway. We sought to investigate the mediators and region of the lysozyme promoter that regulate lysozyme transcription activity. We found that PMA stimulated lysozyme expression in NCI-H292 cells through activation of PKC. Lysozyme up-regulation required activation of extracellular signal-regulated kinase

mitogen-activated protein kinase 2 (ERK2 MAPK), and nuclear transcription factor c-Myb. Specifically, the proximal promoter region contained a consensus c-Myb binding site and was required for c-Myb to transactivate the lysozyme promoter. We also confirmed that c-Myb is an important factor in the induction of lysozyme transcriptional activity without PMA treatment. Given that lysozyme is essential for the innate immunity of the airway epithelium, understanding the signal transduction pathway for PMA-induced lysozyme expression would yield important clues about the secretory defense function of airway epithelial cells.

II. MATERIALS AND METHODS

1. Materials

Anti-phospho-p44/42 MAP kinase (Thr202/Tyr204), anti-phospho-p38 MAP kinase (Thr180/Tyr182), and anti-phospho-SAPK/c-Jun NH2-terminal kinase MAP kinase (Thr183/Tyr185) antibodies were purchased from Cell Signaling (Beverly, MA, USA). Anti-human lysozyme antibody was purchased from AbD Serotec (Oxford, UK). PD98059 and anti-α-tubulin antibody were purchased from Calbiochem (San Diego, CA, USA). Anti-c-Myb antibody was purchased from Abcam (Cambridge, UK).

2. Cell Cultures

The human mucoepidermoid pulmonary carcinoma cell line NCI-H292 was purchased from the American Type Culture Collection (CRL-1848, Manassas, VA, USA) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) in the presence of penicillin-streptomycin at 37 ℃ in a 5% CO_2 humidified chamber.

3. Treatment of cells with PMA

NCI-H292 cells were seeded into 6-well plates, cultured for 24 hr to confluency, and serum-starved in RPMI-1640 with 0.2% FBS for 24 hr. Serum-starved medium was used throughout the experiments. Cells were treated with PMA (Sigma-Aldrich, St. Louis, MO, USA) as indicated in each experiment. For the inhibition studies, cells were pretreated with media containing inhibitors for 30 min before it was replaced with control or PMA-containing medium. After 24 hr, cells were collected to measure lysozyme expression.

4. RNA isolation and polymerase chain reaction (PCR)

Total RNA was extracted from cells using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Reverse transcription reactions were performed with 3 μg of total RNA, random hexamers primers (Applied Biosystems, Foster City, CA, USA), AMV reverse transcriptase (Applied Biosystems), and RNase inhibitor (Applied Biosystems)

in a final volume of 25 μl. The reverse transcription step ran for 30 min at 42℃ and 5 min at 95℃. PCR was performed with a MyCycler .

5. Real-time quantitative PCR

Real time PCR was performed using an Applied Biosystems 7300 Fast Real-Time PCR system with SYBR Green PCR Core Reagents (Applied Biosystems). Reactions were performed in a total volume of 20 μl which included 10 μl of 2XSYBR Green PCR Master Mix, 300 nM of each primer, and 1 μl of previously reverse-transcribed cDNA template. The following primers were used: lysozyme, forward 5`-TGCTGGAGACAGAAGCACTG-3` and reverse 5`-GGAGTTACACTCCACAACCTTG-3`; and β2-microglobulin, used as a reference for normalization, forward 5`-CGCTCCGTGGCCTTAGC-3` and reverse 5`-GAGTACGCTGGATAGCCTCCA-3`. The thermocycler parameters were 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 sec and 60℃ for 1 min. All reactions were performed in triplicate. The relative quantity of lysozyme mRNA was obtained using the comparative cycle threshold method and was normalized using β2-microglobulin as an endogenous control.

6. Western blot analysis

NCI-H292 cells were seeded into 6-well plates and cultured for 24 hr before serum starvation. After starving in 0.2 % FBS medium for 24 hr, cells were treated with PMA and harvested with 2X Lysis buffer (250 nM Tris-Cl, pH

6.5, 2% SDS, 4% β-mercaptoethanol, 0.02% bromphenol blue, and 10% glycerol). Samples (cell lysates) were resuspended in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.0, 10% glycerol, 2% SDS, 100 mM β-mercaptoethanol, and 0.1% bromphenol blue), boiled for 10 min, and analyzed on SDS-PAGE gels. Resolved proteins were transferred to a polyvinylidene difluoride membrane (Millipore). Blots were blocked with 5 % skimmed milk in Tris-buffered saline (TBS, 50 mM Tris-Cl, pH 7.5, and 150 mM NaCl) for 1 hr at room temperature and then incubated overnight with primary antibodies (1:1000) in 0.5% Tween 20 in TBS (TTBS). After thorough washing with TTBS, the blots were further incubated for 1 hr at room temperature with horseradish peroxidase-conjugated secondary antibodies (1:2000) (Cell Signaling) in TTBS and then visualized by ECL (Amersham Biosciences).

7. c-Myb and ERK1/2 siRNA preparation and transfection

c-Myb siRNA oligonucleotides (Stealth^{TM} siRNA) were synthesized by Invitrogen. We screened c-Myb mRNA (GenBank M15024) and selected potential siRNA sequences with high values of knock-down probability. The siRNA sequences in M15024 started at bases 292 (5`-CAGATGACTGGAAAG TTATTGCCAA-3`). Stealth RNAi negative control duplex (Medium GC, Invitrogen) was used as an siRNA negative control. siPKC(catalog no. sc-29449), siERK1 (catalog no. sc-29307), siERK2 (catalog no. sc-35335), and the siRNA negative control (scrambled sequence, catalog no. sc-37007) were

purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). siRNA transfection into NCI-H292 cells was carried out with Lipofectamine™ 2000 (Medium GC, Invitrogen) according to the manufacturer's instructions. NCI-H292 cells were seeded into 6-well plates one day before transfection and co-transfected with 15 nM of each siRNA when the cells reached 30-50% confluence.

8. Construction of lysozyme promoter

pGL3-lysozyme deletion mutants covering the promoter regions of lysozyme were generated by PCR using pairs of primers bearing specific restriction sites. Different sizes of primer pairs were used to construct the 5′ deletion mutant. -1531/+26 forward (5`-CAGTTCACCACAACCTCCGC-3`), -519/+26 forward (5`-TCTAGAGTAAGAAACTAGCT-3`), -350/+26 forward (5`-TGTTACTCAAAAATCACAGCTC-3`), -241/+26 forward (5`-GTGCTAT-GCCCCAATTCTTC-3`), -121/+26 forward (5`-GAGTCAGTGGATCAATAG-ACAG-3`), -91/+26 forward (5`-TTCCACACAACTGAAAGGGTGG-3`), -63 /+26 forward (5`-CAAACCACAAGGGGAAGAAGG-3`), -40/+26 forward (5`-GTTAAAAGATGTTAAATACTGGGGC-3`), and -20/+-26 forward (5`-GGGGCCAGCTCACCCTGG-3`) with *Xho*I sites and reverse primer (5`-GC-ACTCTGACCTAGCAGTCA-3`) with *Hind*Ⅲ sites were used to amplify the 5` flanking region of lysozyme gene. The PCR product was ligated into pGEM® -T Easy Vector (Promega, Madison, WI, USA). The recombinant plasmid DNA

was isolated, cut with *Xho*I (Promega) and *Hind*Ⅲ (Promega), and cloned into the equivalent site of pGL3-basic vector (Promega). To determine the location of the c-Myb response element on the 5′-flanking region of the lysozyme gene, three mutation constructs were generated using mutated oligonucleotides. The amplified fragment was digested with *Xho*I and *Hind*Ⅲ and then cloned into the pGL3-basic vector (Promega). All of the constructs were sequenced.

9. Construction of c-Myb expression plasmid

The expression vector for c-Myb, pcDNA5-c-Myb, was constructed by ligation of human c-Myb cDNA into pcDNA5 (Invitrogen). The c-Myb cDNA region was amplified by PCR from a c-Myb cDNA clone (ImaGenes, Berlin, G-ermany). Primers containing a *Not*I restriction site and Kozak sequence at the 5` end and a *Xho*I restriction site at the 31 end were used to creat full length c-Myb cDNA. The primer sequences used were forward, 5`-GGCCGCGGCCG-CGCCATGGCCCGAAGACCCCG-3`, and reverse, 5`-CCGGCTCGAGTCA-CATGACCAGCGT CCGGG-3`.

10. Transient transfection and luciferase analysis

We previously described the creation of constructs with a deleted promoter of the lysozyme gene. NCI-H292 cells were transiently transfected with pGL3-basic, pGL3-lysozyme (-1531/+26), pGL3- lysozyme (-519/+26), pGL3-

lysozyme (-350/+26), pGL3-lysozyme (-241/+26), pGL3-lysozyme (-121/+26), mutation Ⅰ, mutation Ⅱ, and mutation Ⅲ constructs using FuGENE6 Transfection Reagent (Roche Applied Science) according to the manufacturer's instructions, incubated for 48 hr, treated with 10 ng/ml of PMA for 24 hr, harvested, and assayed for luciferase activity using a luciferase assay system (Promega) according to the manufacturer's instructions. β-galactosidase activity was also assayed to standardize sample transfection efficiencies. To confirm that the luciferase activity of each construct was from PMA, we assayed the activity of each construct in the absence of PMA.

11. Chromatin immunoprecipitation (ChIP) assay

Approximately $2X10^9$ NCI-H292 cells in 150-mm dishes were treated with PBS containing 1% formaldehyde for 10 min, washed twice with PBS, and fixed with 125 mM glycine at room temperature for 5 min. The cells were rinsed twice with PBS and resuspended in 1 ml of solution A (10 mM HEPES, pH 6.5, 0.25% Triton X-100, 10 mM EDTA, and 0.5 mM EGTA) by pipetting. After a short spin (5 min at 3000 rpm), the pellets were resuspended in solution B (10 mM HEPES, pH 6.5, 200 mM NaCl, 1 mM EDTA, and 0.5 mM EGTA) containing protease inhibitors by vigorous pipetting to extract nuclear proteins. After centrifugation at 4,000 rpm for 5 min, the nuclear pellets were resuspended in immunoprecipitation buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 1% SDS, and 0.5% Empigen BB) containing protease inhibitors and

sonicated to break the chromatin into fragments with an average length of 0.2-1 kb. The following antibodies were used in the assay: 1 and 2 μg of anti-c-Myb antibody and 2 μg of normal mouse IgG as a negative control. The putative c-Myb site (-117 to -45) and control site (an upstream site in the lysozyme promoter, -5030/-4954) were used for ChIP analysis. The c-Myb site primer sequences used were forward, 5`-CAGTGGATCAATAGACAGTTCCTG-3`, and reverse, 5`-TTCTTCCCCTTGTGGTTTGG-3`, and control site forward, 5`-ACTTCTCTCTTAGAGTCCCCTT-3`, and reverse, 5`-GAACTCAAAGGA-A ACAGAGGC-3`.

12. Electromobility shift analysis

Nuclear extracts were prepared using the NucBuster protein extraction kit (Novagen). Briefly, cells were washed with ice-cold phosphate-buffered saline and pelleted, then resuspended in NucBuster reagent containing protease inhibitors at 150 μl NucBuster reagent 1 per 50 μl packed cell volume. Intact nuclei were separated from the cytoplasmic fraction by centrifugation and nuclear proteins were collected following addition of 75 μl of Nucbuster Reagent 2. Nuclear extracts were stored at −70 ℃. For the electromobility shift assay, oligonucleotides corresponding to the c-Myb sequence (5`-TGTTTTCC-ACACAACTGAAAGGGTGGAGCC-3`) and mutant c-Myb sequence (5`-TG-TTTTCCACATGGTCAAAAGGGTGGAGCC-3`) were syn thesized, annealed, and end-labeled with ATP using T4-polynucleotidekinase (Promega). 10 μg of

nuclear extract were incubated at room temperature for 30 min with the [^{32}P]-labeled c-Myb probes in binding buffer (Promega). Oligo-nuclear protein complexes were separated from the probes by electrophoresis through 6% nondenaturing polyacrylamide gels in 0.5X Tris borate-EDTA (TBE) buffer. Supershift experiments were conducted using 2 μg of anti-phospho-c-Myb antibody (Millipore). The gels were dried and autoradio graphed using an intensifying screen at -70 ℃.

13. Statistical Analysis

The data are presented as the means ± S.D. of at least three independent experiments. Where appropriate, statistical differences were assessed by Wilcoxon Mann-Whitney tests. A p value less than 0.05 was considered statistically significant.

III. RESULTS

1. PMA up-regulates lysozyme expression in NCI-H292 cells

PMA is known to be one of the PKC activators. PKC activation has been suggested as one of the major pathways involved in inflammation. To determine whether PMA can induce lysozyme expression within cells, we carried out real time PCR and Western blot analysis on NCI-H292 cells treated with PMA. Cells were treated with 2 to 50 ng/ml PMA for 24 hr. PMA induced both lysozyme

gene and protein expression in a dose-dependent manner (Fig. 1A and 1C). Real time PCR revealed that PMA increased mRNA levels with a peak at 10 ng/ml. Based on this observation, 10 ng/ml of PMA was used for all subsequent experiments. In addition, to determine whether PMA induced lysozyme expression in a time-dependent manner, we examined lysozyme gene and protein expression at different time points (Fig. 1B and 1D). Stimulation with 10 ng/ml of PMA led to a significant time-dependent increase with a peak at 6–24 hr after PMA stimulation.

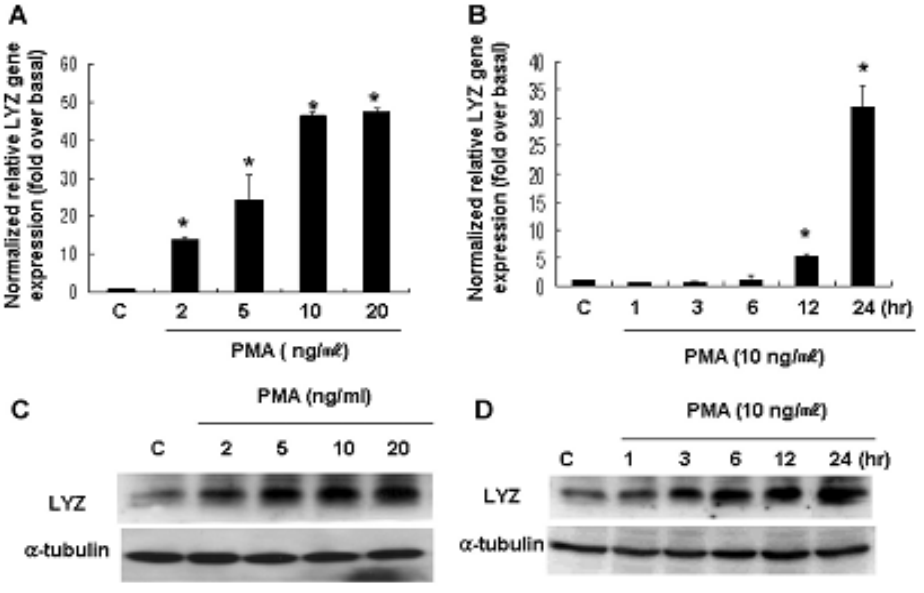

Figure 1. Effects of PMA on lysozyme gene expression in NCI-H292 cells

Confluent cells were treated with increasing doses of PMA (2, 5, 10, and 20 ng/ml) for 24 hr (A and C) or PMA (10 ng/ml) for 1, 3, 6, 12, and 24 hr (B and D). Total RNA was prepared and subjected to quantitative real-time PCR with β2-M (beta2-microglobulin) as an internal control (A and B). Total cell lysates were collected for lysozyme Western blot analysis (C and D). *, $p<0.05$

compared to control (DMSO). Data are presented as mean ± S.D. values of three independent experiments. Figures are representative of three independent experiments.

2. Involvement of ERK2 MAPK in PMA-induced lysozyme expression

Previous report have demonstrated that PKC isoforms are upstream of MAPK signaling cascades and trigger cellular response and cell proliferation in a variety of mammalian tissues or cells including bone marrow-derived mast cells.[14,20] We investigated which MAPK subfamily members are activated by PMA. As shown in Fig. 2A, phospho-ERK1/2 MAPK was maximally activated at 10 min and decreased after 30 min of PMA treatment, but phospho-JNK and phospho-p38 were not induced. PMA-treated A549 cells were used as positive controls for p38 activation. To examine the involvement of ERK1/2 in PMA-induced lysozyme expression, cells were exposed to PD98059 (a MEK1 inhibitor) prior to PMA treatment. Pretreatment with PD98059 for 1 hr clearly inhibited PMA-induced ERK1/2 MAPK and significantly suppressed PMA-induced lysozyme expression (Fig. 2B). To identify the molecules involved in the upstream signaling of ERK1/2 MAPK in PMA-induced lysozyme gene expression, we investigated the role of PKC in initiating PMA-induced lysozyme expression. PMA has been reported to induce PKC activation in several cell types and is known to regulate ERK1/2 activity.[21,22] To determine the role of PKC, cells were pretreated with 10 μM RO-31-8220 (a PKC inhibitor) for one hr before PMA stimulation, followed by Western

blotting (Fig. 2B). RO-31-8220 significantly inhibited PMA-induced ERK1/2 phosphorylation and lysozyme expression. These results indicate that PMA-induced ERK1/2 phosphorylation and lysozyme expression occur via PKC activation. To further confirm the PD98059 and RO-31-8220 results, cells were transiently transfected with ERK1, ERK2, PKC, or control siRNAs (Fig. 2C and D). Western blot analysis showed that ERK1 and ERK2 siRNA constructs clearly inhibited ERK1 or ERK2 MAPK, and PKC siRNA constructs also inhibited PKC indicating that all siRNAs worked well. In addition, PKC and ERK2 siRNA, but not ERK1 siRNA, significantly suppressed PMA-induced lysozyme expression. These results demonstrate that PKC and ERK2 MAPK are essential for PMA-induced lysozyme expression.

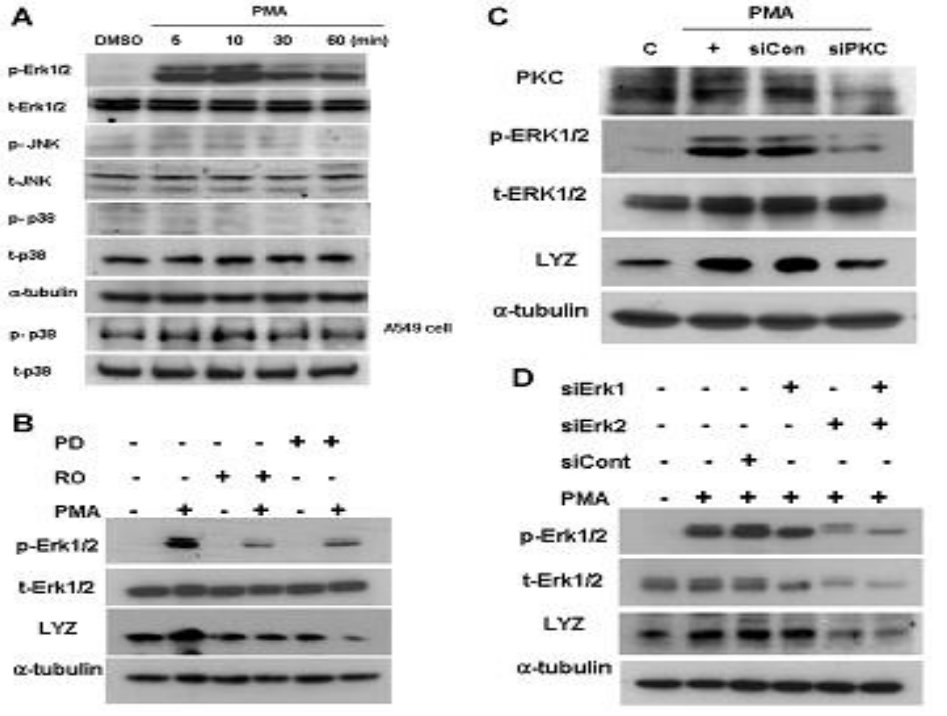

Figure 2. PMA induces lysozyme expression by ERK MAP kinase signaling

Confluent cells were treated with PMA for the indicated periods of time (5, 10, 30, and 60 min), and 30 μg/lane of total cell lysate protein was subjected to Western blot analysis. The blotted proteins were probed with antibodies against phosphorylated or non-phosphorylated forms of MAP kinases (ERK1/2, JNK, and p38) and α-tubulin (as a control) as indicated (A). Confluent cells were pretreated for 1 hr with PD98059 (40 μM) and RO-31-8220 (5 μM), and then stimulated with PMA for 10 min (to detect the phosphorylation of ERK1/2) or 24 hr (to detect lysozyme) prior to the collection of cell lysate for Western blot analysis (B). Cells were transiently transfected with siPKC and control siRNA (siCon) and stimulated with PMA for 10 min (to detect the phosphorylation of ERK1/2) or 24 hr (to detect lysozyme) prior to Western blot analysis (C). Cells were transiently transfected with siERK1, siERK2, siERK1/2, and control siRNA (siCon) and stimulated with PMA for 10 min (to detect the phosphorylation of ERK1/2) or 24 hr (to detect lysozyme) prior to Western blot analysis (D). The figures shown are representative of three independent experiments.

3. PMA-induced c-Myb activation mediates lysozyme transcription via the cis-acting c-Myb regulatory motif

To identify the PMA-response region within the lysozyme promoter, cells were transiently transfected with various deletion mutants and treated with PMA for 24 hr. PMA increased luciferase activity 3- to 5-fold for all promoter constructs, indicating that the -121/+26 region of lysozyme promoter may be necessary for response to PMA (Fig. 3A). To further study the potential transcription factors involved in PMA-induced lysozyme expression, the lysozyme promoter region (-121/-1) was analyzed for putative transcription

factor binding sites using the PATCH^{TM} database (http://gene-regulation.com) and Genomatix/MatInspector database (http://genomatix.de). Two putative transcription factor sites (c-Myb and GATA) on the lysozyme promoter (-121/-1) were identified (Fig. 3B). We performed an additional lysozyme luciferase assay using deletion mutants within the -121/+26 region. PMA selectively increased the luciferase activity of the -91/+26 region of the lysozyme promoter (Fig. 3C). The PMA-induced luciferase activity of the -63/+26 region was lower than that of the -91/+26 region, indicating that the -91/-63 region of the lysozyme promoter may be necessary for its response to PMA. As shown in Fig 3B, the -91/-63 region contains a c-Myb putative site. To further investigate whether c-Myb might act as a cis-element, three constructs for selective mutagenesis of the c-Myb-binding site were generated. The mutant Ⅰand Ⅲ c-Myb site lysozyme promoter constructs decreased the responsiveness relative to the wild-type lysozyme promoter construct (Fig 3D). These results suggest that c-Myb in the regulatory region of the lysozyme promoter may be critical for PMA-induced up-regulation of lysozyme transcriptional activity.

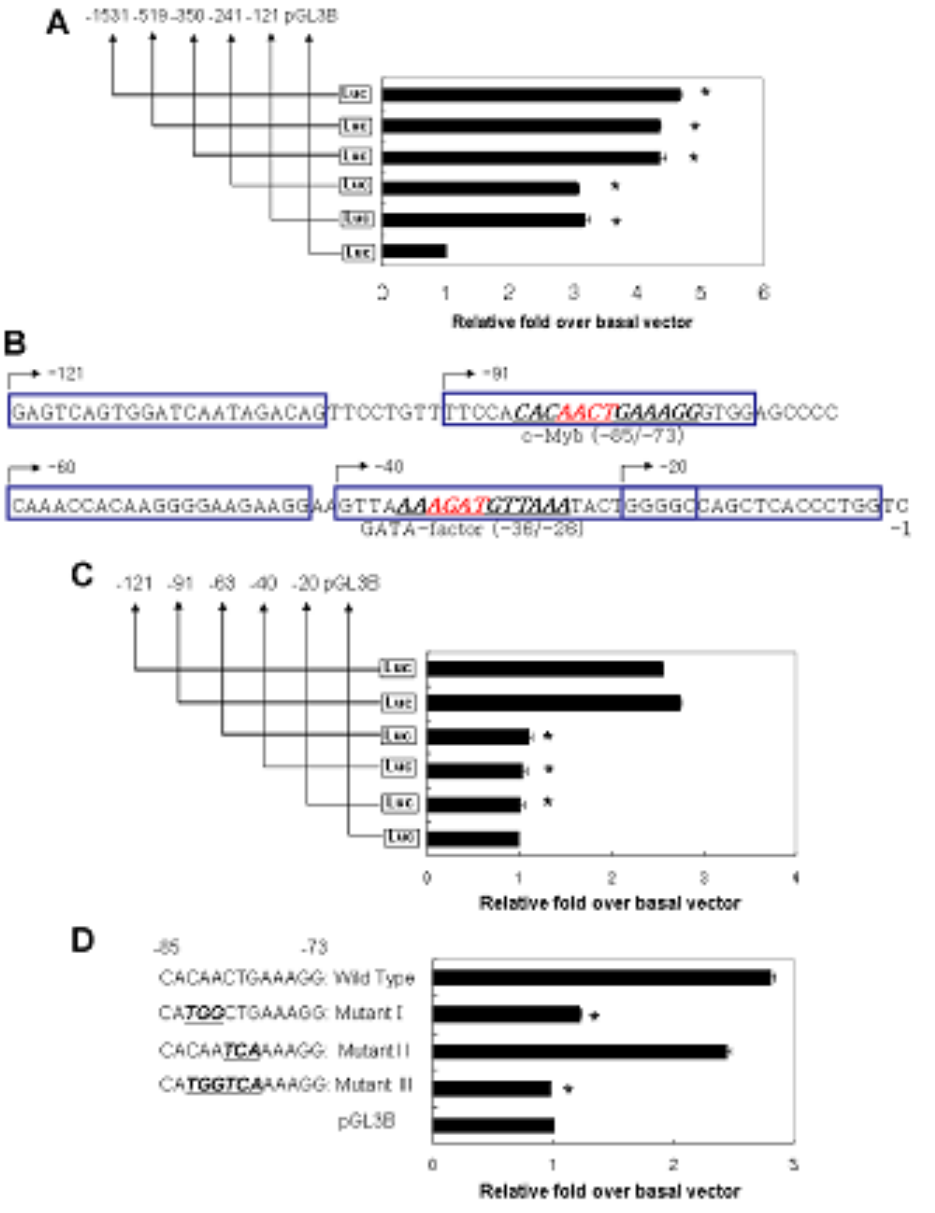

Figure 3. PMA-induced c-Myb activation mediates lysozyme transcription via the cis-acting c-Myb regulatory motif

Cells were transiently transfected with various lysozyme promoter luciferase reporter constructs and stimulated with PMA for 24 hr. Luciferase activity was then measured in PMA-treated or

untreated cells. *, $p < 0.05$ compared with untreated construct. Values shown are means ± S.D. of experiments performed three times. Two putative transcription factor sites (c-Myb and GATA) on the lysozyme promoter (-121/-1) were identified, and each core motif was indicated (B). Cells were transiently transfected with several lysozyme promoter constructs and stimulated with PMA for 24 hr. Cell lysates were harvested and reporter assays were performed. *, $p < 0.05$ compared with -121/+26 reporter construct (C). Site-directed selective mutagenesis was performed to construct c-Myb-binding site mutants as indicated. Values shown are means ± S.D. of experiments performed three times. *, $p<0.05$ compared with wild-type reporter construct. (D). All luciferase activities are shown after correction for transfection efficiency using the β-galactosidase activity of the cell lysates. The figures shown are representative of three independent experiments.

4. PKC, ERK1/2, and c-Myb are essential for PMA-mediated lysozyme promoter activity

To determine whether c-Myb plays a role in lysozyme expression, cells were transiently transfected with c-Myb siRNA or control siRNA. The effects of the siRNA were analyzed by Western blot analysis (Fig. 4A). c-Myb siRNA suppressed PMA-induced lysozyme expression, indicating that c-Myb is required for PMA-induced lysozyme expression. To determine the role of PKC and ERK1/2 in c-Myb expression, cells were transiently transfected with PKC and ERK1 or ERK2 siRNA (Fig. 4B and C). Western blot analysis showed that PKC and ERK2 siRNA, but not ERK1 siRNA, significantly suppressed PMA-induced c-Myb expression. Taken together, these data indicate that PKC and ERK2 MAPK are essential for PMA-induced lysozyme expression via c-Myb. To investigate whether lysozyme promoter activity is affected by PKC

and ERK1/2, we transfected cells with the -91/+26 construct and exposed them to RO318220 and PD98059 for one hr prior to PMA treatment. Pretreatment with RO318220 and PD98059 significantly suppressed PMA-induced lysozyme promoter activity (Fig. 4D). Next, to further investigate the role of ERK1/2 and c-Myb in lysozyme promoter, cells were co-transfected the -91/+26 construct and siERK1, siERK2, or c-Myb siRNA. The activity of the -91/+26 construct decreased considerably in the presence of siERK2 or c-Myb siRNA (Fig. 4E). However, the activity of the -91/+26 construct was not decreased with the siERK1. These results suggest that the activity of the -91+26 construct is mainly regulated by ERK2 or c-Myb.

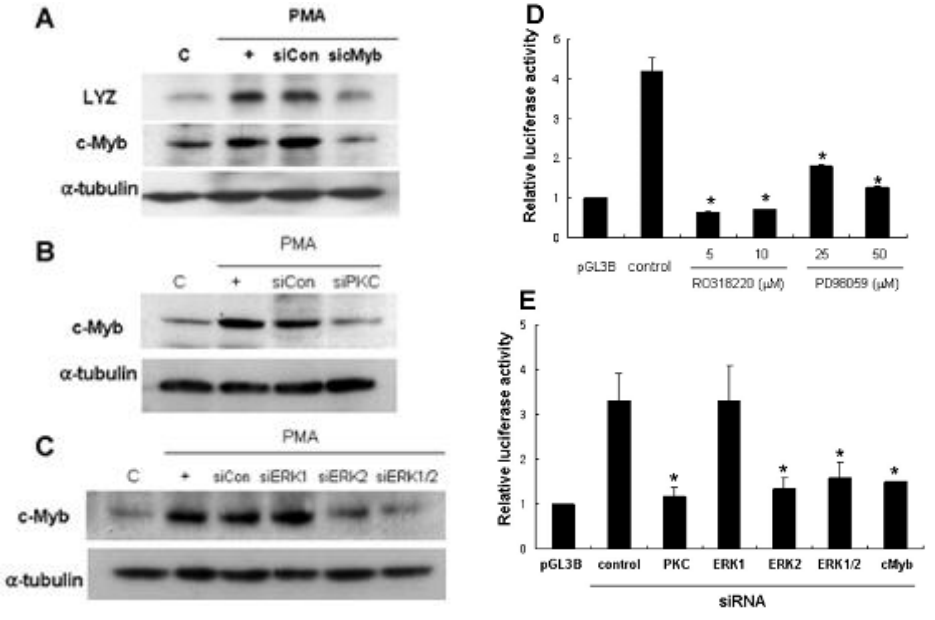

Figure 4. PKC, ERK1/2, and c-Myb are essential for PMA-mediated lysozyme promoter activity

Cells were transiently transfected with a c-Myb siRNA construct. Cells were serum-starved overnight and then treated with PMA for 24 hr, after which cell lysates were harvested for Western blot analysis (A). Cells were transiently transfected with siPKC (B) and ERK1 or ERK2 siRNA (C) and stimulated with PMA for 24 hr prior to Western blot analysis. Cells were transiently transfected with a -91/+26 lysozyme promoter construct. Cells were serum starved overnight, pre-treated with RO318220 and PD98059, and then stimulated with PMA for 24 hr. Luciferase activity was then measured in PMA-treated and -untreated cells (D). *, $p<0.05$ compared with PMA treatment. Cells were transiently transfected with a -91/+26 lysozyme promoter construct in combination with siRNA constructs for PKC, ERK1, ERK2, or c-Myb. Cells were stimulated with PMA for 24 hr. Luciferase activity was then measured in PMA-treated and -untreated cells (E). *, $p<0.05$ compared with control siRNA treated by PMA. Values shown are means ± S.D. of experiments performed three times.

5. c-Myb binding to the lysozyme promoter in response to PMA

To analyze the DNA binding activity of PMA-activated c-Myb, we performed an EMSA assay using the nuclear extracts of PMA-treated NCI-H292 cells (Fig. 5A). The activities of lysozyme specific c-Myb oligonucleotides increased remarkably in response to PMA. To identify specific c-Myb binding complexes, competition and supershift analyses were performed using 50-, and 100-fold excesses of non-radiolabeled (cold) c-Myb oligo-nucleotide or mutant c-Myb oligonucleotide, and anti-phospho-c-Myb antibody. The specific band was found to be selectively inhibited by a consensus c-Myb competitor or anti-c-Myb antibody. However, the specific band was not inhibited by mutant oligonucleotide or rabbit IgG. These results indicate that

activated c-Myb binds to a *cis*-acting element in the lysozyme promoter. Consistent with this conclusion, the binding of c-Myb to the lysozyme promoter was further confirmed by ChIP assay. We further investigated which regions (transcription factor-binding sites) of the lysozyme gene promoter were activated at different time points by PMA-induced c-Myb. Since regions -85 to 73 contain putative c-Myb binding sites, we performed ChIP assays using specific primers (-117/-45) for PCR. We also used the region -5030/-4954 as a negative control. The ChIP assays clearly showed that stimulation with PMA led to a significant increase lysozyme promoter binding activity with a peak at 24 hr after PMA stimulation (Fig. 5B). Anti-rabbit IgG, the negative control, did not yield any signals, confirming the assay specificity. Input chromatins were also used in these assays to indicate that equal amounts of cell lysates were used from PMA-treated cells. In addition, we examined the activity of c-Myb in the lysozyme promoter with c-Myb siRNA knocked down in cells. Twenty-four hours after transfection with c-Myb siRNA or control siRNA, cells were treated with PMA for 24 hr. After purification of DNA in the immunoprecipitate, the abundance of genomic DNA containing a promoter was determined by PCR amplification using sequence-specific primer pairs. The ChIP assays clearly showed that c-Myb directly bound to the lysozyme promoter (-117/-45). When c-Myb expression was knocked down by c-Myb siRNA, but not control siRNA, the recruitment of c-Myb on -117/-45 site was reduced. Based on these results, we conclude that PMA-induced c-Myb protein is strongly recruited to the

chromatin regions of the c-Myb binding site of the lysozyme promoter.

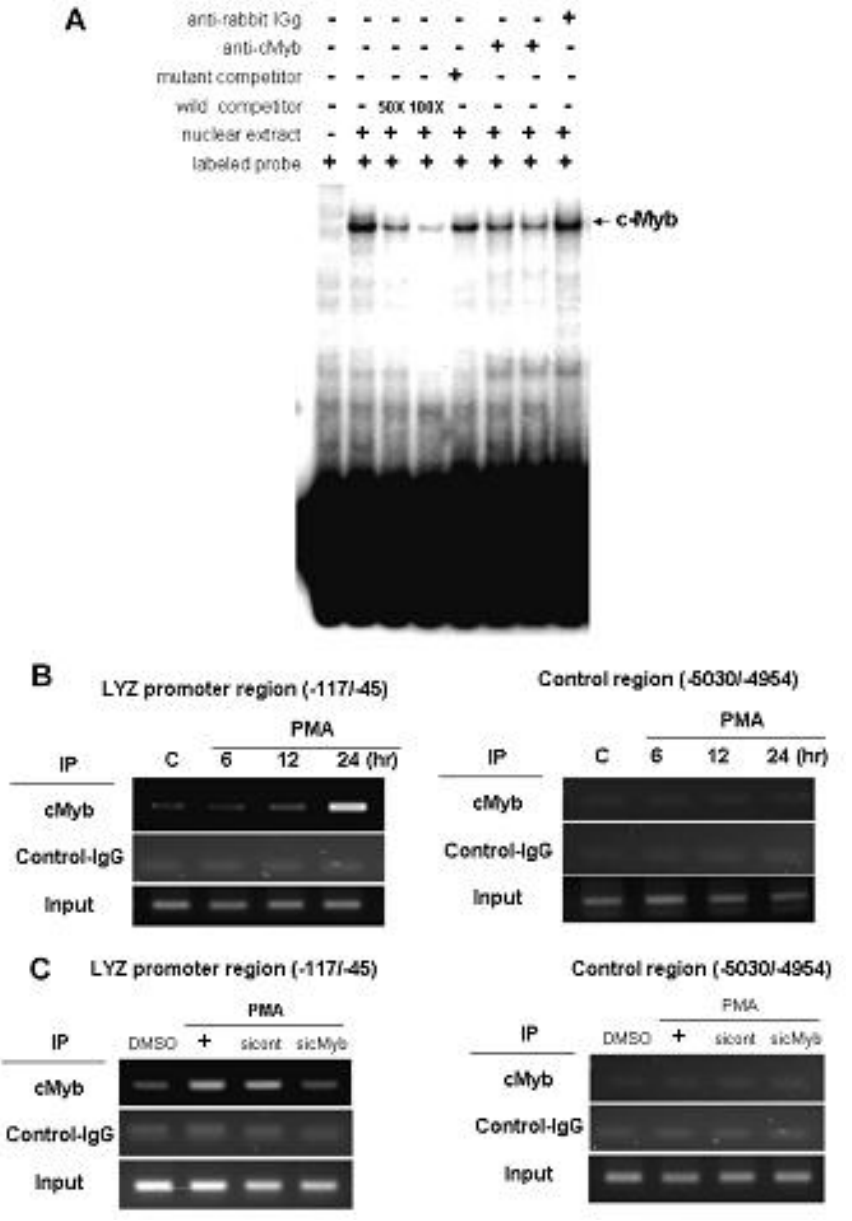

Figure 5. c-Myb binding to the lysozyme promoter in response to PMA

Nuclear protein extract from PMA-treated NCI-H292 cells was subjected to an electromobility shift assay. Nuclear proteins were incubated with wild- type lysozyme probe, 50 or 100-fold

excesses of cold probe, 100-fold excesses of mutant cold probe, and anti-phospho-c-Myb antibody (1 and 2 μg) or control IgG (2 μg) before the electromobility shift assay. The labeled nuclear proteins were separated by electrophoresis on 6% polyacrylamide gels, and the gels were dried and exposed to autoradiography at -70 ℃ (A). Chromatin prepared from PMA-treated cells (6, 12, and 24 hr) was immunoprecipitated using anti-c-Myb antibodies or normal mouse IgG (control). The immunoprecipitated chromatin was analyzed with PCR using primers specific to the indicated site. Input chromatin represents a portion of the sonicated chromatin before immunoprecipitation (B). Cells were transfected with c-Myb or control siRNA and stimulated with PMA for 24 hr. Cross-linked protein-DNA complexes were immunoprecipitated using anti-c-Myb antibody or normal mouse IgG. The immunoprecipitated chromatin was analyzed with PCR using primers specific to the indicated site. Input chromatin represents a portion of the sonicated chromatin before immunoprecipitation (C). The figures shown are representative of three independent experiments.

6. c-Myb overexpression up-regulates lysozyme expression in NCI-H292 cells.

To examine the functional role of c-Myb in lysozyme expression, we used wild-type c-Myb expression vectors in NCI-H292 cells. Efficient transfection of constitutively active c-Myb expression was confirmed by detection of wild-type c-Myb proteins. Overexpression of the wild-type c-Myb affected the level of lysozyme expression, suggesting that c-Myb activity was required for lysozyme expression (Fig. 6A). Next, we examined the functional effects of c-Myb on lysozyme gene promoter activity. To investigate whether c-Myb overexpression mediates lysozyme promoter activity, we co-transfected cells with the -91/+26 construct as a wild-type, a -20/+26 construct that did not contain a c-Myb site,

or mutant constructs (M1, M2, and M3) as site-directed mutations of the c-Myb site with wild-type c-Myb expression vectors. The activity of the -91/+26 and M2 construct increased when paired with wild-type c-Myb, but the activity of the -20/+26, M1, and M3 constructs were not affected by wild-type c-Myb. These results suggest that the activity of the -91/+26 construct was mainly regulated by c-Myb. To analyze the DNA binding activity of c-Myb overexpression, we performed an EMSA assay using the nuclear extracts of c-Myb overexpressed NCI-H292 cells (Fig. 6C). The activities of lysozyme-specific c-Myb oligonucleotides remarkably increased in response to c-Myb. However, the specific band was selectively inhibited by a consensus c-Myb competitor or anti-c-Myb antibody. The specific band was not affected by mutant oligonucleotide or rabbit IgG. In addition, we further confirmed this conclusion by ChIP assay. We investigated which regions (transcription factor-binding sites) of the lysozyme gene promoter were activated at different time points by c-Myb. The ChIP assays clearly showed that c-Myb overexpression led to a significant increase (with a peak at 8 hr after pcDNA5-c-Myb transfection) (Fig. 6D). Input chromatins were also used in these assays to indicate that equal amounts of cell lysates were used. Taken together, these findings suggest that c-Myb protein is strongly recruited to the chromatin regions of the c-Myb binding site of the lysozyme promoter to augment lysozyme transcriptional activation.

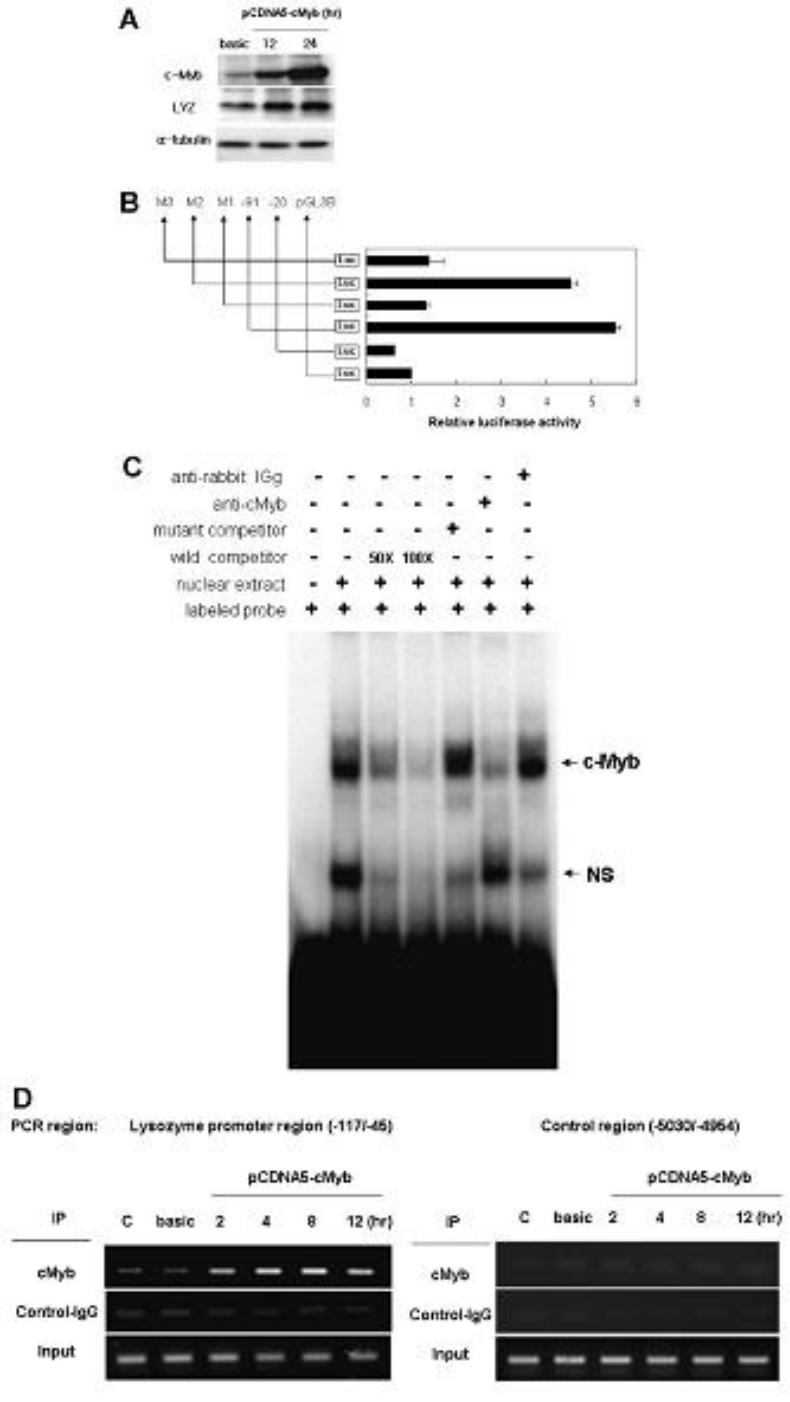

Figure 6. c-Myb overexpression up-regulates lysozyme expression

NCI-H292 cells were transiently transfected with basic (empty) and wild- type c-Myb expression vectors for the indicated times, and total cell lysates were collected for c-Myb and lysozyme Western blot analysis (A). Cells were transiently transfected with a 20/+26, 91/+26, mutation Ⅰ, Ⅱ, and Ⅲ reporter constructs in combination with either c-Myb expression vector or basic vector. Cell lysates were harvested and reporter assays performed (B). Nuclear protein extract from c-Myb transfected NCI-H292 cells was subjected to an electromobility shift assay. Nuclear proteins were incubated with wild-type lysozyme probe, 50 or 100-fold excesses of cold probe, 100-fold excesses of mutant cold probe, and anti-phospho-c-Myb antibody (2 μg) or control IgG (2 μg) before the electromobility shift assay. The labeled nuclear proteins were separated by electrophoresis on 6% polyacrylamide gels, and the gels were dried and exposed to autoradiography at -70 ℃ (C). Chromatin prepared from c-Myb-transfected cells (2, 4, 8, and 12 hr) was immunoprecipitated using anti-c-Myb antibodies or normal mouse IgG (control). The immunoprecipitated chromatin was analyzed with PCR using primers specific to the indicated site. Input chromatin represents a portion of the sonicated chromatin before immunoprecipitation (D). 'NS' indicates a non-specific complex.

IV. DISCUSSION

Lysozyme, an important antimicrobial peptide derived from serous cells and the surface epithelium.[23] It is expressed constitutively in a variety of cells and tissues.[2] The predominant localization of lysozyme in immune cells, including monocytes, macrophages, and leukocytes, as well as mucosal surfaces and secretions, implies an important role for lysozyme in the prevention of infection.[24] Epithelial cells are one important cellular source of lysozyme.[2] In a

previous study, we showed that lysozyme is expressed in human nasal epithelium and that lysozyme accumulates in retinoic acid-deficient human tracheobronchial epithelial cells.[25,26] To further extend our study, we investigated the transcriptional mechanism of lysozyme expression in a human airway epithelia cell line (NCI-H292) using PMA, a potent PKC activator. NCI-H292 is widely used as a model for airway epithelial cells because the cells retain their mucoepidermoid structural characteristics in culture. In our experience, we have obtained the same results using NCI-H292 cells or nasal epithelial cells. Furthermore, siRNA or plasmid transfection efficiency in primary nasal epithelial cells is very low. For these reasons, we selected NCI-H292 cells as a model of the human airway epithelial cell system. Previous reports have demonstrated a critical role for PKC-mediated activation of the MAPK signaling pathway in cellular responses or cell proliferation,[14,20] but the exact order of events leading to lysozyme expression has not been elucidated. Using inhibitors and siRNA assays, we demonstrated that PMA-induced ERK1/2 phosphorylation was controlled by PKC. The specific knock-down of ERK2 revealed the important role of ERK2 in PMA-induced lysozyme expression and c-Myb transcription factor activation, whereas ERK1 did not play a major role in lysozyme expression (Fig. 4E). Although ERK1 and ERK2 are the main isoforms of MAPK and these two kinases have similar functions, divergent roles for ERK1 and ERK2 have been shown in previous reports. $ERK1^{-/-}$ mice are viable and fertile, whereas $ERK2^{-/-}$ mice experience

embryonic lethality.[27] PKC stimulation is involved in ERK2 activation in fetal cerebral arteries, but ERK1 activation in adult cerebral arteries.[28] In addition, Warren *et al*. reported that ERK1 is dispensable, but ERK2 required, for $CD8^+$ T cell activation.[29] A recent study further proposed that ERK1 antagonizes the positive signaling provided by ERK2 in Ras-mediated signaling.[30] The different effects of ERK1 and ERK2 on signaling may be due to functional and quantitative differences or to the significant role in their regulation.[29,30] To date, the signal molecules involved in the signaling of PMA-induced lysozyme expression have not been reported. In addition, there is very little information about which transcription factors are involved in lysozyme gene regulation. Our transient transfection analysis indicated that the presence of a *cis*-regulatory element in the 5` flanking region of the lysozyme promoter could potentially influence lysozyme expression in human airway epithelial cells. The -85/-73 region of the lysozyme promoter was involved in the response to PMA and c-Myb was important for lysozyme up-regulation. c-Myb is a transcriptional activator that contains three functional domains, including an amino-terminal DNA binding region, centrally located acidic transcriptional activation region, and a carboxyl-terminal negative regulatory domain.[31-34] We found that the PMA-induced c-Myb had both DNA binding and transcriptional activity in the -85/-73 region. In addition, specific c-Myb knock-down with siRNA completely blocked PMA-induced lysozyme expression. To examine whether c-Myb overexpression mediates transcription and translation of lysozyme without PMA

treatment, we constructed a pcDNA5-c-Myb expression vector. Overexpressing c-Myb significantly increased lysozyme promoter activity as well as protein expression (Fig. 6). Interestingly, we found that c-Myb both with and without PMA treatment employed the same mechanisms for increasing lysozyme promoter activity. Taken together, these results indicate that c-Myb activity could regulate lysozyme expression in human airway epithelial cells.

V. CONCLUSION

In summary, we demonstrated that treatment of NCI-H292 cells with PMA results in activation of PKC, stimulating ERK2 MAPK signaling and up-regulating lysozyme expression. c-Myb is also required for transcriptional mechanisms that mediate lysozyme up-regulation. Further studies are needed to assess the roles of c-Myb in the regulation of endogenous lysozyme and interaction with other transcription factors in normal human airway cells.

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ABSTRACT(IN KOREAN)

PMA를 처치한 기도 상피 세포에서 c-Myb 전사 인자의 활성화를 통한 라이소자임의 발현

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배 정 호

목적. 라이소자임은 기도 상피에서 분비되는 항균 단백질로 기도 상피의 선천성 면역에 필수적인 역할을 담당하고 있다. 기도 상피의 염증 반응 시 라이소자임의 분비가 증가하는 것으로 알려져 있으나 라이소자임 유전자의 발현 기전, 특히 전사 과정에 대해서는 명확히 규명되어 있지 않다. 본 연구에서는 기도 상피 세포주인 NCI-H292 cell에서 protein kinase C (PKC) 활성인자인 phorbol 12-myristate 13-acetate (PMA) 처치 후 라이소자임 발현 양상 및 발현에 관여하는 세포 내 신호전달과 전사 과정을 알아보려고 하였다.

결과. 기도 상피 세포에서 PMA에 의한 라이소자임 발현이 PKC와 mitogen actvating protein/ERK2 kinase 활성화를 통해 유도되는 것을 확인하였고 전사 과정에서 c-Myb 전사인자가 중요한 역할을 하는 것을 프로모터 스터디로 규명하였다. c-Myb 전사인자는 PMA에 의해 활성화되어 라이소자임 프로모터의 프록시말 부위(-85/-73) 에 결합하여 라이소자임 발현을 증가시킨다. 또한 PMA 없이 c-Myb 전사인자 과발현만으로도 기도 상피 세포에서 라이소자임 프로모터 활성과 단백질 발현이 증가함을 확인하였다.

결론. NCI-H292 세포주에서 PMA에 의한 라이소자임 발현이 ERK1/2 MAPK - c-Myb 신호전달을 통하여 이루어지는 것을 규명하였다.

핵심되는 말 : 라이소자임, PMA, ERK MAP kinase, c-Myb 전사인자

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